Dysfunction in the nigrostriatal system: effects of L-DOPA and GDNF

Nina Nevalainen
To my family
ABSTRACT

Parkinson’s disease is a common neurodegenerative disorder caused by nigrostriatal dopamine loss, with motor deficiencies as the primary outcome. To increase the striatal dopamine content, patients are treated with 3,4-dihydroxyphenyl-L-alanine (L-DOPA). Beneficial relief of the motor symptoms is achieved initially, although the efficacy is lost with time and severe side effects, referred to as L-DOPA-induced dyskinesia, manifest in the majority of patients. Biological mechanisms responsible for the dopaminergic degeneration and the upning of dyskinesia are still unclear, and thus knowledge regarding critical factors for maintenance of the nigrostriatal system as well as neurochemical changes upon chronic L-DOPA is urgent. The present work aims at studying the importance of glial cell line-derived neurotrophic factor (GDNF) for nigrostriatal preservation, and the involvement of the dopaminergic, serotonergic, and glutamatergic systems in L-DOPA-induced dyskinesia. Effects from different levels of GDNF expression were evaluated on fetal mouse nigrostriatal tissue in a grafting study. In GDNF gene-deleted grafts, degeneration of the entire nigrostriatal system was evident at 6 months. In grafts with partial GDNF expression, significant loss of dopamine neurons was observed at later time points, although deviant findings in the dopamine integrity such as reduced innervation capacity and presence of intracellular inclusions-like structures were already present at earlier stages. The results emphasize GDNF as a crucial factor for long-term maintenance of the nigrostriatal system. Furthermore, striatal neurochemical alterations upon chronic L-DOPA treatment were studied in hemiparkinsonian rats using in vivo voltametry. The findings demonstrated impaired dopamine as well as glutamate releases in dyskinetic subjects, with no effects from acute L-DOPA administration. Conversely, in L-DOPA naïve dopamine-lesioned animals, dopamine release
was increased and glutamate release attenuated upon a L-DOPA challenge. Moreover, L-DOPA-derived dopamine release was demonstrated to originate from serotonergic nerve fibers in the dopamine-lesioned striatum, an event that contributes significantly to dopamine levels also in intact striatum, and thus, is not a consequence from dopamine depletion. Assessment of serotonergic nerve fibers in L-DOPA treated animals and in a grafting study concluded that nerve fiber density was not affected by chronic L-DOPA treatment, nevertheless, dysfunction of this system can be suspected in dyskinetic animals since dopamine release was impaired and regulation of glutamate release by serotonergic 5-HT$_{1A}$ receptor activation was achieved in normal but not in dyskinetic animals. Furthermore, the selective serotonin reuptake inhibitor, fluoxetine, attenuated L-DOPA-induced dyskinetic behavior, an effect that was demonstrated to be mediated via 5-HT$_{1A}$ receptors. In conclusion, dysmodulation of multiple transmitter systems is evident in LID.
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ABBREVIATIONS

AADC  \( \text{L-\text{aromatic amino acid dopa decarboxylase}} \)
AIM  \( \text{abnormal involuntary movement} \)
ALDH1  \( \text{aldehyde dehydrogenase1A1} \)
AMPA  \( \text{\( \alpha \)}\text{-amino-3-hydroxy-5-methylisoxazole-4-propinoic acid} \)
BBB  \( \text{blood-brain barrier} \)
BSA  \( \text{bovine serum albumin} \)
CNS  \( \text{central nervous system} \)
COMT  \( \text{catechol-O-methyl transferase} \)
DARPP-32  \( \text{dopamine and cyclic AMP-regulated phosphoprotein of relative molecular mass 32,000} \)
DAT  \( \text{dopamine transporter} \)
5,7-DHT  \( \text{5,7-dihydroxytryptamine} \)
DMEM  \( \text{Dulbecco’s modified Eagle’s medium} \)
DOPAC  \( \text{3,4-dihydroxyphenylacetic acid} \)
E  \( \text{embryonic day} \)
FAST-16  \( \text{Fast Analytical Sensing Technology with 16-bit resolution} \)
GABA  \( \text{\( \gamma \)}\text{-aminobutyric acid} \)
GDNF  \( \text{glial cell line-derived neurotrophic factor} \)
GIRK2  \( \text{G-protein Activated Inwardly Rectifying Potassium Channel 2} \)
Glut1  \( \text{Glucose transporter 1} \)
5-HT  \( \text{5-hydroxytryptamine, serotonin} \)
KCl  \( \text{potassium chloride} \)
L-DOPA  \( \text{3,4-dihydroxyphenyl-L-alanine} \)
LGE  \( \text{lateral ganglionic eminence} \)
LID  \( \text{L-DOPA-induced dyskinesia} \)
MAO  \( \text{monoamine oxidase} \)
MEA  \( \text{multielectrode array} \)
MFB  \( \text{medial forebrain bundle} \)
MSN  \( \text{medium spiny neuron} \)
NMDA  \( \text{N-methyl-D-aspartate} \)
6-OHDA  \( \text{6-hydroxydopamine} \)
8-OHDPAT  \( \text{(2R)-(+)\text{-8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide}} \)
PBS  \( \text{phosphate buffered saline} \)
PD  \( \text{Parkinson’s disease} \)
SERT  \( \text{serotonin transporter} \)
SSRI  \( \text{selective serotonin reuptake inhibitor} \)
TH  \( \text{tyrosine hydroxylase} \)
VM
ventral mesencephalon

VMAT
vesicular monoamine transporter

WAY-100635
N-[2-{4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridylcyclohexanecarboxamide
SAMMANFATTNING PÅ SVENSKA

Parkinsons sjukdom är en av de vanligast förkommande neurodegenerativa sjukdomarna och drabbar ca 1-2% av befolkningen i övre medelåldern. Vid sjukdomen sker en specifik celldöd i substantia nigra i hjärnan, vilket leder till brist på signalsubstansen dopamin i striatum och funktionsnedsättande konsekvenser för det motoriska systemet. Karaktäristiska symptom är främst långsam motorik, tremor, stelhet samt balanssvårigheter. För att öka dopaminnivåerna i striatum och på så sätt återställa motorisk funktion behandlas patienter i regel med 3,4-dihydroxyphenyl-L-alanine (L-DOPA). Denna behandling är till en början mycket effektiv, men efter några års kronisk behandling avtar läkemedlets effekt och en stor andel av patienterna drabbas av biverkningar som yttrar sig som ofrivilliga rörelser s.k. dyskinesier. Två angelägna områden att utforska inom detta forskningsfält är dels identifiering av faktorer avgörande för dopamincellers överlevnad, men även optimering av den befintliga medicineringen och förstå uppkomst av dyskinesier.

I denna avhandling framgår att nervtillväxtfaktorn GDNF är avgörande för långvarig dopaminerg och striatal cellöverlevnad. I en transplantationsstudie påvisades degeneration av båda cellslagen redan efter 6 månader vid frånvaro av GDNF. I transplantat med sänkta GDNF-nivåer överlevde dopamincellerna längre tidsperioder. Emellertid kunde inklusionskroppar och försämrad innerveringskapacitet av striatal vävnad bevitnas redan vid tidigare tidpunkter. Detta arbete understryker vikten av normalt GDNF-uttryck för långsiktig nigrostriatal cellöverlevnad.

Vidare utvärderades effekter av långvarig L-DOPA-behandling på olika cerebrala signalsystem. Denna del av avhandlingsarbetet påvisar att serotonerga neuron har en framstående roll vid dopaminsyntes efter intag av
L-DOPA, och bidrar till förhöjda dopaminkoncentrationer i striatum såväl vid frånvaro av dopaminerga nervfibrer som vid normala tillstånd. I dyskinetiska djur däremot, kunde ingen ökning av dopaminivåer registreras efter tillförsel av L-DOPA och lägre dopaminivåer uppmättes än i obehandlade dopaminlesionerade djur. Dessa fynd tyder på att L-DOPA konverteras i serotonerga neuron under hela L-DOPA-behandlingens tidsförlopp, men att detta system på sikt kan påverkas negativt av L-DOPA. Dock kunde ingen förlust av serotonerga nervfibrer påvisas och därmed torde effekten vara av annan karaktär. Extracellulära glutamativåer var oförändrade i dyskinetiska djur, men däremot var koncentrationerna vid frisättning från nervterminalerna betydligt lägre än i normala djur. I ickebehandlade dopaminlesionerade djur var även de extracellulära basalivåerna lägre, vilket indikerar att basalivåerna blir återställda vid kronisk L-DOPA behandling, emedan glutamatfrisättningen förblir sänkt. Glutamativåerna kunde sänkas då 5-HT₁A receptorer aktiverades farmakologiskt i normala djur, dock ej i dopaminlesionerade djur, oberoende av behandling med L-DOPA. Fluoxetine, en selektiv serotoninåterupptagshämmare, reducerade dyskinesierna, förmodligen via 5-HT₁A receptorer, då blockad av de senare ledde till återkomst av dyskinesier. Sammantaget verkar långvarig L-DOPA-behandling påverka flera signalsystem i hjärnan och därigenom bidra till uppkomst av dyskinesier.
This thesis is based on the following papers, which are referred to in the text by their roman numerals:


II. Nevalainen N., af Bjerkén S., Gerhardt G. A., Strömberg I. The role of serotonergic nerve fibers in conversion of L-DOPA to dopamine. *Manuscript*


INTRODUCTION

The neurotransmitter dopamine

Dopamine is synthesized by hydroxylation of the amino acid tyrosine and decarboxylation of L-DOPA, and was initially believed to merely be a precursor to noradrenaline and adrenaline (Fig. 1). In the 1950’s it was demonstrated to be an independent transmitter with a specific distribution within the brain separated from noradrenaline (Carlsson et al. 1958). Today it is well known to be critical for several functions such as motivation, memory, learning, reward and movement control, and that disturbances within the dopaminergic systems of CNS is implicated in disorders and psychological conditions such as Parkinson’s disease (PD), schizophrenia, attention deficit hyperactivity disorder, and in addiction.

![Figure 1: Synthesis of dopamine, noradrenaline, and adrenaline from the amino acid tyrosine by the enzymes tyrosine hydroxylase (TH), L-aromatic amino acid dopa decarboxylase (AADC), dopamine-β-hydroxylase (DBH), and phenylethanolamine-N-methyltransferase (PNMT).](image)

Dopamine exerts its effect via dopamine D₁ (D₁ and D₃)- and D₂ (D₂, D₃, D₄)-like receptors (Kebabian & Calne 1979, Missale et al. 1998). The receptors are G-protein coupled slow-acting receptors and found abundantly in the striatum on MSNs, and in limbic (ventral striatum, septum, amygdala, hippocampus) and cortical (prefrontal, motor, cingulate) areas. In the striatum, the binding of dopamine to D₁ receptors excites MSNs via
activation of adenyl cyclase and phosphorylation of DARPP-32, while activation of D$_2$ receptor hyperpolarizes MSNs via inhibition of adenyl cyclase and/or increasing Ca$^{2+}$ content within the cell, leading to dephosphorylation of the DARPP-32 protein (reviewed in (Greengard et al. 1999)). Moreover, D$_2$ receptors are also located on the dopamine neurons themselves as autoreceptors, and upon increased extracellular dopamine levels, D$_2$ autoreceptor activation leads to inhibition of dopamine release and synthesis via tyrosine hydroxylase inactivation, and decreased neuronal activity. Dopamine is cleared from the extracellular space by reuptake into the presynaptic terminal via DAT or degraded via MAO and COMT into the main metabolites DOPAC and homovanillic acid.

The nigrostriatal dopamine system
The majority of the dopaminergic neurons in CNS are located in the mesencephalon. The neurons are found in three nuclei: the retrorubal area (A8), the substantia nigra (A9), and the ventral tegmental area (A10; (Dahlström & Fuxe 1964)). These areas give rise to the mesolimbic and mesocortical (A8 and A10) and mesostriatal (nigrostriatal, A9) pathways. The nigrostriatal system primarily innervates dorsal striatum (caudatus and putamen), an area that control motor behavior (Ungerstedt 1971b, Björklund & Lindvall 1984).

During early development midbrain dopamine neurons are generated in the proliferative zone, with peak ontogeny previously considered at days E13-15 in rat (Hanaway et al. 1971, Altman & Bayer 1981), but might already occur at E12 according to more recent data (Gates et al. 2006). This is also around the date when they initially express TH (Foster et al. 1988, Di Porzio et al. 1990, Shults et al. 1990). Dopamine can be visualized approximately one day after the expression of TH (Olson & Seiger 1972, Voorn et al. 1988). Following, the neurons migrate towards their final location in the VM where
they are settled around E18 (Hanaway et al. 1971, Specht et al. 1981, Voorn et al. 1988, Shults et al. 1990). When at their final position, the nigral neurons form axons and innervate the striatal anlage LGE, where dopamine nerve fibers can be detected already at E14 in rat (Olson & Seiger 1972, Seiger & Olson 1973, Voorn et al. 1988) although the innervation is not complete until postnatally. The innervation occurs along the lateral borders of the developing striatum and then in patchy dopamine-dense islands (Olson & Seiger 1972, Voorn et al. 1988) that overlaps with developing striosomes (Graybiel 1984, van der Kooy & Fishell 1987). Synapses are formed during late prenatal and early postnatal stages. A portion of the dopamine cells becomes apoptotic after birth, resulting in a refined and mature dopamine system at postnatal day 21 in rat (Voorn et al. 1988, Kalsbeek et al. 1992).

To roughly distinguish between A9 and A10 dopamine neurons, which is desirable in developmental studies since dissections of the VM include both types of neurons, a few markers are available. A9 dopamine neurons are typically identified by co-expression of TH with ALDH1 and GIRK2, whereas the A10 neurons, can be distinguished by co-expression of TH and calbindin (Gerfen et al. 1987a, Gerfen et al. 1987b, German & Liang 1993, McCaffery & Drager 1994, Haque et al. 1997, Schein et al. 1998).

**Basal ganglia**

The basal ganglia are a subcortical system of interconnected nuclei that regulate extrapyramidal motor control via cortical activation and inhibition. The system constitutes of four nuclei: the substantia nigra (pars compacta and pars reticulata), the striatum (nucleus caudatus and putamen), the subthalamic nucleus, and the globus pallidus (internal and external segments), where the striatum is the main input station, and substantia nigra pars reticulata along with globus pallidus interna the main output stations.
According to the classical basal ganglia model, which describes the functional anatomy and circuitry of the system (Albin et al. 1989, DeLong 1990), the striatal activity is regulated by glutamatergic input from cortex and dopaminergic input from substantia nigra pars compacta. Although, it should be noted that the basal ganglia contain a wide variety of neurotransmitters and modulators, and therefore, the striatal activity is in reality controlled by several additional actors than dopamine and glutamate.

The signaling is mediated via receptors located on the MSNs, which are the major striatal neuronal population (90-95%) and utilize GABA as neurotransmitter (Gerfen & Wilson 1996). The MSNs are segregated by the expression of different dopamine receptors and opioid peptides and thereby forms two separate ascending pathways: (i) the direct striatonigral expressing D₁ receptors, dynorphin, and substance P, and (ii) the indirect striatopallidal pathway expressing D₂ receptors, enkephalin, and neurotensin (Gerfen et al. 1990, Gerfen & Wilson 1996). Dopaminergic activation of the two separate pathways balances the net activation of the basal ganglia output nuclei and the cortical activation via thalamus. In further detail, dopamine binding to D₁ receptors in the direct pathway activates MSNs and promotes movement by disinhibiting thalamocortical projections via attenuated GABA release in thalamus, whereas dopamine binding to the D₂ receptors of the indirect pathway inhibits MSNs and suppress or terminate movements by inhibiting thalamocortical activity (Fig. 2). Although the model is considered to be correct, it is complicated by observations of co-expression of both types of dopamine receptors on a subset of MSNs (Surmeier et al. 1992, Lester et al. 1993, Aizman et al. 2000).
Parkinson’s disease

PD is a neurological disorder characterised by progressive degeneration of the dopamine neurons in the substantia nigra pars compacta, with the primary outcome motor dysfunction. The motor symptoms of the disease was formally described for the first time in 1817 by the physician James Parkinson (Parkinson 1817), whereas the correlation of the motor symptoms and death of nigral neurons was established about a hundred years later (Trétiakoff 1919). Postmortem studies thereafter concluded that dopamine levels are reduced in the basal ganglia of patients (Ehringer & Hornykiewicz 1960) and that the degree of nigral degeneration parallel with the reduction of striatal dopamine levels (Bernheimer et al. 1973). The prevalence of the disease is approximately 1-2% of the population over 60 years of age, and thus qualifying as the second most common neurological disorder (Tanner 1996, Langston 1998, Shastry 2001). Motor symptoms manifest when more than 50% of the dopamine neurons have degenerated or striatal dopamine levels.
levels have been reduced with 80% (Bernheimer et al. 1973, Kish et al. 1988, Agid 1991), consequently, diagnosis is often set late in the disease progression. The cardinal symptoms are bradykinesia, tremor at rest, akinesia, muscular rigidity, flexed posture and loss of postural reflexes. The symptoms are initially often asymmetrical with one side of the body being more affected, although as the disease progresses, symptoms are seen bilaterally. One characteristic postmortem finding in PD patients is cytoplasmic inclusions referred to as Lewy bodies (Lewy 1912, Trétiakoff 1919).

Hypokinesia and akinesia is predicted by the basal ganglia model, as lost striatal dopamine input results in disinhibition of the indirect pathway and lost activation of the direct pathway (Fig. 2), with the outcome of decreased cortical activation. Nevertheless, striatal dopamine depletion does not cover the wide range of motor symptoms found in PD patients, tremor and rigidity being two examples. These symptoms could instead arise from the parallel degenerating processes found in several other neuronal systems. Indeed, the pathology of PD has been reassessed over the years, and is today known to include loss of the serotonergic neurons of the raphe nuclei (Hornykiewicz 1975, Jellinger & Paulus 1992, Kish et al. 2008), the noradrenergic neurons of locus coeruleus (Mann & Yates 1983, Braak et al. 2003, Zarow et al. 2003), and the cholinergic neurons of nucleus basalis (Nakano & Hirano 1984). The broad array of neuronal structures affected could serve explanatory to the non-motor symptoms, such as cognitive decline, dementia, and depression (reviewed in (Fahn 2003)).

**Disease etiology**

Loss of dopamine neurons and receptors is part of normal aging. Reports testify that a neuronal reduction of 36-48% occurs between ages 20-90
(Hirai 1968, Mann et al. 1984), that striatal dopamine levels are 50-60% reduced between ages 14-92 (Kish et al. 1992), and that approximately 10% of pre- and post-synaptic dopaminergic markers are lost per decade between young and old adulthood (reviewed in (Backman et al. 2006)). Thus, increased age is a strong risk factor for developing PD, although the neuronal cell death is accelerated in PD due to unknown causes and the pattern of degeneration deviates from normal aging and is more pronounced in putamen compared to caudate nucleus (Nyberg et al. 1983, Kish et al. 1988, Kish et al. 1992).

Parkinsonism is a collective term for several neurodegenerative states in which at least two of the cardinal symptoms of PD are expressed. It is divided into primary and secondary parkinsonism, the Parkinson plus syndromes, and heredodegenerative disease. Primary parkinsonism i.e. PD has an unknown or a genetic etiology, and is thus separated from secondary parkinsonism that has a known origin, typically from trauma to the brain from injuries, stroke, and toxins. Parkinson plus syndromes are characterized by extended neurodegeneration affecting several brain areas, whereas heredodegenerative diseases are inherited neurological conditions that give rise to symptoms resembling of PD. Idiopathic PD can be distinguished from the other forms of parkinsonism by characteristics such as unsymmetric onset of symptoms, presence of at least two of the cardinal symptoms with bradykinesia and/or tremor being one of them, and symptomatic relief from L-DOPA treatment (Fahn 2003).

Even though the origin of idiopathic PD remains unclear, several risk factors have been identified and correlated with the upcoming of the disease. A genetic factor exists, although it is estimated to give rise to only a few percentages of the total number of PD cases and seems not to play a major
role in causing typical PD, but might, on the other hand, be a critical factor in cases with an early disease onset (Olanow & Tatton 1999, Tanner et al. 1999, Wirdefeldt et al. 2004). Nevertheless, mutations in several genes such as α-synuclein, ubiquitin, and parkin have been found in both familial and sporadic cases of PD, and could result in impaired protein degradation and buildup of misfolded proteins (reviewed in (Fahn 2003, Farrer 2006, Olanow 2007)). In accordance, the main content of Lewy bodies that are found in the remaining dopamine neurons of patients is aggregated α-synuclein (Spillantini & Goedert 2000). Aside from proteolytic stress, factors such as excitotoxicity, oxidative stress, mitochondrial dysfunction, and inflammatory changes in the nigra might be crucial and could result in nigral cell death (reviewed in (Olanow 2007)). One interesting theory suggests that the PD pathology begins in the enteric nervous system and progresses via the vagus nerve to the brain stem and its nuclei (Braak et al. 2004). Indeed, several brainstem nuclei degenerate during the disease progression, and in addition, α-synuclein-positive inclusions have also been found in the intestinal submucosa (Braak et al. 2006, Shannon et al. 2012). Furthermore, the discovery of Lewy bodies in a subset of dopaminergic grafts from transplanted patients has raised the possibility of PD having a prion-disease-like feature (Kordower et al. 2008, Li et al. 2008).

The 6-OHDA hemiparkinsonian rat model
6-OHDA is a hydroxylated dopamine analogue (Blum et al. 2001), and was found to exert specific neurotoxic effects in catecholaminergic neurons (Tranzer & Thoenen 1968). These findings lead to development of an animal model of PD (Ungerstedt 1968, Ungerstedt & Arbuthnott 1970). Like dopamine, 6-OHDA is not able to cross the BBB, and is therefore injected into the brain. When in the brain, it can be uptaken by catecholaminergic transporters, and causes oxidative damage, mitochondrial failure, and
ultimately neuoronal death via apoptosis (Cohen & Heikkila 1974, Graham et al. 1978, Choi et al. 1999, Lotharius et al. 1999). The lesion is performed unilaterally, and the model is hence referred to as a hemiparkinsonian model (Perese et al. 1989). To produce this dopamine degeneration, 6-OHDA can be injected into the substantia nigra, the MFB, or into the striatum (Perese et al. 1989, Sauer & Oertel 1994, Przedborski et al. 1995). Injection into the nigra and MFB produces severe, rapid lesions (Faull & Laverty 1969, Hökfelt & Ungerstedt 1969), whereas striatal injections results in partial, slowly progressing lesions that are typically ongoing during weeks (Berger et al. 1991, Sauer & Oertel 1994, Przedborski et al. 1995). Upon severe dopamine depletion, dopamine D_2 receptors become supersensitive, a state that can be taken advantage of in order to behaviorally determine the lesion extent (Ungerstedt 1971a). This is performed with administration of amphetamine or apomorphine. The amphetamine approach serves as an indirect indicator, and releases dopamine in the intact striatum, giving rise to overactivation within this hemisphere that results in the animals rotating in an ipsilateral direction to the lesioned hemisphere (Ungerstedt & Arbuthnott 1970, Ungerstedt 1971a, Schwarting & Huston 1996). In opposite, when utilizing the dopamine D_1/D_2 agonist apomorphine, the low doses applied will activate the supersensitive dopamine receptors in the lesioned hemisphere, whereas having minimal effect in the intact hemisphere, and thus animals starts to rotate in a contralateral direction to the lesioned hemisphere (Herrera-Marschitz & Ungerstedt 1984). Depending on the number of rotations performed by the animals, conclusions can be made regarding the status of the lesion. Apomorphine might be preferable as a predictor of severe lesions, since animals do not rotate in response to this drug when submaximally denervated, whereas amphetamine induces rotational behavior in animals with partial lesions (Hudson et al. 1993) and
is poorly correlated to the number of TH-positive neurons in the denervated hemisphere (Tronci et al. 2012).

**Treatment of Parkinson’s disease**

At the present, there is no curing treatment available. Therapeutic strategies are focused on increasing striatal dopamine levels and thereby achieving alleviation of motor symptoms. Dopamine agonists can be administered to stimulate postsynaptic dopamine receptors and thereby increase motor activity. Unfortunately treatment with dopamine agonists is suboptimal due to low efficacy and appearance of prominent side effects, such as confusion and psychosis. MAO- and COMT-inhibitors can be provided to prolong dopamine life cycle in the synaptic cleft, and muscle-relaxing agents can be administered to counteract the rigidity in muscles. Severe cases with prominent motor disability can be relieved with the surgical procedure deep brain stimulation, where an electrode is inserted into the subthalamic nucleus, the globus pallidus, or the thalamus, depending on the features of the motor symptoms. The targeted nucleus is inactivated by high-frequency stimulation, thus causing a reversible lesion and balancing the basal ganglia networks. Although this approach often is very successful, it is limited due to cost-factors, its invasive character, and unsuitability to all patients.

**L-DOPA-treatment**

L-DOPA is the precursor of dopamine and is given to patients in order to increase the striatal dopamine content, since it, unlike dopamine itself, can cross the BBB and thereafter be converted to dopamine in the brain. In the late 1950s, it was demonstrated that motor deficiencies result when dopamine levels are attenuated in the striatum, but can be counteracted by L-DOPA administration (Carlsson et al. 1957, Ehringer & Hornykiewicz 1960). The poor results from the first clinical trials (Barbeau 1961, Birkmayer & Hornykiewicz 1961) were improved when using the L-form of
DOPA, rather than the racemic D,L-DOPA, at higher doses that were introduced in a stepwise manner (Cotzias 	extit{et al.} 1967, Cotzias 1968) and combined with peripheral carboxylase inhibitors such as benserazide or carbidopa (Bartholini & Pletscher 1968, Tissot 	extit{et al.} 1969, Dunner 	extit{et al.} 1971). The addition of carboxylase inhibitors increased the CNS availability of L-DOPA 10-fold (Cederbaum 1987), compared to when administered alone and merely 1% reaches the brain (Nutt & Fellman 1984, Jankovic 2002). Ever since, L-DOPA has become the gold standard treatment of the motor symptoms in PD. During the initial period of L-DOPA-treatment, high efficacy and restored motor function is achieved, but unfortunately, the efficacy is reduced upon long-term use (Granérus 1978). In addition, side effects emerge in the majority of patients, which manifest as motor fluctuations and involuntary movements referred to as L-DOPA-induced dyskineisa (LID). It has been established that 40-60% of the treated patients develop LID within 4 years of L-DOPA treatment and 90% after 9-15 years of treatment (Ahlskog & Muenter 2001). Despite the high incidence of LID, L-DOPA treatment is still the most successful treatment of PD, and therefore highlights the importance of identifying the key biological mechanism(s) in order to improve treatment and avoid side effects.


dopamine grafts

The most desireable approach when it comes to treatment of PD would be to restore the nigrostriatal pathway. With this goal in mind, preclinical studies were performed in which dopamine-depleted rats received fetal mesencephalic dopamine grafts into the striatum, resulting in motor recovery (Björklund & Stenevi 1979, Perlow 	extit{et al.} 1979). In addition, an organotypic innervation pattern of the striatum (Björklund 	extit{et al.} 1981, Strömberg 	extit{et al.} 1992), long-term graft survival (Freed 	extit{et al.} 1980, Strömberg & Bickford 1996), as well as formation of new dopamine synapses and restored dopamine release was reported (Mahalik 	extit{et al.} 1985, Rose 	extit{et al.} 1985,
Zetterström et al. 1986, Bolam et al. 1987, Clarke et al. 1988, Strömberg et al. 1988). As a result of the promising findings, grafting trials were initiated in patients with PD (Lindvall et al. 1988, Madrazo et al. 1988). In these trials, fetal mesencephalic dopaminergic grafts were transplanted into the striatum of patients and the outcome was successful in a subset of cases in terms of symptomatic improvement, long-term graft survival, striatal reinnervation, and functional integration of grafts (Lindvall et al. 1990, Freed et al. 1992, Kordower et al. 1995, Wenning et al. 1997, Kordower et al. 1998, Hagell et al. 1999, Piccini et al. 1999, Cochen et al. 2003). Some of the patients recovered to such an extent that L-DOPA was no longer required. Nonetheless, the outcome was variable and some patients experienced only marginal or no symptomatic relief (Freed et al. 2001, Olanow et al. 2003). In addition, prominent side effects emerged, referred to as graft-induced dyskinesia (Freed et al. 2001, Hagell et al. 2002). Identification of the detrimental factors has been difficult in retrospect since the trials were performed at various clinics and with different protocols used. Some important key factors for the poor outcome have been attributed to low cell survival and hampered innervation of the host striatum, as well as graft characteristics, immunosupression, disease progression, and pregrafting LID (Barker et al. 1996, Brundin & Bjorklund 1998, Björklund et al. 2003, Lane et al. 2010, Lane & Smith 2010). Despite the negative outcome, the high response found in several patients is considered proof-of-concept and therefore patients are being recruited for a new transplantation trial (TRANSEURO) funded by the European Union. This trial aims at achieving successful grafting in carefully selected patients in order to set up a template protocol for future use.
Glial cell line-derived neurotrophic factor (GDNF)

The neurotrophic factor GDNF is a member of the transforming growth factor-β superfamily, along with other members such as neurturin (Kotzbauer et al. 1996), artemin (Baloh et al. 1998), and persephin (Milbrandt et al. 1998). As the name implies, GDNF was derived from glial cells and is expressed by astrocytes, and also by target cells. It was purified and cloned by Lin and colleagues in 1993 and was demonstrated to promote mesencephalic dopamine cell survival in vitro (Lin et al. 1993). GDNF is produced in the striatum and can be retrogradely transported to the mesencephalic dopamine neurons (Strömberg et al. 1993, Tomac et al. 1995b), consequently, the GDNF protein and mRNA are found in the striatum, whilst the receptors are localized on the nigral neurons (Trupp et al. 1997). It excerts its action via GDNF-family receptor-α1 (GFRα1) receptors, which after binding to GDNF, forms a complex with the transmembrane protein receptor tyrosine kinase (RET; (Durbec et al. 1996, Buj-Bello et al. 1997)), although GDNF might not initiate the RET-GFRα1-formation, but rather bind into pre-assembled complexes (Eketjall et al. 1999). GDNF could also exert signaling via GFRα1 alone (Poteryaev et al. 1999, Trupp et al. 1999), or via GFRα1 and neural cell adhesion molecule (NCAM; (Paratcha et al. 2003)). Via different intracellular signaling cascades, GDNF promotes cell survival, proliferation, neuritogenesis, and enhancement of neurotransmission (reviewed in (Takahashi 2001, Airaksinen & Saarma 2002)).

The neurorestorative and neuroprotective potential of GDNF has been extensively demonstrated in vivo, resulting in numerous reports describing its abilities in promoting dopaminergic cell survival (Strömberg et al. 1993, Hoffer et al. 1994, Beck et al. 1995, Tomac et al. 1995a, Kirik et al. 2000, Georgievska et al. 2002), and in increasing dopamine graft survival and
axonal outgrowth (Rosenblad et al. 1996, Sinclair et al. 1996, Granholm et al. 1997, Yurek 1998) in animal models of PD. In addition, it is essential for development of peripheral organs, and GDNF knockout mice fail to develop kidneys, ureters, and the enteric nervous system and therefore die during the first postnatal day (Moore et al. 1996, Pichel et al. 1996, Sánchez et al. 1996). Further on, decreased levels have been shown to make dopamine neurons more vulnerable to drug-induced toxicity, and to accelerate dopamine cell death and deterioration of motor function during aging studies (Boger et al. 2006, Boger et al. 2007, Boger et al. 2008). Apart from shielding the dopamine neurons, GDNF also enhances dopamine release (Hebert et al. 1996, Xu & Dluzen 2000), a mechanism that could take place due to increased formation of dopaminergic nerve terminals (Bourque & Trudeau 2000). Due to its life-promoting and nurturing effects on dopamine neurons, it has been evaluated for clinical use. When infusing GDNF directly into the putamen of PD patients, sprouting of remaining dopamine nerve terminals, increased $[^{18}\text{F}]-$dopamine uptake, and increased motor activity was achieved (Gill et al. 2003, Love et al. 2005, Slevin et al. 2005), although, another study reported lack of efficacy and adverse events such as formation of antibodies against GDNF (Lang et al. 2006). Taken together, GDNF could be useful to retard the progression of PD and in combination with dopamine grafts, but more effort is required to optimize administration, dosage, and benefit.

**1-DOPA-induced dyskinesia**

LID manifests as choreic and dystonic movements that can affect any skeletal muscle group, but are often most severe in the upper body. The movements are involuntary and therefore disabling for the patients from a functional and social aspect. LID is often seen when L-DOPA dose is high and hence referred to as peak dose dyskinesia that occurs typically at 40-100
minutes after intake of L-DOPA (reviewed in (Fahn 2000)). Dyskinesia can also be seen when L-DOPA concentration is low or when levels are rising and falling and is then referred to as off or biphasic dyskinesia (reviewed in (Giron & Koller 1996)). Depending of the type of LID, symptoms may manifest differently. Peak dose dyskinesia typically affects the upper limbs, the jaw and tongue, and trunk, whereas off- and biphasic dyskinesia mainly affect the lower limbs. The upcoming of dyskinesia is still elusive, although several years of effort in this issue have lead to some important conclusions: (i) LID is observed when dopamine depletion is severe (Winkler et al. 2002, Carlsson et al. 2009), (ii) L-DOPA itself does not cause dyskinesia but requires conversion to dopamine (Buck & Ferger 2008), (iii) overactivity of the dopamine D1-receptor-mediated direct pathway is correlated to expression of LID (Aubert et al. 2005, Santini et al. 2007, Westin et al. 2007, Darmopil et al. 2009, Mela et al. 2012), and (iv) the involvement of several neurotransmitter systems is evident (Brotchie 2005).

**Risk factors**

Three main risk factors for developing LID have been identified: young age at disease onset, disease severity, and high doses of L-DOPA (Schrag & Quinn 2000). Moreover, preclinical studies have indicated pulsatile stimulation of dopamine receptors to be a critical factor for development of LID (Jenner 2000). In support of this, long-acting dopamine agonists are less prone than L-DOPA to give rise to dyskinesia, and in addition, administration of L-DOPA in a continuous fashion reduces the risk of LID (Poewe 1998, Olanow et al. 2000, Albin & Frey 2003, Nyholm & Aquilonius 2004). When it comes to treatment with dopamine agonists, the tolerability and efficacy is less than for L-DOPA, and it is therefore not preferred. One exception is early-onset PD, a group of patients in which L-
DOPA treatment is postponed as long as possible since young age is one of the main risk factors for LID.

Animal models

In order to study the neurochemical alteration during LID, animal models that mimic peak-dose dyskinesia have been developed (Cenci et al. 1998, Lundblad et al. 2002, Steece-Collier et al. 2003). In the rat model of LID, severely dopamine-lesioned animals are given daily L-DOPA injections until stable status of dyskinesia is seen, typically after a couple of weeks. As in patients, the severity of LID is increased with time. Due to the unilateral lesion approach, dyskinetic behavior is only present in one side of the body i.e. contralaterally to the lesioned hemisphere.

Involvement of non-dopaminergic systems in L-DOPA-induced dyskinesia

Several neurotransmitter systems have been indicated to contribute to the upcoming of LID, the serotoninergic and glutamatergic being two of the most investigated. The striatum is innervated by serotoninergic nerve fibers originating from the serotonin neurons in the dorsal raphe nucleus and receives glutamatergic afferents from the cortex (Dahlström & Fuxe 1964, Steinbusch 1981, Gerfen 1992). Serotonergic neurons exert their action (mainly inhibitory) via numerous heterogenous subtypes of 5-HT receptors, several of them located in the basal ganglia (Barnes & Sharp 1999). Glutamate is the main excitatory neurotransmitter in the CNS, and exerts its effects via ionotrophic NMDA, AMPA, and kainate receptors and metabotrophic glutamate receptors.

In similarity with the dopaminergic neurons, serotoninergic neurons express the enzyme AADC (Arai et al. 1996) and the transporter protein VMAT (Nirenberg et al. 1995, Peter et al. 1995), thus enabling conversion of L-DOPA and storage of dopamine in serotoninergic neurons. In support,
dopamine has been localised in serotonergic nerve endings, and L-DOPA-derived dopamine is indicated to originate from the serotonergic neurons in the dopamine-depleted striatum (Hollister et al. 1979, Arai et al. 1995, Tanaka et al. 1999, Maeda et al. 2005, Yamada et al. 2007). It is suggested that dopamine, derived from serotonergic neurons, might give rise to LID due to lack of dopamine-regulating mechanisms on the serotonergic neurons, with the consequence of fluctuating dopamine levels and pulsatile postsynaptic stimulation and LID (Cenci & Lundblad 2006, Cenci & Lindgren 2007, Carta et al. 2010). A strong indicator for this view is that LID was attenuated when silencing the serotonin system, either by lesion or activation of 5-HT1A autoreceptors (Carta et al. 2007, Munoz et al. 2008, Eskow et al. 2009).

Increased corticostriatal signaling and receptor activation has been associated with LID in animals and patients (Chase & Oh 2000, Calon et al. 2003, Robelet et al. 2004, Silverdale et al. 2010, Ahmed et al. 2011), and could be detrimental to motor performance by creating imbalance between striatal output signalling pathways. Indeed, D1-receptor mediated signaling is increased in LID (reviewed in (Cenci & Konradi 2010)). In addition, it could contribute to loss of dendritic spines on MSNs (Garcia et al. 2010), which are critical for striatal synaptic connections, and when preserved have been correlated to alleviation of LID in animals receiving dopaminergic grafts (Soderstrom et al. 2010).

Antidyskinetic treatment strategies
There are methods available to prolong the onset of LID, for example by postponing initiation of L-DOPA treatment and instead utilize dopamine agonists, or by administration of L-DOPA in a continuous manner via subcutaneous or intraduodenal routes, as stated in the previous section.
above. Moreover, deep brain stimulation can be performed to alleviate dyskinesia and has indeed high success rate when targeting the subthalamic nucleus or globus pallidus (Benabid et al. 1998, Kumar et al. 1998a, Kumar et al. 1998b). Nevertheless the invasive, costly, and symptom-dependent characteristics prevent it from being applied in all patients. When it comes to antidyskinetic drugs, the NMDA receptor antagonist amantadine is the single clinically available drug at the moment and provides modest (40-60%) alleviation of LID (Verhagen Metman et al. 1998, Luginger et al. 2000, Crosby et al. 2003). 5-HT agonists have demonstrated promising results in alleviating LID in animal models and in patients (Bonifati et al. 1994, Bibbiani et al. 2001, Olanow et al. 2004, Bara-Jimenez et al. 2005, Gregoire et al. 2009), but are still under investigation due to challenges such as achieving high efficacy without prominent side effects, before suitable for future clinical use. Overall, understanding the underlying mechanisms for development of LID is required in order to prevent and effectively suppress dyskinetic behavior and optimize L-DOPA treatment, which despite its shortcomings, still provides the best relief of motor symptoms in PD.
SPECIFIC AIMS

- To investigate the role of serotonergic neurons in L-DOPA conversion and dopamine release in the dopamine-depleted (Paper I) and normal striatum (Paper II)

- To study dopamine release in dyskinetic animals (Paper I)

- Evaluate the effects of L-DOPA on serotonergic nerve fiber density (Papers I and II)

- Quantify striatal basal glutamate concentration and KCl-evoked glutamate release in dyskinetic animals (Paper III)

- Study serotonergic 5-HT$_{1A}$ receptor-mediated effects on KCl-evoked glutamate release and in SSRI-induced inhibition of dyskinetic behavior (Papers II and III)

- Assess effects of decreased and absent GDNF expression on nigral and striatal tissue with focus on neuronal survival and axonal innervation pattern (Paper IV)
MATERIALS AND METHODS

Animals
Female Sprague Dawley rats (B&K, Sollentuna, Sverige or Taconic Inc., Denmark) and GDNF gene-deleted mice on B6/C57/Bl background (Pichel et al. 1996) were used in the studies of this thesis. All animals were housed under a 12:12 h light-dark cycle and provided with food and water ad libitum. Handling of animals occurred according to internationally accepted guidelines and all experiments had been approved by the local ethics committee.

Chemicals
Information regarding retailers for the chemicals used is stated in original Papers I-IV.

Dopamine lesions
In order to perform severe unilateral dopamine lesions, the right MFB was targeted with a single injection of the neurotoxin 6-OHDA in young rats, weighing approx. 150 g. When performing the procedure, animals were anesthetized with 4% isoflurane, which was then gradually stepped down to approximately 2% to maintain surgical anesthesia. The animals were placed in a stereotaxic frame with the toothbar set at -2.3 mm. An incision was made in the scalp and a hole was drilled in the skull so that a stereotaxic injection could be performed at coordinates anterior-posterior (AP): -4.4 and medial-lateral (ML): -1.2 in mm, calculated from bregma. The needle was lowered -7.8 mm below the dural surface and 8 µg of 6-OHDA (2 µg/µl in 0.9% sodium chloride containing 0.02% ascorbic acid) was administered at a rate of 1 µl/min. The needle was left in place for additional 3 min prior to retraction.
Serotonin lesions
The neurotoxin 5,7-DHT was administered into the right lateral ventricle in order to produce serotonergic lesions. After inducing anesthesia with isoflurane, placing the animal in a stereotaxic frame, and drilling a hole in the skull, the animals received a 5,7-DHT injection into the right lateral ventricle at coordinates AP: +0.5, ML: -1.2, and DV: -4.5 mm with tooth bar set at -2.3 mm for young animals (150-200 grams) and -3.3 mm for older animals (>200 gram). Each animal received 100 µg 5,7-DHT (10 µg/µl in 0.9% sodium chloride containing 0.02% ascorbic acid) at an injection rate of 1 µl/minute. After the neurotoxin injection, the syringe was left in the brain for 3 min before being withdrawn.

Apomorphine-induced rotational behavior
At two weeks after the 6-OHDA lesion, apomorphine-induced rotational behavior was monitored to screen for severe dopamine lesions. Animals were placed in separate bowls and left for 15 min to adjust before receiving a subcutaneous injection of apomorphine (0.05 mg/kg). Whole turns in a contralateral direction from the denervated striatum were registered during 70 min. Animals rotating 450 turns or more were selected for further studies, as it has been demonstrated to correspond to at least 90% of dopamine depletion (Hudson et al. 1993).

L-DOPA treatment and rating dyskinesia
For the chronic L-DOPA treatment, rats were administered 4 mg/kg L-DOPA in combination with 15 mg/kg benserazide hydrochloride that was dissolved in 0.9% sodium chloride at 2 ml/kg body weight. Control animals received injections of vehicle. In paper II, normal and 5-HT-lesioned animals received daily subcutaneous injections of L-DOPA or vehicle for 14 or 28
days, and the graft recipients in the *in oculo* grafting study received daily intraperitoneal L-DOPA or vehicle injections for 21 days. No behavioral assessments were performed in these animals.

In Papers I-III, severely dopamine-depleted animals were given daily subcutaneous L-DOPA injections for 14 days and screened at days 1, 3, 7, 10, and 14 for expression of AIMs. Ratings were performed in accordance to the validated basic rating scale as previously described (Cenci et al. 1998, Lundblad et al. 2002, Cenci & Lundblad 2007). Briefly, animals were placed in separate cages and evaluated for 1 min every 20th min during 3 h, starting 20 min after the injection. Consequently, each animal was evaluated nine times during a test session. When evaluating, the occurrence of axial, forelimb, and orolingual AIMs were scored from 0 to 4 depending on their severity and persistence (1=occasional, 2=frequent, 3=continuous but interrupted by external distraction, 4=continuous and not interrupted by external distraction), and thus the theoretical total maximum score for one animal during one test session is 108. Animals displaying scores of ≥2 on at least two of the three subtypes of AIMs during one observation were considered as dyskinetic and included in the studies. Those with scores of 0-1 were labeled mildly dyskinetic and excluded. Following 14 days of chronic L-DOPA treatment, rats were given 2-3 injections per week and ratings were performed every 10th day in order to assure stable status of dyskinesia. The mean AIMs scores for dyskinetic animals at their last testing session preceding electrochemical recordings (Papers I and III) or trial with fluoxetine (Paper II) and 8-OHDPAT (Paper III) were 32.80±2.06 in paper I, 57.00±5.64 in paper II, and 38.71±6.89 (amperometric study) and 41.67±5.53 (behavioral study with 8-OHDPAT) in paper III. For animals recruited to the electrochemical studies, a regular dose of L-DOPA was administered two days before recordings.
Electrochemical detection of dopamine and glutamate

In all studies, control recordings were performed where vehicle was ejected instead of L-DOPA to demonstrate that the effects seen after L-DOPA injection are induced by the drug and are not an experimental artifact.

Carbon fiber electrodes for dopamine recordings

In papers I and II, single carbon fiber electrodes (Quanteon, Nicholasville, KY, USA) were utilized for dopamine recordings with high-speed in vivo chronoamperometry. With this recording technique, electrochemically active compounds can be detected, i.e. analytes that can undergo oxidation and reduction. In the striatum, a limited number of analytes are electrochemically active. These constitute mainly by the biogenic amines and ascorbic acid. In order to increase the selectivity for the analyte of interest, here dopamine, and to minimize disturbance from the major interferent, ascorbic acid, a coating procedure with the teflon derivate Nafion was undertaken. The Nafion layer repels anions and is impermeable to ascorbic acid and anionic biogenic amine metabolites, whilst concentrating cations, such as biogenic amines at the electrode surface, thereby resulting in highly selective electrodes for catecholamines (Gerhardt et al. 1984).

Microelectrode arrays for glutamate recordings

Glutamate is a strictly regulated neurotransmitter with a rapid onset and reuptake, and thus requires a technique that enables subsecond detection (Hu et al. 1994, Burmeister & Gerhardt 2001). This task can be achieved with in vivo amperometry, enabling determination of the basal extracellular glutamate levels and KCl-evoked glutamate release. A detailed description of the methodological process of handling electrodes has been published previously (Hascup et al. 2007) and was followed here with minor modification. The recordings were conducted with enzyme-coated ceramic-
based MEAs (Burmeister et al. 2000, Burmeister et al. 2002) of the model S2 (Thin-Film Technologies, Inc., Buellton, CA, USA) to monitor basal glutamate levels and glutamate release. Glutamate itself is not electrochemically active, although it can be indirectly measured upon degradation by L-glutamate oxidase into α-ketoglutarate and hydrogen peroxide ($\text{H}_2\text{O}_2$), with the electrons freed from oxidation of $\text{H}_2\text{O}_2$ as a reporter molecule (Day et al. 2006). For this purpose, MEAs were prepared so that one pair of the recording sites were coated with a solution of L-glutamate oxidase (1%), BSA (1%), and glutaraldehyde (0.125%), and the other pair acted as sentinel sites and were coated with BSA/glutaraldehyde, that is, a protein layer with similar properties as at the recording sites but lacking the ability to record glutamate (Fig. 3). To selectively measure the concentration of glutamate, the current recorded by the sentinel sites is subtracted from the current recorded by the glutamate detecting sites, thus enabling a self-reference recording technique and removal of background noise from interfering molecules (Burmeister & Gerhardt 2001).

**Figure 3:** S2 MEAs have four recording channels, which were coated so that one pair ($1+2$) could record glutamate (Glu) via glutamate oxidase, and the other pair ($3+4$) acted as sentinel sites.
After the protein-coating procedure, electrodes were let to dry in a clean atmosphere for at least 72 h before all four recording sites were electroplated with 1,3-phenylenediamine to block interfering molecules, in particular ascorbic acid, from reaching the recording sites. This coating layer excludes molecules by size, and only small molecules such as H₂O₂ can pass through the layer. The electrodes were utilized for amperometric recordings earliest 24 h after the electroplating procedure.

**Striatal drug delivery - mounting micropipettes to the recording electrode**

In order to apply KCl and L-DOPA at the striatal recording sites during experiments, two glass micropipettes (with tip diameters of 10-15 µm) were mounted to the recording electrode. When mounting the pipettes to the carbon fiber electrode, one pipette was attached at each side of the electrode, and all tips were placed parallel and each micropipette was separated by 130-160 µm from the tip of the electrode. For the MEAs, the pipettes were first attached adjacent to each other, so that tips were in contact, and then attached to the MEA so that the tips were separated 50-100 µm from the electrode surface and placed in a centered position between the four recording sites. The micropipettes were filled with solutions of KCl (70 mM for glutamate recordings and 120 mM for dopamine recordings) and L-DOPA (2.5 mM, except for dopamine recordings in dopamine-lesioned striata where 12.5 mM was used), respectively, and connected to a micropressure system. During recordings, the volume ejected was monitored through a scale fitted in the ocular of an operation microscope. The microelectrodes were kept in PBS (0.05 M) until used for *in vivo* recordings.

**Animal preparation**

Animals were anesthetized with urethane (1.25-1.5 g urethane/kg body weight), tracheotomized to facilitate spontaneous breathing, and placed in a
stereotaxic frame. During the operation procedure and recordings, animals were kept on a heating pad and body temperature was monitored and maintained at ~37°C. Following, the scalp and the bone overlying the striatum was removed either bilaterally (Papers I and III) or over the right striatum (Paper II). An additional burr hole was made caudally for insertion of an Ag/AgCl reference electrode.

In vivo chronoamperometry – detection of dopamine

High speed in vivo chronoamperometry enables monitoring of dopamine release and clearance at a subsecond time resolution (van Horne et al. 1992), and was employed in Papers I and II for quantification of extracellular dopamine concentration increments upon KCl stimulation. Recordings were performed using the FAST-16 system (Quanteon, (Hoffman & Gerhardt 1998)) and Nafion-coated carbon fiber electrodes. When recording, a square wave potential was applied to the system that stepped from 0 to 0.55 V at a rate of 5 Hz, causing oxidation and subsequent reduction of analytes in close proximity of the electrode tip. The FAST software integrates the signal so that an average signal per second is achieved for the oxidation and reduction reactions, respectively. Increased extracellular levels of electrochemically active compounds induce a rapid change in the current recorded by the electrode that is directly proportional to analyte concentration (Gerhardt & Burmeister 2000). Increments in analyte concentration results in a peak formation, in which the highest point corresponds to the maximum extracellular concentration reached (calculated from the baseline), also referred to as the maximum peak amplitude (µM). KCl was applied at the recording site to induce dopamine release from surrounding nerve terminals by disrupting the potassium gradient. From the peak formation, the time for onset ($T_{\text{rise, sec}}$) and reuptake ($T_{50\text{ or }T_{80\text{ sec}}}$) can be calculated. $T_{\text{rise}}$ is defined as the time from the KCl ejection until the peak has reached its peak
amplitude, whereas the reuptake describes the time required for the peak amplitude to decrease to 50% or 20% of its maximum peak value. To determine the identity of the biogenic amine, the reduction/oxidation ratio at the peak maximum is utilized, as it has been demonstrated that dopamine exhibits higher ratios (0.8) than serotonin (0.2, (Strömberg et al. 1991)). The contribution of noradrenaline is expected to be minimal within the striatum, as this area is not a main target of the locus coeruleus neurons.

Preceding in vivo recordings, electrodes were calibrated according to previously described protocols (Gerhardt et al. 1984), and included when recording dopamine in a linear manner ($R^2 > 0.995$), with high selectivity over ascorbic acid (>200:1), and limit of detection <0.05 µM when signal-to-noise ratio was set at 3:1. Electrochemical recordings were performed at stereotaxic coordinates $ML: \pm 2.6$ mm, $AP: 0/1$ mm, and $DV: -3.5, -4.5, and -5.5$ mm in Paper I, and at $ML: -2.6$ mm, $AP: 0/1$ mm, and $DV: -3.0, -3.5, -4.0, and -4.5$ mm in Paper II. At each striatal recording site, KCl (240 nl in Paper I and 100 nl in Paper II) was ejected twice before and twice after the L-DOPA ejection (240 nl in intact and 480 nl in dopamine-lesioned striata in Paper I, and 150 nl in all groups in Paper II) at intervals of 5 min at each site, and the second KCl ejection after the L-DOPA infusion was performed after 10 min. The second KCl stimulation was performed to study recovery capacity of the system. The striatal location for the first measurements was alternated and balanced within each treatment group of each study.

In vivo amperometry – detection of glutamate

Amperometric recordings were preceded by a calibration procedure where the electrode was challenged for detection of glutamate. Electrodes were calibrated in 40 ml PBS (0.05 M, pH=7.4) at 37°C against an Ag/AgCl reference electrode. A constant potential of 0.7 V was applied at 2 Hz using the FAST-16 system and software and the signal was amplified 500x (2
nA/V) via the headstage. Standard solutions of ascorbic acid and glutamic acid were added to the beaker to produce a final concentration of 250 µM of ascorbic acid and 60 µM of glutamic acid, where the latter was achieved by three subsequent 20 µM increments. The change in signal was measured upon each addition and electrodes were included when detecting glutamate selectively over ascorbic acid (>20:1) in a linear manner (R²>0.99), with a limit of detection <2 µM when the signal-to-noise level was set at 3:1. At the end of the calibration, standard solutions of dopamine and H₂O₂ were added to the beaker to produce a final concentration of 2 and 8.8 mM, respectively. This was performed to ensure impermeability of larger molecules and to test electrode sensitivity to the reporter molecule H₂O₂.

Recordings were performed at stereotaxic coordinates (with bregma as the reference) ML:±2.6 mm, AP:0/+1 mm, and DV: -3.5 mm and -4.5 mm below the dural surface. For each site, a stable baseline was acquired before starting the recordings. Basal extracellular glutamate concentration was determined before KCl stimulations and after local L-DOPA administration, respectively, by averaging the subtracted baseline values over 30 sec. To provoke glutamate release, calibrated volumes of KCl (100 nl) was pressure-ejected at the recording sites 4-5 times in 2-min intervals before and after the L-DOPA ejection (100 nl). Upon the L-DOPA ejection, 10 min was let to pass before re-stimulating with KCl. At the end of the recordings in a subset of normal and dopamine-lesioned animals, L-DOPA loading was preceded (5 min) by an injection of the 5-HT₁A receptor agonist 8-OHDPAT (1 mg/kg, s.c.). The first measurement was alternated between hemisphere and striatal position for subjects of each experimental group.
Grafting techniques

In oculo study: creating raphe-striatal microcircuits

The in oculo grafting model (Strömberg 1999) was undertaken to evaluate effects of chronic L-DOPA treatment on serotonergic nerve fiber density. For this purpose, 8 adult rats received a cograft consisting of fetal dorsal raphe nucleus and LGE into each eye. The grafting procedure was performed at two separate occasions and fetal LGE tissue was inserted 6 weeks prior to fetal raphe tissue. For each procedure, tissue was dissected from rat fetuses of E14 and kept in DMEM until inserted into the anterior eye chamber (Fig. 4). When performing the procedures, the recipient rat (~200g) was atropinized, anesthetized with isoflurane, and the tissue to be grafted was inserted into the anterior eye chamber utilizing a glass pipette after making an incision in the cornea with a razor blade. Fetal raphe dorsalis was grafted and placed adjacent to the LGE. Two weeks later, daily injections of L-DOPA (4 mg/kg) or vehicle were given to the graft hosts for 3 weeks. After this time, the tissue was fixed, dissected and stored in sucrose solution (10% sucrose in 0.1 M phosphate buffer with 0.01% sodium azide) until evaluated immunohistochemically.

Intraventricular grafting: creating nigrostriatal microcircuits

GDNF heterozygous mice were mated to obtain fetuses with different GDNF expression, that is, wildtype fetuses with full GDNF expression, heterozygous with reduced (~50%) (Airavaara et al. 2004) GDNF expression, and knockouts lacking the GDNF gene. At E14, fetuses were obtained from the pregnant mouse and VM and LGE were dissected bilaterally and kept in DMEM until transplanted. Meanwhile, wildtype recipient mice were anesthetized with isoflurane and placed in a stereotaxic frame. A burr hole was made in the cranium 0.8 mm mediolaterally from the bregma, and unilateral VM/LGE tissue pieces originating from the same
fetus were inserted using a pull-push cannula into the right lateral ventricle and ejected at -3.5 mm below the dural surface (Fig. 4).

![Image](image-url)

**Figure 4:** VM, LGE, and raphe were dissected from fetuses at embryonic day 14 and grafted into the anterior eye chamber or right lateral ventricle of adult recipients to create raphe-LGE and VM-LGE microcircuits.

During the dissection procedure, tail-tips from the fetuses were collected and later analysed using polymerase chain reaction with primers specific to the GDNF gene and the knockout nonsense DNA sequence, respectively (for a detailed description of the genotyping process, see Paper IV). Expression of each allele was determined by electrophoresis and the presence of a 344 base pair long DNA fragment for the wildtype gene and 255 base pair long for the knockout gene. Heterozygous mice were identified by the presence of both products. The grafts were evaluated at 3, 6, and 12 months postgrafting (see Table 1 for total number of grafts evaluated at each time point).
Table 1: Number of VM/LGE co-grafts at the different time points included in the study of Paper IV.

<table>
<thead>
<tr>
<th>gdnf genotype</th>
<th>3 months</th>
<th>6 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>wildtype</td>
<td>16</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>heterozygous</td>
<td>7</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>knockout</td>
<td>8</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

**Tissue preparation and immunohistochemical evaluation**

For fixation of brain tissue, animals were transcardially perfused with Ca\(^{2+}\)-free Tyrode solution followed by immersion fixation with paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4; Papers I-III) for 48 h, or perfusion of paraformaldehyde through the vascular system followed by postfixation of the brains for 1-2 h (Paper IV). The brains from fetuses of embryonic day 19 (Paper IV) were fixed by placing the whole fetal heads in paraformaldehyde overnight. After fixation, brain tissue was thoroughly rinsed and stored in sucrose solution (10% sucrose in 0.1 M phosphate buffer with 0.01% sodium azide).

*Immunohistochemistry*

Tissue was freeze-dried with gasous carbon dioxide and cut into 14 \(\mu\)m thick sections using a cryostat. The sections were attached to chromealun-gelatin-coated glass slides and placed in PBS (0.1 M) for 15 min. Primary antibodies for visualization of dopaminergic, serotonergic, and striatal neurons, microglia, blood capillaries, and \(\alpha\)-synuclein were used in the studies (for details of the primary antibodies, see Table 2). Alexa Fluor\textsuperscript{®} conjugates (A594 and A488, raised against mouse and rabbit antibodies in goat, diluted 1:500, Molecular Probes Inc., Eugene, OR, USA) were used as secondary antibodies. Sections were incubated with primary antibodies for 48 h at 4°C.
and with secondary antibodies for 1 h at room temperature. All antibodies were diluted in PBS (0.1 M) with 0.3% Triton-X-100, except the SERT antibody in Paper I, where Triton-X-100 was excluded. Instead, the SERT antibody incubation was preceded by an additional incubation with fetal calf serum (10% in 0.3% Triton-X-100 in PBS) for 1 hour at room temperature. Before applying the secondary antibodies, sections were incubated for 15 min in goat serum (5% in 0.1 M PBS) at room temperature. All antibody incubations were performed in a humid chamber and sections were rinsed in PBS (0.1 M; 3x10 min) between incubations. When finished, the sections were cover-slipped in 90% glycerol in PBS.

<table>
<thead>
<tr>
<th><strong>Target protein</strong></th>
<th><strong>Primary antibody</strong></th>
<th><strong>Source</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AADC</strong></td>
<td>rabbit anti-AADC</td>
<td>Millipore AB&lt;br&gt;<strong>Solna, Sweden</strong></td>
</tr>
<tr>
<td><strong>ALDH1</strong></td>
<td>Rabbit anti-ALDH1</td>
<td>Abcam      &lt;br&gt;<strong>Cambridge, UK</strong></td>
</tr>
<tr>
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<td>Mouse anti-calbindin</td>
<td>Sigma      &lt;br&gt;<strong>Saint Louis, MO, USA</strong></td>
</tr>
<tr>
<td><strong>DARPP-32</strong></td>
<td>Rabbit anti-DARPP-32</td>
<td>Cell Signaling Technology,&lt;br&gt;<strong>Danvers, MA, USA</strong></td>
</tr>
<tr>
<td><strong>GIRK2</strong></td>
<td>Rabbit anti-GIRK2</td>
<td>Millipore  &lt;br&gt;<strong>Solna, Sweden</strong></td>
</tr>
<tr>
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<td>Rabbit anti-Glut1</td>
<td>Abcam      &lt;br&gt;<strong>Cambridge, UK</strong></td>
</tr>
<tr>
<td><strong>Iba1</strong></td>
<td>Rabbit anti-Iba1</td>
<td>Wako Pure Chemical Industries, Ltd, &lt;br&gt;<strong>Osaka, Japan</strong></td>
</tr>
<tr>
<td><strong>SERT</strong></td>
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<tr>
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</tr>
<tr>
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<td>Immunostar, Inc. &lt;br&gt;<strong>Hudson, WI, USA</strong></td>
</tr>
<tr>
<td></td>
<td>rabbit anti-TH (1:300)</td>
<td>Pel-Freeze  &lt;br&gt;<strong>Rogers, AR, USA</strong></td>
</tr>
</tbody>
</table>

**Table 2:** Primary antibodies used in Papers I-IV.
Measurement of serotonergic nerve fiber density

SERT-positive nerve fiber density was assessed to evaluate the extent of the serotonergic lesions and effects of L-DOPA treatment in Papers I and II. The estimations were performed at every striatal recordings site in the brains collected from the subjects included in the chronoamperometric studies. In the raphe-striatal intraocular co-grafts, density measurements were performed in the striatal, i.e. DARPP-32-positive, part of the graft for evaluation of serotonergic innervation of the striatal tissue. Images of the area of interest were captured with a digital camera (Jenoptic, Jena, Germany) connected to a fluorescence microscope. Thereafter, all images were made binary and the density measurements were performed in a set frame using the NIH Image software. The density evaluations were expressed as mean grey values.

Dopamine cell counts

TH-positive cells were counted manually in every fourth section throughout each transplant in Paper IV. Since the diameter of a dopamine neuron is ~20 µm and the sections were cut 14 µm thick, each neuron was with high probability only counted once, and the procedure gave an estimation of TH-positive cells survival in transplants derived from GDNF wildtype, heterozygous, and knockout tissue. To estimate the proportions of A9 and A10 neurons in the transplants, the TH-positive neurons that co-expressed GIRK2, ALDH1, and calbindin were counted.

Statistical evaluation

Data were analyzed with one- or two-factor analysis of variance (ANOVA), or repeated measurements ANOVA, followed by Bonferroni post hoc test or contrasts. Student’s t-test was used when only two groups were compared. Two-factor ANOVA was performed when two factors were assessed on the outcome variable and in order to detect possible interaction effects between
these factors. One-way ANOVA was applied when evaluating effects of a single factor within or between groups. The repeated measurements ANOVA was utilized for assessment of AIMs in animals that were rated at several occasions. All data are expressed as mean values±standard error of mean (SEM). The significance level was set at p<0.05.

RESULTS AND DISCUSSION

Quantification of dopamine release using in vivo chronoamperometry

*In vivo* chronoamperometry allows second-by-second monitoring of extracellular concentration of catecholamines upon KCl-evoked release from nerve terminals (described in (Gerhardt & Burmeister 2000)). The KCl ejection disrupts the intracellular/extracellular potassium gradient that normally is higher inside the cells, causing an influx of potassium into the nerve fibers surrounding the recording electrode, and thus, inducing transmitter release as a result. In the striatum, noradrenergic nerve fibers originating from the locus coeruleus are very few, and therefore their contribution can be ignored. Dopamine can be distinguished from serotonin by calculating the reduction/oxidation ratio at the peak maximum, which is typically higher for dopamine (~0.8) than serotonin (~0.2). The amplitude (or maximum concentration) of KCl-evoked dopamine release is calculated with the baseline as a reference. Unfortunately, the baseline itself cannot be interpreted as basal dopamine concentration, since this would require a self-reference system to remove background noise. In the normal striatum, typically increments of around 5 µM were noted when stimulating with KCl (120 mM, 100 or 240 nl). Upon local L-DOPA administration, a robust increase in dopamine release was observed (Fig. 5, and Papers I, II). This was expected, since it is well known that L-DOPA can be converted in dopamine nerve fibers (Hefti *et al.* 1981). Furthermore, the onset and
reuptake can be readily followed upon inducing dopamine release in the normal striatum, and occurs typically around 1-1.5 min. In the dopamine-lesioned striatum, the dopamine peak amplitudes were in the nanomolar range, which complicated reliable measurements of kinetics, and are for this reason not presented for these cases (Paper I).

![Graph](image)

**Figure 5:** KCl-evoked dopamine release is increased in normal striatum when L-DOPA is applied at the recording site. KCl is abbreviated K+.

**Measuring striatal glutamate concentration using in vivo amperometry**

Glutamate concentration can be quantified utilizing *in vivo* amperometry. The recordings are performed with multielectrode arrays that provide a self-reference system and allows determination of extracellular basal levels as well as KCl-evoked glutamate release (Day et al. 2006). Whereas the extracellular levels correspond to the overall levels, provided by neuronal and non-neuronal sources (around 4 µM in normal striatum, Paper III), the KCl-evoked glutamate release indicates concentrations released by the nerve terminals (approx. 10 µM in normal striatum, Paper III). Glutamate is a fast-regulated neurotransmitter (Hu et al. 1994, Burmeister & Gerhardt 2001). Thus, onset and reuptake occurs very rapidly, typically within 10 sec when stimulating with KCl. Due to its high temporal resolution, *in vivo* amperometry is well suited for studying glutamate release. The glutamate
concentration and kinetics vary in accordance to the age of the animal, the area for recordings, animal species, and of course also to the concentration of KCl applied (Nickell et al. 2005, Day et al. 2006, Hascup et al. 2007, Quintero et al. 2007). Furthermore, when stimulating several times at one recording site, similar peak amplitudes are produced even though stimulating in short intervals (here 2 minutes, see Fig. 6).

Figure 6: KCl-evoked glutamate release (µM) in normal striatum. Ejections of KCl in 2-min intervals results in peaks with similar amplitudes. Each KCl ejection is indicated by K+.

Sites for carboxylation of L-DOPA in the dopamine-lesioned striatum

Striatal AADC expression has been correlated to dyskinetic behavior (Bankiewicz et al. 2006), and consequently, inhibition of AADC attenuates dyskinesia (Buck & Ferger 2008). Except for being expressed in the biogenic amines in the brain, AADC is present in non-neuronal cells such glial and endothelial cells (Hardebo et al. 1979, Melamed et al. 1981). To assess the possibility of L-DOPA being converted in striatal neurons, AADC-expression was immunohistochemically evaluated at the striatal recording sites of L-DOPA naïve and dyskinetic dopamine-lesioned animals and compared to normal controls (Paper I). Sparsely scattered AADC-positive neurons were found in the striata of all animals, in consensus with previous reports (Melamed et al. 1980, Mura et al. 1995, Lopez-Real et al.
2003), and have been suggested to be striatal output neurons (Darmopil et al. 2008). Although, due to the fact that similar numbers of AADC-positive neurons were found in L-DOPA naïve and dyskinetic animals, and their sparse distribution in medial portions of the striatum, these neurons might not be the determining factor for the upcoming of LID. It is rather the lateral portions of the rat striatum that have been implicated as the critical area for motor control, especially for tongue and forelimb movements (Pisa 1988).

**L-DOPA-derived dopamine release from serotonergic nerve fibers**

Previous studies have indicated that L-DOPA-derived dopamine release can be derived from serotonergic nerve fibers in the dopamine-depleted striatum (Hollister et al. 1979, Tanaka et al. 1999, Yamada et al. 2007), due to expression of AADC and VMAT-2 (Nirenberg et al. 1995, Peter et al. 1995, Arai et al. 1996).

*In dopamine-lesioned striatum*

The present work confirms that dopamine release can be increased by L-DOPA administration in severely dopamine-depleted animals (Paper I). The dopamine lesion severity (>90%) in these animals was indicated by the profound apomorphine-induced rotational behavior, and strengthened by immunohistochemical evaluations where no or very few sparsely distributed TH-positive nerve fibers were found in the lesioned striatum (Fig. 6, Paper I). The increased dopamine release in dopamine-depleted animals after L-DOPA administration was most likely derived from serotonergic neurons. This is based on findings illustrating that dopamine release was no longer potentiated by L-DOPA administration in serotonin- and dopamine-lesioned animals (Fig. 4, Paper I). In support, decreased L-DOPA-derived dopamine levels following a serotonergic lesion have been demonstrated previously (Hollister et al. 1979, Tanaka et al. 1999, Navailles et al. 2010). Notably,
dopamine release from serotonergic nerve fibers in dopamine-lesioned animals gave rise to small increments in extracellular dopamine concentration (Fig. 2, Paper I). In the dopamine-depleted animals, higher concentrations of L-DOPA (480 nl of 12.5 mM solution) were applied than in normal striatum (240 nl of 2.5 mM solution). Still, very low levels of dopamine release were observed in the denervated striatum (~400 nM) compared to the robust increase in dopamine release observed in normal animals. Another indicator of the poor capacity of serotonergic nerve fibers in enhancing striatal dopamine concentration in the denervated striatum is that no correlation have been found between serotonergic nerve fiber density and dopamine concentration (Lundblad et al. 2009), and is further supported here (Fig. 7). Since 5-HT nerve fiber density increases in a dorsoventral direction within the striatum, one would expect that L-DOPA-derived dopamine release would be more robust at deeper striatal recording sites. This seems not to be the case, especially not in dyskinetic animals.

![Figure 7](image.png)

**Figure 7**: No effects were found from increased serotonergic nerve fiber density on L-DOPA-derived dopamine release when advancing dorsoventrally within the lesioned striatum. Dopamine is abbreviated DA.
In normal striatum

The intraventricular procedure for serotonergic denervation resulted in near complete striatal lesion (Fig. 6e, Paper I; Fig. 5a, Paper II), which was actually seen bilaterally (Paper I). Thus, when injecting 5,7-DHT into the right lateral ventricle, bilateral striatal serotonergic lesions were achieved, probably due to distribution of the neurotoxin via the cerebrospinal fluid to the contralateral hemisphere. Hence, in Paper I, the contralateral (left) striatum of the dopamine-serotonin-lesioned animals was serotonin-depleted but had intact dopamine nerve fibers. When applying L-DOPA into this environment, dopamine release was moderately (statistically non-significantly) increased (Fig. 4a, Paper I). This finding implies that serotonergic nerve fiber integrity might be of importance also for L-DOPA-derived dopamine synthesis in the intact striatum. For this reason, in Paper II, the contribution of serotonergic nerve fibers in L-DOPA-derived dopamine synthesis was evaluated. This task was achieved by comparing KCl-evoked dopamine release provoked before and after local L-DOPA application in normal and serotonin-lesioned animals. It was found that upon a striatal serotonin lesion, L-DOPA-derived dopamine release was decreased with approx. 35% (Fig. 3a, Paper II). Thus, even in normal animals with an intact dopamine system, serotonergic nerve fibers contribute with a significant proportion of dopamine upon L-DOPA administration. This finding suggests that (i) the contribution of serotonergic nerve fibers in converting L-DOPA and releasing dopamine occurs also when dopamine nerve fibers are available, and consequently that (ii) serotonergic nerve fibers probably contribute with L-DOPA-derived dopamine during the whole course of the L-DOPA treatment i.e. not only in severely dopamine-depleted and dyskinetic stages as earlier suggested (Carta et al. 2007), but also when L-DOPA efficacy is high. Removal of serotonergic nerve fibers also affected the dynamics of dopamine reuptake and consisted initially of up-regulated
clearance of dopamine, whereas at later time points clearance was prolonged in the serotonin-lesioned animals, the latter especially in L-DOPA treated animals (Paper II). This could imply effects from loss of the serotonin transporter, which might be involved in clearance of dopamine (Kannari et al. 2006, Larsen et al. 2011). Nevertheless, the serotonergic neurons may still be involved in the development and expression of LID, but possibly via other mechanisms. These could include alterations in serotonergic transmission as a consequence of the uptake of L-DOPA into the raphe neurons. Serving as examples for this, studies have demonstrated that L-DOPA administration alters cerebral serotonin tissue levels, resulting in attenuated levels in striatal and extrastriatal areas (Bartholini et al. 1968, Navailles et al. 2010, Navailles et al. 2011, Eskow Jaunarajs et al. 2012).

Dopamine release in dyskinetic animals
The capacity of the serotonergic nerve fibers to produce dopamine from L-DOPA has lead to the suggestion that this is the primary factor for the upcoming of LID (reviewed in (Cenci & Lundblad 2006, Carta et al. 2010)). Larger swings in dopamine levels have been reported in dyskinetic compared to non-dyskinetic subjects (de la Fuente-Fernandez et al. 2004, Lindgren et al. 2010), thus indicating that the serotonergic neurons would be more prominent in their dopamine synthesis during dyskinetic stages. At odds with these statements, the findings in Paper I, demonstrate the opposite scenario. In dyskinetic animals, no increase in dopamine release was observed after L-DOPA administration, neither in the dopamine-lesioned nor in the intact striatum (Fig. 5, Paper I). Consequently, the dopamine release after acute L-DOPA was significantly lower in dyskinetic than in L-DOPA naïve animals. Re-stimulating a second dopamine release after L-DOPA injection did not always produce a response in the intact striatum of dyskinetic animals. These results could not be assigned differences in
serotonergic nerve fiber density, since dyskinetic animals had similar nerve fiber density as L-DOPA naïve animals (Fig. 6, Paper I). The 6-OHDA-lesioning procedure is known to affect the serotonergic nerve fiber density negatively, but reduced nerve fiber density to a similar extent in L-DOPA naïve dopamine-lesioned and dyskinetic animals.

The basal extracellular dopamine levels have been reported to be increased in dyskinetic subjects (Tedroff et al. 1996, Pavese et al. 2006, Lindgren et al. 2010), and therefore, increased dopamine D₂ autoreceptor activation might be expected in these animals. However, it would not affect dopamine release from serotonin neurons in the dopamine-lesioned striatum. Consequently, as the serotonergic nerve fibers seem to be the main structure responsible for increased dopamine release after L-DOPA administration in severely dopamine-depleted animals, hampered dopamine release in dyskinetic animals is probably due to disadvantages from L-DOPA treatment on the serotonergic neurons. Detrimental effects on dopamine levels from chronic L-DOPA treatment has been reported by other investigators (Lundblad et al. 2009, Navailles et al. 2011) and one possible explanation could be poor L-DOPA conversion. Decreased activity of AADC (King et al. 2011) and several-fold higher L-DOPA levels in the denervated striatum of dyskinetic animals (Carta et al. 2006) certainly witnesses of this. Although high L-DOPA levels per se are most likely not the dyskinesia-inducing factor, because conversion to dopamine is required (Buck & Ferger 2008). Another critical factor for counteracting LID might be to retain normal ratios between serotonin and dopamine levels, in order to balance the output signals from the basal ganglia to cortex. It has been demonstrated that when grafting serotonin neurons alone to the dopamine-depleted striatum, pre-grafting LID was aggravated (Carlsson et al. 2007), whereas if dopamine neurons are included in the grafts, LID is attenuated (Garcia et al. 2012). An increased
serotonin-to-dopamine ratio has also been suggested as an important factor in the upcoming of graft-induced dyskinesia (Politis et al. 2011). Balancing of the two systems could be the mechanism by which serotonergic 5-HT$_{1A}$ agonist can counteract dyskinesia induced by dopamine D$_1$ receptor agonists (Dupre et al. 2011).

**Effects of l-DOPA on serotonergic nerve fiber density**

Effects of L-DOPA treatment on serotonergic nerve fiber density have been investigated previously, although the conclusion is still elusive. Several investigators report increased nerve fiber density in dyskinetic animals (Lundblad et al. 2009, Gil et al. 2010, Rylander et al. 2010b, Zeng et al. 2010) and interpret the findings as the mechanism that would give rise to LID. In Paper I, untreated dopamine-lesioned animals and L-DOPA-treated dyskinetic animals exhibited similar striatal levels of nerve fibers. This was further strengthened by the findings in Paper II, where an in oculo grafting study was undertaken to study whether the density of serotonergic nerve fibers in the striatal part of a raphe-LGE cograft was affected by chronic L-DOPA treatment of graft recipients. The results of this study revealed a trend of lost nerve fiber density after L-DOPA treatment (Fig. 5b, Paper II). In support, data from PD patients revealed no differences between dyskinetic and non-dyskinetic subject (Kish et al. 2008). Interestingly, PD patients have an ongoing serotonin degeneration (Hornykiewicz 1975, Jenner et al. 1983, Kish et al. 2008, Bedard et al. 2011). Since patients in general receive L-DOPA treatment, it is difficult to establish whether the nerve fiber loss is an effect from the disease or the treatment. Furthermore, no effects were found by L-DOPA treatment on serotonergic fiber density in normal or serotonin-lesioned animals receiving chronic L-DOPA treatment for 2 and 4 weeks (Fig. 5a, Paper II). Since the serotonin-lesioned animals had a near complete depletion, possible sprouting effects would have been difficult to distinguish, although it should have been evident in normal animals. The different effects
reported by various investigators could reflect the dose of L-DOPA used and the treatment duration. In the studies declared here, a dose of 4 mg/kg was applied, since it is sufficient to produce dyskinetic behavior.

**Striatal glutamate concentration**

The striatum receives glutamate afferents from cortex that synapse onto the striatal neurons. The corticostriatal glutamate influx can be regulated by dopamine and serotonin, since dopamine D₂ (Bamford et al. 2004) and 5-HT₁A (DeFelipe et al. 2001) receptors are expressed on the glutamate projections. In Paper III, in vivo amperometry was utilized to investigate the effects of dopamine depletion and L-DOPA treatment on striatal glutamate concentration. This task was achieved by utilizing MEAs, which have been demonstrated to create minimal tissue damage and microglia activation (Rutherford et al. 2007).

**Dopamine-lesioned striatum of L-DOPA naïve animals**

The findings in Paper III illustrate that basal extracellular glutamate concentration and KCl-evoked glutamate release was significantly decreased upon a unilateral dopamine lesion, when compared to normal animals. The glutamate drive has been suggested to increase after dopamine denervation due to lost dopaminergic inhibition via D₂ receptors located on the glutamate nerve terminals. In reality, inconsistent data has been provided in this issue and glutamate levels have been reported to be increased, decreased or to remain similar to pre-lesion states after a dopamine depletion (Lindfors & Ungerstedt 1990, Jonkers et al. 2002, Bianchi et al. 2003, Corsi et al. 2003, Robelet et al. 2004, Holmer et al. 2005, Bido et al. 2011). The varying results could have its explanation in the time point chosen for evaluation following dopamine depletion, since glutamate levels are decreased at later time points compared to earlier (Meshul et al. 1999). We chose to perform
the recordings 4-8 months post-lesion, which could explain the findings of this study. The long-term effects of a dopamine lesion are interesting since the parkinsonian patients typically live with their disease for long time periods, even decades, and it is at the later stages that both motor disability as well as dyskinesia are most troublesome and severe. Decreased glutamate signaling would lead to long-term depression of the striatal synapses and could explain the cognitive deficits found in these patients, since aside from motor control, the striatum is also involved in memory and learning. In support, long-term potentiation, as well as long-term depression, has been demonstrated to be impaired upon severe dopamine-depletion (Paille et al. 2010). Moreover, glutamate concentration was attenuated bilaterally in the dopamine-depleted animals, although the dopamine lesion itself was performed in one cerebral hemisphere. Bilateral changes have been reported before, both on glutamate concentration and activity within the basal ganglia (Lindefors & Ungerstedt 1990, Breit et al. 2008), and are most probably due to interhemispheric connections.

**Dyskinetic animals**

After chronic L-DOPA treatment and induction of LID in dopamine-depleted animals, basal glutamate concentration was increased marginally and reached similar levels as in normal controls (Fig. 2, Paper III). Interestingly, L-DOPA treatment did not affect KCl-evoked glutamate release in these animals, and thus, similar concentrations were detected as in L-DOPA naïve animals. The basal glutamate concentration and concentrations released from nerve terminals are related, although the basal extracellular levels reflect the total extracellular glutamate concentration that is derived not only from neuronal but also non-neural sources. It has been of general view that glutamate levels are increased during LID (Robelet et al. 2004), although, a great bulk of the existing literature demonstrate alteration of glutamate receptors upon LID, rather than actual glutamate levels (Oh et al. 1998,
Chase & Oh 2000, Calon et al. 2003, Konradi et al. 2004, Silverdale et al. 2010, Ahmed et al. 2011). These receptor alterations consist largely of changes in subunit composition and mRNA expression, hyperphosphorylation of subunits, and increased receptor binding. Whereas phosphorylation indeed indicates increased activation, increased binding could in fact reflect receptor supersensitivity and indirectly point at decreased glutamate levels. Therefore, to achieve a clear view of the glutamate signaling during development and maintenance of LID, more effort should be put into this issue. One should also keep in mind that glutamate receptors on MSNs might be affected by the altered signaling within the basal ganglia system, and serving as an example of this, striatal dopamine D₁/NMDA receptor complexes were reduced in dyskinetic animals, whereas maintained at similar levels in L-DOPA-treated nondyskinetic animals (Fiorentini et al. 2006). The interaction of these receptors have been demonstrated to be important for trafficking, desensitization, and signaling (Lee et al. 2002, Fiorentini et al. 2003), and might therefore be a critical event in LID.

It has been extensively demonstrated that LID is attenuated upon antagonism of glutamate receptors (Papa & Chase 1996, Blanchet et al. 1998, Verhagen Metman et al. 1998, Konitsiotis et al. 2000, Luginger et al. 2000, Kobylecki et al. 2010, Rylander et al. 2010a), which is a strong indicator of that modulation of glutamate levels are needed in order to achieve alleviation of dyskinesia. In Paper III, it was demonstrated that administration of L-DOPA could decrease glutamate concentration in normal, bilaterally in L-DOPA naïve dopamine-lesioned animals, but not in the dopamine-lesioned striatum of dyskinetic animals (Fig. 2, Paper III). This finding could be interpreted as loss of L-DOPA-induced regulation of glutamate concentration at dyskinetic stages, and could arise due to poor L-DOPA conversion since conversion of L-DOPA into dopamine can reduce glutamate release via dopamine D₂.
receptors. In accordance, it was reported in Paper I that striatal L-DOPA administration increased dopamine release in normal and L-DOPA naïve dopamine-lesioned, but not in dyskinetic animals. Moreover, it has been shown that the NMDA receptor antagonist amantadine increases dopamine levels (Mizoguchi et al. 1994, Arai et al. 2003), and consequently, the antidyskinetic effect of NMDA antagonist might not only be due to antagonistic action on postsynaptic glutamate receptors.

**Antidyskinetic effects from targeting the serotonin system**

When it comes to the involvement of the serotonergic system in generation of LID, the present work has up until now presented and discussed current opinions and perceptions. From our point of view, it is apparent that even though the striatal serotonergic nerve fibers do not seem to give rise to abnormally high dopamine concentrations upon acute L-DOPA loading in the dopamine-lesioned dyskinetic subjects, this system is clearly involved in LID. Therefore, pharmacological approaches were used, such as increasing serotonergic concentration and stimulating and blocking 5-HT$_{1A}$ receptors, in an attempt to identify crucial factors.

**Modulating the extracellular serotonin concentration**

To evaluate the effects of increased extracellular serotonin levels, the SSRI fluoxetine (20 mg/kg) was co-administered with L-DOPA (Fig. 6, Paper III). Upon this approach, alleviation of LID was achieved, and the animals exhibited similar AIMs scores as on the first day with L-DOPA. Another recent study has demonstrated the same effect, although the mechanism behind the effect was not established (Bishop et al. 2012). To determine whether the effect was mediated via 5-HT$_{1A}$ receptors, a new trial with fluoxetine was performed in the same animals 2 weeks later, when the 5-HT$_{1A}$ antagonist WAY-100635 was applied in combination with L-DOPA. Indeed, upon this event LID re-emerged with similar severity as on the last
L-DOPA trial. The finding indicates that increased serotonin levels blocks dyskinesia by activating 5-HT$_{1A}$ receptors, although the location of the receptors is elusive. 5-HT$_{1A}$ receptors are most abundantly expressed in limbic (hippocampus and septum) and cortical areas and in the mesencephalic raphe nuclei, whereas binding and RNA expression in the basal ganglia is very low and barely detectable (Barnes & Sharp 1999). Since the limbic areas are involved in cognitive function rather than controlling movements, the presynaptic autoreceptors situated on the raphe neurons and the postsynaptic cortical receptors seem to be the most promising candidates for alleviation of LID. Indeed, activation of 5-HT$_{1A}$ autoreceptors on raphe neurons silences serotonergic activity (Sprouse & Aghajanian 1987) and is indicated to decrease L-DOPA-derived dopamine release (Kannari et al. 2001, Lindgren et al. 2010, Nahimi et al. 2012), whereas postsynaptic cortical 5-HT$_{1A}$ receptor activation is indicated to reduce striatal glutamate influx and LID (Antonelli et al. 2005, Mignon & Wolf 2005, Dupre et al. 2011, Ostock et al. 2011).

Activation of 5-HT$_{1A}$ receptors

It has been reported that LID is alleviated upon administration of 5-HT$_{1A}$ agonist, both in animal models of LID (Bibbiani et al. 2001, Carta et al. 2007, Munoz et al. 2008, Eskow et al. 2009, Gregoire et al. 2009, Dupre et al. 2011) and in dyskinetic patients (Bonifati et al. 1994, Olanow et al. 2004, Bara-Jimenez et al. 2005). Similar results were achieved in Paper III, where co-administration of 8-OHDPAT (1 mg/kg s.c.) and L-DOPA reduced LID in severely dyskinetic animals. Furthermore, when administering the same dose of 8-OHDPAT prior to striatal L-DOPA loading during electrochemical glutamate recordings, decreased KCl-evoked glutamate release was observed in normal animals (Fig. 3, Paper III). In contrast, when the same approach was executed in dopamine-depleted animals, no effects were registered on
glutamate release, neither in drug naïve nor in dyskinetic subjects. Based on these findings, it becomes difficult to argue that the main antidyskinetic mechanism of activation of 5-HT$_{1A}$ receptors would be a consequence of decreased striatal glutamate levels. Moreover, the dose chosen ought to have been sufficient to achieve postsynaptic 5-HT$_{1A}$ receptor activation, since previous studies have shown that doses ≥ 0.2 mg gives rise to serotonin syndrome (Iravani et al. 2006, Carta et al. 2007), a state that arises due to cortical 5-HT$_{1A}$ receptor activation (Goodwin et al. 1987, Yamada et al. 1988, DeFelipe et al. 2001). The anti-LID effect from 8-OHDPAT as well as fluoxetine is therefore interpreted to be exerted via action on serotonergic neurons, or alternatively, via postsynaptic targets that do not affect striatal glutamate concentration. Indeed, a direct projection connecting the medial prefrontal cortex with the raphe nuclei has been demonstrated (Sesack et al. 1989, Takagishi & Chiba 1991, Buchanan et al. 1994, Hajos et al. 1998). It seems to exert a significant regulatory action on raphe neuronal activity, and lesioning this pathway inhibited the influence of 8-OHDPAT on raphe neurons (Ceci et al. 1994, Hajos et al. 1999). Furthermore, cortical 5-HT$_{1A}$ activation could potentially also inhibit cortical output to the brain stem and spinal cord (see basal ganglia model, Fig. 2). Additionally, the antidyskinetic action of 8-OHDPAT could be exerted by altered dopamine D$_1$ receptor signaling pathway in striatal output neurons (Dupre et al. 2011).

**GDNF - a crucial factor for nigrostriatal maintenance**

In Paper IV, the effects of reduction and deletion of GDNF tissue levels were evaluated on VM dopamine neuronal survival and fetal striatal development. The obstacle of evaluating the long-term effects from GDNF depletion on nigrostriatal development, due to the limited survival of GDNF knockout mice, was overcome by grafting fetal VM and LGE tissue into the lateral cerebral ventricles of adult wildtype mice. The nigrostriatal
microcircuits were evaluated at 3, 6, and 12 months to study short- and long-term effects of reduced and depleted GDNF tissue expression.

**Early effects from reduced GDNF levels in the nigrostriatal tissue**

At 3 months postgrafting, no difference was found in TH-positive neuronal survival in grafts with different GDNF expression (Fig. 1, Paper IV). Although, in knockout tissue, autofluorescent debris and unidentified intraneuronal inclusion-like structures were observed already at this early time point in the VM portion of the grafts (Fig 2, Paper IV). Immunohistochemical evaluation confirmed the presence of α-synuclein-positive dots in the cytoplasm of these cells, although they seldomly overlapped with the inclusions. For this reason it is not possible to state whether these are in fact Lewy bodies, or something else.

In grafts with normal GDNF expression, the grafted TH-positive neurons innervated the LGE portion by forming dense dopamine-rich patches that overlapped with DARPP-32-positive areas (Fig. 3, paper IV). In heterozygous tissue, the TH-positive and DARPP-32-positive areas were somewhat mismatched. In the knockout tissue, DARPP-32 expression was poor and TH-positive nerve fibers innervated the LGE in a sparse and widespread manner, not forming the patchy distribution of TH-positive nerve fibers as seen in tissue with GDNF expression. Although, when investigating GDNF knockout fetuses at E19, TH-positive dense areas were found that overlapped with striatal neurons (Fig. 3, Paper IV). This is in accordance with previous reports demonstrating no morphological changes in the dopamine system in newborn GDNF gene-deleted mice (Pichel et al. 1996). Interestingly, it has previously been demonstrated that GDNF is expressed in a patchy distribution in normal tissue (Lopez-Martin et al. 1999), and it could therefore be an important guiding cue in order to achieve
the dense striatal dopamine innervation. Furthermore, GDNF mRNA levels are reduced in the adult compared to the developing striatum (Schaar et al. 1993, Strömberg et al. 1993), and consequently, when grafting fetal dopamine neurons into the adult dopamine-lesioned striatum, a non-patchy innervation of the host striatum is achieved (Strömberg et al. 1997). Even though striatal GDNF levels are upregulated upon dopamine depletion, the upregulation was only found in young animals (Nakajima et al. 2001, Yurek & Fletcher-Turner 2001). Consequently, the age-dependent reduction of GDNF levels in the brains of parkinsonian patients might contribute to poor dopamine cell survival seen in some patients after receiving fetal grafts. The findings strongly indicate that dopaminergic neurons require similar GDNF expression as during development in order to properly innervate the striatum.

Long-term effects from reduced GDNF levels in the nigrostriatal tissue
When assessing grafts after 6 and 12 months, significantly fewer surviving neurons were found in the GDNF knockout than in wildtype grafts. Previous studies have reported that grafted VM dopamine neurons lacking GDNF expression degenerate if not preincubated with GDNF before transplantation (Granholm et al. 2000). Furthermore, loss of dopamine neurons are found in conditional GDNF knockout mice when GDNF expression is suppressed during adulthood (Pascual et al. 2008). Here, the whole grafts derived from GDNF knockout tissue had often degenerated already at 6 months (Fig. 1, Paper IV), and if remaining, the graft volume was drastically reduced and TH/DARPP-32 expression was poor. Thus, both the striatal and dopaminergic parts were negatively affected by the GDNF depletion. In the grafts with reduced GDNF levels from heterozygous fetuses, reduced TH-positive cell survival (Fig. 1, Paper IV) and neuronal inclusion-like structures were found at 12 months (Fig. 2, Paper IV). This indicates that at
longer time points, dopamine neurons become more vulnerable when GDNF levels are reduced (Boger et al. 2007). In accordance, earlier dopamine degenerating onset and age-related behavioral changes have been found in these mice (Boger et al. 2006). At earlier time points, the GDNF shortage could be compensated for by other neurotrophic factors belonging to the same family. The wildtype grafts, on the other hand, maintained high cell numbers even at 12 months and no inclusion-like structures were found in the TH-positive neurons at any time point. It should also be noted that the density of activated microglia in the VM parts was similar in wildtype and knockout grafts at 3 months and in heterozygous and wildtype tissue at 12 months (Fig. 4, Paper IV). In addition, blood vessel support was evenly distributed and similar in wildtype and knockout tissue. Therefore, the effects are probably not due to poor blood supply or increased inflammation, but rather to a specific effect of the reduced/absence of GDNF expression.

The TH-positive neurons were labeled with antibodies against GIRK2 and ALDH1 to identify A9 dopamine neurons (Gerfen et al. 1985, McCaffery & Drager 1994, Schein et al. 1998, Inanobe et al. 1999, Chung et al. 2005) and calbindin to label A10 neurons (Johansson et al. 1995, Meyer et al. 1999). It is of interest to assess which subpopulation of dopamine neurons that is affected by reduced/lost GDNF expression, since it was previously shown that both A9 and A10 neurons respond to GDNF supplementation, but at different doses (Borgal et al. 2007). Here, no difference in A9 and A10 dopamine neuronal distribution was found between genotypes and approximately 10% of the TH-positive neurons expressed GIRK2 and ALDH1, respectively, and around 20% of the TH-positive neurons co-expressed calbindin. Therefore, the TH-positive cell loss seemed not to affect a specific subpopulation of dopamine neurons, but rather reflects an overall loss. In support, it was previously shown that both subtypes are lost in conditional GDNF knockout mice (Pascual et al. 2008). It is remarkable
that such a small number of TH-positive neurons stained positive against GIRK2/ALDH1/calbindin, but could also point out the unspecificity of these antibodies.

CONCLUDING REMARKS

L-DOPA-derived dopamine release from serotonergic nerve fibers
A significant amount of L-DOPA-derived dopamine release originate from serotonergic nerve fibers in normal striatum, thus, a serotonergic denervation resulted in decreased dopamine release and altered dynamics of onset and clearance (Paper II). Serotonergic nerve fibers seem to be the main source of L-DOPA-derived dopamine release in the dopamine-lesioned striatum, but gave rise to small increments in extracellular dopamine levels that were several folds lower than in the normal striatum (Paper I).

Dopamine release in dyskinetic animals
Striatal L-DOPA administration did not potentiate dopamine release in dyskinetic animals, and dopamine concentration was significantly attenuated in dyskinetic subjects compared to L-DOPA naïve animals. Consequently, serotonergic nerve fiber function seems to be affected negatively by chronic L-DOPA treatment (Paper I).

Effects of L-DOPA on serotonergic nerve fiber density
Chronic L-DOPA treatment did not affect serotonergic nerve fiber density, neither in dopamine-lesioned (Paper I), nor serotonin-lesioned or normal animals (Paper II). A negative trend was found on serotonergic nerve fiber density in raphe-LGE co-grafts when graft recipients were treated with L-DOPA (Paper II). The L-DOPA dose utilized (4 mg/kg) was sufficient to promote dyskinetic behavior in severely dopamine-depleted animals.
**Striatal glutamate concentration in dyskinetic animals**

A dopamine depletion resulted in a reduction of striatal basal extracellular glutamate concentration and KCl-evoked glutamate release. Upon chronic L-DOPA treatment, basal extracellular glutamate concentration was comparable to normal levels, whereas KCl-evoked glutamate release was unaffected and reduced to similar extents as in L-DOPA naïve dopamine-depleted animals. Striatal L-DOPA administration attenuated glutamate release in normal and L-DOPA naïve dopamine-depleted animals, but not in the dopamine-lesioned striatum of dyskinetic animals (Paper III).

**Anti-dyskinetic effects of fluoxetine and 8-OHDPAT**

Co-administration of fluoxetine and L-DOPA significantly attenuated dyskinetic behavior. This effect was likely mediated via 5-HT₁A receptors, since administration of the 5-HT₁A antagonist WAY-100635 preceding fluoxetine and L-DOPA completely blocked the effects of fluoxetine and dyskinesias re-emerged with full potential (Paper II). The antidyskinetic mechanism of 5-HT₁A receptor activation with 8-OHDPAT might not consist of inhibition of striatal glutamate concentration, since glutamate release remained unaffected upon this event in dyskinetic animals.

**Effects of GDNF on the nigrostriatal system**

GDNF gene deletion resulted in early degeneration of VM dopaminergic and striatal tissue. Preceding the time point of degeneration, cytoplasmic inclusion-like structures were found in the dopamine neurons, and poor striatal innervation was evident. Similar findings were made in nigrostriatal tissue with partial GDNF expression (~50%), but dopamine degeneration occurred at later time points and the striatal innervation was denser than in knockout tissue, although less organized than wildtype tissue. Thus, GDNF
is needed for long-term nigrostriatal neuronal maintenance and axonal guidance (Paper IV).
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