Deuterium-L-DOPA: a Novel Means to Improve Treatment of Parkinson’s Disease

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Stockholm 2012
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Till mamma och pappa
ABSTRACT

L-DOPA, the precursor of dopamine, is administered to restore dopamine deficiency in Parkinson’s disease (PD) patients. L-DOPA initially provides a sustained symptomatic relief with superior efficacy as compared to other treatments, while long term treatment is complicated by the gradual emergence of troublesome motor complications i.e. fluctuations in therapeutic effect and L-DOPA-induced dyskinesia. The risk for motor complications is associated with disease duration and total L-DOPA load. While the underlying mechanisms remain to be fully elucidated, the inability of the remaining dopaminergic neurons to buffer exogenously applied L-DOPA and pulsatle stimulation of dopamine receptors resulting from the short half-life of the drug seem critical. An improved treatment strategy with similar efficacy as L-DOPA and reduced side effects is therefore highly warranted.

Deuterium-L-DOPA was expected to yield dopamine more resistant to enzymatic degradation, as deuterium, heavy hydrogen, forms a stronger bond with carbon. Four isoforms of deuterium-L-DOPA, carrying different combinations of α and β carbon substitutions, were screened for isotope effects on striatal dopamine metabolism by means of in vivo microdialysis in intact rats. The triple substituted isoform, αββ-D3-L-DOPA (D3-L-DOPA), dramatically increased the duration of dopamine output and reduced noradrenaline output as compared to L-DOPA. These effects most likely reflect reduced activity of the dopamine metabolizing enzymes MAO and DβH towards the deuterium substituted α- and β- carbons, respectively. Deuterium substitutions thus increase the half-life of dopamine formed from L-DOPA, which may reduce pulsatile stimulation of dopamine receptors as well as the total L-DOPA load in PD patients. The improved central kinetics of D3-L-DOPA may thereby significantly reduce the risk for L-DOPA induced motor complications. Reduced output of noradrenaline from D3-L-DOPA may additionally contribute to reduce the side effect profile, as noradrenaline released from L-DOPA may be involved in the expression of dyskinesias.

The neurochemical and behavioral effects of D3-L-DOPA were subsequently evaluated in two, well-established animal models of PD, the reserpine and the 6-OHDA-lesioned model. D3-L-DOPA produced an increased dopamine output as compared to L-DOPA in the 6-OHDA-lesioned striatum; an effect which closely resembled that of L-DOPA in combination the MAO-B inhibitor selegiline; used in clinical practice to potentiate the symptomatic effect of L-DOPA and reduce motor fluctuations. Moreover, selegiline pre-treatment did not potentiate the effect of D3-L-DOPA. The enhanced output of dopamine from D3-L-DOPA and selegiline/L-DOPA may thus be attributed to decreased metabolism of dopamine at MAO-B containing sites.

An acute challenge with D3-L-DOPA was shown to produce an increased motor activation as compared to L-DOPA in both models of PD, indicating an increased behavioral potency. In addition, the behavioral effect produced by D3-L-DOPA was found to be of similar magnitude as the combination of selegiline/L-DOPA. Our data hence provide experimental support for the potential clinical advantage of D3-L-DOPA and suggest that monotherapy with D3-L-DOPA may provide equal benefit as the combination of selegiline/L-DOPA. The effects of D3-L-DOPA and L-DOPA were also compared in a chronic treatment design. Significantly, a lower dose of D3-L-DOPA, 60% of the equivalent L-DOPA dose, produced similar anti-parkinsonian benefit while the expression of dyskinesias was markedly reduced. The equivalent dose of D3-L-DOPA, as compared to L-DOPA, produced a more pronounced anti-parkinsonian effect and similar expression of dyskinesia. Taken together, these findings indicate that deuterium substitutions offer the advantage of a wider therapeutic window.

In conclusion, the increased half-life of dopamine formed from D3-L-DOPA may serve to protect dopamine receptors from pulsatile stimulation and the increased behavioral potency of D3-L-DOPA may allow for adequate control of parkinsonian symptoms at an overall lower dosage. Altogether, a reduced L-DOPA load and more sustained stimulation of dopamine receptors may substantially improve PD treatment by reducing the risk for motor fluctuations and dyskinesias. Our preclinical data thus provide support for the utility of deuterium-substitutions in the L-DOPA molecule as a means to improve the therapeutic effect and reduce the side effects of L-DOPA therapy.
LIST OF PUBLICATIONS


IV. Malmlöf T, Svensson TH, Schilström B. (2012) Deuterium substitutions in the L-DOPA molecule increase dopamine but reduce noradrenaline output in the striatum. *Manuscript*
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LIST OF ABBREVIATIONS

3-OMD 3-O-methyldopa
3-MT 3-metoxityramine
5-HIAA 5-hydroxyindole acetic acid
5-HT 5-hydroxytryptamine
6-OHDA 6-hydroxydopamine
AADC Aromatic amino acid decarboxylase
AIM Abnormal involuntary movement
ALDH Aldehyde dehydrogenase
AMPA α-Amino-3-hydroxy-5-methylisoxasole-4-propionic acid
ANOVA Analysis of variance
BBB Blood brain barrier
DBH Dopamine-β-hydroxylase
DOPAC 3,4-dihydroxyphenylacetic acid
DOPAL 3,4-dihydroxyphenylacetaldehyde
CNS Central nervous system
COMT Catechol-O-methyltransferase
D3-L-DOPA α,β,β-D3-L-DOPA
DAT Dopamine transporter
GABA γ-amino butyric acid
GPe Globus pallidus externa
GPi Globus pallidus interna
HPLC High performance liquid chromatography
HVA Homovanillic acid
i.e. That is (id est)
i.p. Intraperitoneal
L-DOPA L-3,4-dihydroxyphenylalanine
L-DOPS L-threo-3, 4-dihydroxyphenylserine
LC Locus coeruleus
LDR Long duration response
LID L-DOPA-induced dyskinesia
LNAA Large neutral amino acid
LTD Long-term depression
LTP Long-term potentiation
MSN Medium spiny neuron
MAO Monoamine oxidase
MFB Median forebrain bundle
MPTP 1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine
NMDA N-methyl-D-aspartate
PEA Phenylethylamine
PD Parkinson´s disease
PDI Peripheral decarboxylase inhibitor
PKA Protein kinase A
ROS Reactive oxygen species
s.c. Subcutaneous
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>SDR</td>
<td>Short duration response</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>Substantia nigra pars reticulata</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic nucleus</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>VMAT</td>
<td>Vesicular monoamine transporter</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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1 INTRODUCTION

1.1 PARKINSON’S DISEASE

Parkinson’s disease (PD) is a chronic and progressive movement disorder caused by degeneration of dopaminergic neurons in the central nervous system (CNS) and the average age of onset is 60 years. The prevalence is reported to be 1 % in the population aged over 60 years (de Lau and Breteler, 2006) and 5 million people all over the world are estimated to suffer from PD. In 1817, the English physician James Parkinson published the first extensive medical description of the disease, “An Essay on the Shaking Palsy” (Parkinson, re-published 2002), in which observations of six affected patients were presented. The shaking palsy was characterized by “involuntary tremulous motion, with lessened muscular power... with a propensity to bend the trunk forwards”; the shaking palsy was thereafter named Parkinson’s disease. PD may however well have existed for thousands of years before it was described by James Parkinson, in fact there are reports of a Parkinson-like disease that dates back to 1000 before Christ in the “Ayurveda”, the ancient Indian medical system (Manyam, 1990). The cardinal motor symptoms of PD include bradykinesia, rigidity, resting tremor and postural instability. The motor symptoms typically affect one side of the body at the early stage, to extend bilaterally at later stages. Postural instability is commonly observed at a later phase in disease progression (Hoehn and Yahr, 1967). The clinical diagnosis of PD is based on the manifestation of at least two cardinal symptoms and a positive response to dopamine replacement therapy. Non-motor symptoms such as orthostatic hypotension, sleep disorders, depression, cognitive dysfunction and disturbed autonomic function are also common (Olanow et al., 2009b). Additionally, there is a high co-morbidity between PD and dementia (Aarsland et al., 2005).

1.1.1 Discovery of the primary pathophysiology of PD and dopamine replacement therapy

The scientific basis for the discovery of the primary cause of PD symptoms and its treatment was laid by Arvid Carlsson and colleagues in the late 1950’s. At the time, dopamine was considered to be a physiologically inactive intermediate in the enzymatic conversion of the catecholamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) to noradrenaline (Carlsson, 2002). The administration of reserpine, an antipsychotic drug, to experimental animals became helpful in characterizing the role of central catecholamines. Reserpine was shown to induce a parkinsonian-like state that could be reversed by administration of L-DOPA (Carlsson et al., 1957). Following the identification of dopamine in the CNS, at equal amounts as those of noradrenaline, Carlsson and colleagues also showed a depletion of both dopamine and noradrenaline by reserpine. In addition, L-DOPA was shown to dramatically increase central levels of dopamine but to only slightly increase the levels of noradrenaline (Carlsson et al., 1958). Taken together these pivotal findings indicated that the reserpine-induced suppression of motor function was related to dopamine as was its reversal by L-DOPA. Shortly thereafter, dopamine was found to be specifically localized to the basal ganglia system (Bertler and Rosengren, 1959, Sano et al., 1959) which is involved in the control of movement (see section 1.2). Dopamine was, as opposed to being an inactive
intermediate in the production of noradrenaline, therefore suggested to be involved in the control of movement (Carlsson, 1959). Spurred by these observations Hornykiewicz and colleagues investigated brains of PD-patients and found significantly lower levels of dopamine in the basal ganglia compared to control subjects (Ehringer and Hornykiewicz, 1960). Thereby, it could be established that PD symptoms were associated with dopamine deficiency in the basal ganglia. The first attempts to supplement PD-patients with L-DOPA were initiated soon thereafter and L-DOPA was shown to improve motor function (Birkmayer and Hornykiewicz, 1961, Sano, re-published 2000). The clinical effectiveness of L-DOPA was further confirmed during long-term treatment (Cotzias et al., 1967, Barbeau, 1969) and in placebo controlled trials (Cotzias et al., 1969, Yahr et al., 1969). L-DOPA revolutionized pharmacological treatment of PD and still remains the most effective treatment more than 50 years after its introduction. Side effects associated with L-DOPA treatment, as observed in the first clinical trials, included nausea, vomiting, postural hypotension and psychiatric disturbances. An additional side effect, appreciated to represent the most limiting factor for the use of L-DOPA, was the appearance of abnormal involuntary movements following its administration. Indeed, L-DOPA-induced dyskinesia (LID) is a yet to be overcome challenge in PD-treatment (see section 1.4).

1.1.2 Pathophysiology of PD

The motor symptoms of PD are the result of a severe degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) which project to the striatum, the input zone of the basal ganglia. The accelerated neurodegeneration is suggested to occur for many years before symptoms appear (Marsden, 1990). PD symptoms present when 50-60% of SNpc cell bodies are lost and striatal tissue levels of dopamine have been reduced by about 80% (Bernheimer et al., 1973, Agid, 1991). This indicates a remarkable capacity of the dopaminergic system to compensate up until this point (Hornykiewicz and Kish, 1987). In fact, the remaining dopaminergic neurons may compensate by increasing their synthesis and release (Zigmond et al., 1990). In addition to the loss of dopaminergic neurons in the SNpc, a prominent pathological hallmark of PD is the presence of neuronal inclusions, Lewy bodies, which are associated with excessive neuronal cell death (Gibb and Lees, 1988). Lewy bodies were shown to contain high amounts of α-synuclein protein (Spillantini et al., 1997). The distribution of Lewy bodies in PD was extensively studied by Braak and subsequently divided into six temporal stages of severity. Since Lewy bodies appear in brain stem nuclei such as the olfactory nucleus before it appears in the substantia nigra (Braak et al., 2003), it has been suggested that cell loss at other sites precede degeneration in the SNpc. Much effort has therefore been devoted to identify “pre-motor” symptoms to predict the onset of the disease and deficits in olfaction, REM-sleep and autonomic function are potential candidates (Postuma et al., 2012). Other transmitter systems are also affected in PD, albeit to variable extent, i.e. noradrenergic neurons in the locus coeruleus (LC) (Hornykiewicz and Kish, 1987, Zarow et al., 2003, McMillan et al., 2011), cholinergic neurons in the nucleus basalis of Meynert (Zarow et al., 2003) and serotonergic neurons in the median raphe nucleus (Halliday et al., 1990). The degeneration of other transmitter systems could thus contribute to non-motor symptoms of the disease (Olanow et al., 2009b).
1.1.3 Etiology of PD

Parkinsonism refers to an acquired condition, with known etiology such as induced by head trauma, infection or intake of neuroleptics. The etiology of idiopathic Parkinson’s disease, however, remains largely unknown and likely depends on a complex interaction between genetic and environmental risk factors. A genetic component of PD is implicated by the identification of several monogenic familiar variants of PD, *i.e.* where mutations in a single inherited gene can be said to cause the disease and additionally by several other gene polymorphisms/mutations which are associated with an increased risk for acquiring the disease (Klein and Westenberger, 2012). The familiar variants of PD usually have an early disease onset and account for approximately 5% of PD cases (Klein and Westenberger, 2012). Twin studies, however, do not support a genetic component in PD with an onset after 50 years of age (Tanner et al., 1999). Environmental risk factors with varying epidemiological support include age, exposure to certain herbicides, pesticides and heavy metals, rural living and well water drinking. Interestingly, tobacco and caffeine intake have been shown to reduce the risk of acquiring PD (Swanson et al., 2009, Wirdefeldt et al., 2011).

1.1.4 Pathogenic mechanisms involved in dopaminergic cell death

Several lines of evidence support mitochondrial dysfunction and increased oxidative stress as pathogenic mechanisms in PD. Mitochondrial dysfunction is tightly coupled to oxidative stress and excessive generation of reactive oxygen species (ROS) and free radicals, which cause damage to membrane lipids, proteins and DNA, all of which were found to be affected in PD (Sherer et al., 2002). Specifically, the first electron transfer chain in the mitochondria involving NADH (complex 1) has been found to be impaired in the substantia nigra (SN) of PD patients (Schapira and Jenner, 2011). The cause of the mitochondrial deficit and oxidative stress could be related to environmental as well as genetic risk factors to which dopaminergic neurons have an increased vulnerability. As mentioned above, exposure to environmental toxins such as pesticides is associated with an increased risk of developing PD, and several pesticides have been shown to inhibit the mitochondrial complex 1 (Sherer et al., 2002). In addition, the synthetic opiate 1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) can induce irreversible Parkinsonism in humans (Langston et al., 1983). MPTP is metabolized to MPP⁺ by monoamine oxidase (MAO) -B (Chiba et al., 1984) located in glial cells and taken up into the dopaminergic neurons (Javitch et al., 1985) where it interferes with the mitochondrial complex 1 (Nicklas et al., 1985). Genetic risk factors, which are associated early onset familiar PD, are mutations in the PINK1, DJ1, and Parkin genes, all with a suggested involvement in mitochondrial function (Greenamyre and Hastings, 2004).

The vulnerability of dopaminergic neurons to the above mentioned pathogenic factors has been hypothesized to relate to several aspects of dopamine metabolism, which in itself may contribute to oxidative stress. Hydrogen peroxide (H₂O₂), a ROS, is formed both in oxidative metabolism of dopamine by MAO and via non-enzymatic autoxidation of dopamine into dopamine-quinone (Stokes et al., 1999). Additionally, in the presence of ferrous iron (Fe⁺⁺), which is increased in the SN of PD patients (Dexter et al., 1989), the free hydroxyl radical (·OH) may be formed from hydrogen peroxide.
via the Fenton reaction. MAO metabolism of dopamine generates 3, 4-dihydroxyphenylacetacetaldehyde (DOPAL) which is potentially toxic to dopaminergic neurons (Panneton et al., 2010). Under normal circumstances DOPAL is rapidly converted to the non-toxic metabolite 3, 4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase (ALDH). However, products of oxidative stress and deficiencies in the mitochondrial complex-1 could decrease the activity of ALDH (Eisenhofer et al., 2004, Jinsmaa et al., 2011) resulting in accumulation of DOPAL in PD patients. All of the above mentioned mechanisms would occur in the cytosolic fraction of dopamine, since the vesicular fraction is protected from metabolism via the activity of the vesicular monoamine transporter (VMAT). VMAT, however, operates on ATP generated by the mitochondria and mitochondrial dysfunction could thus potentially increase the cytosolic contents of dopamine and generate excessive oxidative stress via oxidation of dopamine (Sulzer, 2007). A remaining controversy as regards L-DOPA treatment of PD is the suggestion that the drug may accelerate the degenerative process via increased formation of ROS from autooxidation and oxidative metabolism of dopamine. This hypothesis has derived some support from in vitro studies, while in vivo studies do not support a toxic effect of L-DOPA in PD (Olanow et al., 2004a, Schapira, 2008, Parkkinen et al., 2011, Zesiewicz, 2012).

Other pathogenic mechanisms which are hypothesized to contribute to dopaminergic cell death are excessive protein aggregation and accumulation, as shown by the α-synuclein protein aggregations in Lewy bodies (Greenamyre and Hastings, 2004), overstimulation by glutamate and calcium accumulation (excitotoxicity) as well as neuroinflammation. The cause and effect relationship between these pathogenic mechanisms is far from clear (Jenner and Olanow, 2006).

### 1.2 THE BASAL GANGLIA, DOPAMINE AND PARKINSON’S DISEASE

Our movements are categorized in reflexive, rhythmic and voluntary movements, which involve different hierarchical levels of the motor system. Unlike reflexes, only involving the lower levels of the motor system, the initiation and control of goal-directed voluntary movements engages all levels of the motor system starting with neurons of cerebral cortex to terminate in the motor neurons of spinal cord which regulate muscle contraction and relaxation. The basal ganglia constitute a group of interconnected subcortical nuclei which receive input from cortical motor, limbic, sensory and associative areas and modulate the final output of the same cortical areas via a feedback loop relaying in the thalamus. The basal ganglia nuclei also receive input from the thalamus, hippocampus and amygdala and send direct projections to the brain stem (DeLong, 2000). The basal ganglia-cortical feedback system is organized in segregated but parallel circuits connecting specific regions of the cortex to the basal ganglia and back, and are functionally divided depending on the cortical origin of the circuit (Alexander et al., 1986). The basal ganglia thereby regulate many types of behaviors including planning, initiation and execution of voluntary movements as well as non-motor behaviors such as cognition and emotion. Dopamine serves to modulate basal ganglia function via its prominent innervation of the basal ganglia input zone, the striatum.
1.2.1 Functional organization of the striatum

The striatum consists to 95% of medium spiny neurons (MSNs), which function like a relay station for all converging inputs to the basal ganglia system (Smith and Bolam, 1990). In humans, the striatum is divided into the dorsal striatum (caudate nucleus and putamen) and the ventral striatum (nucleus accumbens). Based on the cortical and subcortical input to the primate striatum it can be functionally divided into modulating sensorimotor (dorsal parts of the caudate and putamen), associative (caudate nucleus and ventral parts of the putamen) and limbic (nucleus accumbens and most ventral parts of the caudate and putamen) functions (Groenewegen, 2003). In rodents, which is the animal species studied in the present thesis work, the dorsal striatum is not separated in two nuclei and is termed caudate-putamen. Functionally, the rat striatum is divided into motor (lateral caudate-putamen), associative (medial caudate-putamen) and limbic (nucleus accumbens) (Joel and Weiner, 2000). The hypokinetic symptoms of PD have been attributed to dysfunction of the motor circuits of the basal ganglia arising from the loss of dopaminergic input to the dorsal striatum (Hornykiewicz and Kish, 1987), therefore the following chapters will focus on the motor circuits of the system.

1.2.2 Basal ganglia connections and function

A simplified scheme of the connections and function of the motor circuit is presented below (Albin et al., 1989, DeLong, 1990). Glutamatergic efferents from the motor, premotor and somatosensory cortices converge onto dendrites and spines of striatal MSNs (Smith and Bolam, 1990). The MSNs are inhibitory projection neurons utilizing the transmitter γ-aminobutyric acid (GABA). Striatal MSNs form two functionally different pathways, the direct and the indirect, connecting the rest of the basal ganglia nuclei to the output structures, the globus pallidus interna (GPi) and the substantia nigra pars reticulata (SNr). The output of the basal ganglia is GABAergic and provides a tonic inhibition over thalamic neurons which form the excitatory feedback projection to the cortex (Bolam et al., 2000). Due to the involvement of different basal ganglia nuclei in the direct and the indirect pathway these two circuits mediate antagonistic effects on the inhibitory outflow from GPi and SNr (see Figure 1). Striatal MSNs, with a direct GABAergic projection to the output structures, will upon activation disinhibit thalamocortical neurons thereby increasing the excitability of cortical motor neurons. MSNs of the indirect pathway project to the globus pallidus externa (GPe), which in turn exerts tonic inhibitory control of the subthalamic nucleus (STN). The STN in turn projects to the output nuclei via glutamatergic efferents. Activation of the striatal MSNs in the indirect pathway will thus disinhibit the STN resulting in increased inhibitory outflow to the thalamus and decreased excitability of cortical motor neurons. In this way, selective activation of the two pathways will modulate the firing rate of basal ganglia output nuclei to facilitate or inhibit movement (Albin et al., 1989, DeLong, 1990, Gerfen, 1992). Dopamine selectively activates and inhibits the direct and indirect pathway, respectively (see section 1.2.3.4). A more complex view of the basal ganglia output structures have emerged indicating that not only firing rate but firing pattern of output structures are important for the selection of proper motor programs (Obeso et al., 2000b).
1.2.3 Dopamine and the basal ganglia

1.2.3.1 Dopaminergic pathways of the CNS

There are four major dopaminergic systems in the brain, the mesolimbic, mesocortical, tuberoinfundibular and nigrostriatal which are categorized based on the nuclei of origin and projection area (see Figure 2). The dopaminergic pathways were first mapped in the rodent brain (Dahlström and Fuxe, 1964). The mesolimbic and mesocortical system both originate in the ventral tegmental area (VTA) but their projections differ. The mesolimbic system innervates the ventral striatum (nucleus accumbens), the amygdala and the hippocampus whereas the mesocortical system preferentially projects to cortical regions, e.g. the prefrontal cortex. The mesolimbic system and mesocortical systems thereby regulate many types of behaviors such as reward, motivation, emotion and cognition and several psychiatric conditions are related to alterations in these systems, for example schizophrenia and addiction. The tuberoinfundibular system originates in the hypothalamus and projects to the pituitary; it is involved in endocrine control. The nigrostriatal system originates in the substantia nigra, specifically in the pars compacta (Andén et al., 1964), and projects to the striatum. The SNpc is rich in neuromelanin pigment, a product formed from autoxidation of dopamine (Sulzer and Zecca, 2000), and was hence given the Latin name from black nigra. The striatum receives a prominent dopaminergic input from cells located in the SN, VTA and retrorubral area, midbrain projections to other basal ganglia nuclei have also been detected (Björklund and Dunnett, 2007). In both rats and primates a simplified functional subdivision of midbrain dopamine projections can be made based on their striatal targets, SNpc neurons mainly target the sensorimotor dorsal striatum (caudate-putamen) and VTA.
neurons mainly target the limbic ventral striatum (nucleus accumbens) (Joel and Weiner, 2000, Björklund and Dunnett, 2007). In PD, dopaminergic cell loss is most severe in the SNpc but also the VTA neurons are affected (German et al., 1989) which may contribute to non-motor symptoms such as impaired cognition, motivation and depression. The present thesis work has been focused on the motor aspects of PD and all neurochemical measurements were therefore performed in the caudate-putamen.

**Figure 2.** Dopaminergic pathways in the human CNS. Am: amygdala; Hip: hippocampus; Hyp: hypothalamus; NAC: nucleus accumbens; P: pituitary; PFC: prefrontal cortex; SN: substantia nigra; Th: thalamus; VTA: ventral tegmental area. Modified from (Rang et al., 2012).

1.2.3.2 **Dopamine receptors in the striatum**

There are five different G-protein-coupled dopamine receptors (D1-D5). They are functionally divided into two families, the D1-like (D1 and D5) and the D2-like (D2, D3 and D4) by their association with different G-proteins with opposing effects on the membrane enzyme adenylyl cyclase. D1-receptors are coupled to the \( G_{olf} \) protein; the subunit \( G_{olf} \) stimulates adenylyl cyclase which increases cAMP formation with subsequent activation of protein kinase A (PKA). PKA further regulates protein function by phosphorylation and targets voltage gated ion-channels and glutamate receptors (see section 1.2.3.4). D2 receptors are coupled to the \( G_{i/o} \) protein and the subunit \( G_{i/o} \) inhibits adenylyl cyclase which decreases the formation of cAMP. In addition, the \( G_{olf} \) subunit can influence excitability of MSNs through direct effects on various ion channels (Neve et al., 2004). Both D1 and D2 receptors are found postsynaptically on MSNs but are also expressed at extrasynaptic sites (Sesack et al., 1995, Yung et al., 1995). D2 receptors are also expressed presynaptically in the terminal and function as synthesis- and release-modulating autoreceptors (Andén et al., 1967, Kehr et al., 1972). The MSNs of the direct pathway express D1 receptors, the neuropeptides dynorphin and substance P and the MSNs of the indirect pathway express D2 receptors and enkephalin (Gerfen et al., 1990, Surmeier et al., 1996).

1.2.3.3 **Nigrostriatal transmission**

Dopaminergic cells fire action potentials in two distinct modes, tonic single spike and phasic burst firing (Grace and Bunney, 1984a, 1984b). The release of dopamine occurs at the terminal and somatodendritic level (Nissbrandt et al., 1985) and is regulated by firing rate and pattern of the neuron; phasic firing elicits large transient increases in terminal dopamine levels at certain “hot spots”, while tonic firing results in temporally and spatially uniform concentrations of dopamine (Venton et al., 2003). The tonic
activity of midbrain dopamine neurons thus contribute to constant dopaminergic stimulation of dopamine receptors and to the basal concentrations of the transmitter as measured by means of in vivo microdialysis (Grace, 2008). The clearance of dopamine from the site of its release is mainly governed by diffusion whereas the high-affinity reuptake of dopamine via the dopamine transporter (DAT) regulates the concentration of diffused dopamine, i.e. the extracellular levels of the transmitter (Cragg and Rice, 2004). Temporal changes in dopamine cell firing, i.e. transient increases in burst firing is important for reward-driven learning and behavior, however the tonic stimulation of dopamine receptors, elicited by single spike firing mode, is important for facilitation of motor activity (Schultz, 2007). The firing pattern of dopamine neurons is modulated by glutamatergic afferents from the cortex and STN (Nieoullon et al., 1978, Chergui et al., 1991, Smith and Grace, 1992), by GABAergic afferents from STR, GP and SNr (Grace and Bunney, 1985, Tepper and Lee, 2007) and by somatodendritic D2 autoreceptors (Lacey et al., 1988). In addition, several other transmitter systems projecting to the SNpc including noradrenaline, acetylcholine and serotonin are also involved in this modulation (Parent et al., 1981, Grenhoff and Svensson, 1988, Kitai et al., 1999).

1.2.3.4 Dopaminergic modulation of striatal MSNs

The dopaminergic neurons impinge on MSNs to form symmetric synapses on the necks of the same dendritic spines that receive cortical input (Bolam et al., 2000). One single SNpc cell has been estimated to influence approximately 75 000 MSNs by forming huge axonal arborizations (Andén et al., 1966, Matsuda et al., 2009). The classic mode of signal transduction is based on synaptic release of dopamine to target postsynaptic receptors in the same synapse. However, striatal dopamine receptor activation also occurs via volume transmission (Agnati et al., 1986, Garris and Wightman, 1994) where dopamine diffuses away from the site of release to target receptors at distant sites (Zoli et al., 1998). This mode of transmission may also explain the large number of asynaptic contacts found in the striatum (Descarries et al., 1996). The MSN are quiescent unless stimulated (Bolam et al., 2000) and exist in a so called “down state”. Glutamate released from the corticostriatal neurons activates postsynaptic AMPA and NMDA receptors and depolarizes the neuron. If there is sufficient convergent excitatory drive from the cortex, the neuron will switch into an “up state” which is near spike threshold and during this “up state” the neuron fires. The modulatory role of the D1 and D2 receptor on MSN excitability relates to the fact that D1 receptor activation increases spiking and D2 receptor activation decreases spiking of MSNs in the “up state”. D1-receptor activation will, through the activation of PKA, modulate potassium channels, voltage-gated L-type calcium channels as well as the glutamate receptors α-Amino-3-hydroxy-5-methylisoxasole-4-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) to increase excitability (Hernandez-Lopez et al., 1997). D2 receptor activation will reduce the likelihood of firing by suppressing inward calcium currents through L-type channels, dendritic voltage-gated channels and NMDA receptors in addition to an increase in K⁺ outflow (Hernandez-Lopez et al., 2000, Surmeier et al., 2007, Gerfen and Surmeier, 2011). Additionally, D2-receptor activation may reduce the release of glutamate in the striatum (Bamford et al., 2004), thereby further decreasing the excitatory drive to the MSN of the indirect pathway. The direct interaction of D1 and D2 receptor coupled G-proteins will mediate an increased activity of MSNs in the
Introduction

direct pathway and a reduced activity of MSNs in the indirect pathway, respectively, hence dopamine signaling will ultimately increase the excitability of cortical motor neurons and facilitate movement. Dopamine also modulates long-term changes in corticostriatal synaptic strength, which contributes to the storage of information in neuronal circuits and may form a base for acquisition and extinction of motor learning. The ability of a synapse to alter its strength depending on the input activity is termed plasticity. Plasticity at the corticostriatal synapse is implicated in motor learning and reward mechanisms. Plasticity can be studied and induced experimentally and has been classified into long-term potentiation (LTP) and long-term depression (LTD). Dopamine is required for the induction of both forms of plasticity acting together with other transmitters and neuromodulators such as acetylcholine, glutamate, adenosine and endocannabinoids (Calabresi et al., 2007, Surmeier et al., 2007, Wickens, 2009).

1.2.3.5 Interneurons and other transmitter systems in the basal ganglia

The modulatory role of dopamine in striatal MSN responsiveness is a complex interplay with other types of neurons and chemical mediators. For example, adenosine modulates the effects of dopamine in the direct and indirect pathway via A1 receptors and A2A receptors, respectively (Ferre et al., 1997). A2A receptors are expressed in the indirect pathway MSNs where they can antagonize the effects of D2 receptor activation and thus reduce motor activity (Fuxe et al., 2007). Additionally, both cholinergic and GABAergic interneurons which are involved in inhibition and activation of specific subsets of MSNs express dopamine receptors (Gerfen and Surmeier, 2011). Other neurotransmitter systems which project to the striatum include noradrenaline (Lindvall and Björklund, 1974, Mason and Fibiger, 1979, Jones and Yang, 1985) and serotonin (Parent et al., 2011).

1.2.4 Basal ganglia dysfunction in PD

From the above depicted basal ganglia model and based on the modulatory role of dopamine in the basal ganglia system we can predict that loss of dopaminergic input to the MSNs in the striatum will result in hypoactivity of the direct pathway and hyperactivity in the indirect pathway. This will result in an excessive inhibitory output from the GPi/SNr to the thalamus and decreased excitation of cortical motor neurons (Albin et al., 1989, DeLong, 1990) (see Figure 1). In support of this model, lesions of the STN of Gpi improve motor symptoms of PD in primate models and patients (Obeso et al., 2000b, Obeso et al., 2008a). Depletion of dopaminergic projections to the striatum has been found to reduce the expression of direct pathway-associated mRNA (dynorphin, substance P) and to increase the expression of enkephalin mRNA associated with the indirect pathway (Gerfen et al., 1990). Treatment with L-DOPA or dopamine agonists will thus reduce the excessive inhibitory output of the basal ganglia system and thereby improve motor function.

The above depicted model of basal ganglia function has helped to understand how profound dopamine deficiency results in motor dysfunction and how dopamine supplementation may improve PD symptoms. However, it is simplified and in order to fully understand all aspects of dysfunction in PD the extended connections between the basal ganglia nuclei and the cortex, thalamus and brain stem as well as a complex feed-
back circuitry within the system have to be taken into consideration (Bolam et al., 2000, Obeso et al., 2000b, Obeso et al., 2008a, Obeso et al., 2008b).

### 1.3 PHARMACOLOGICAL TREATMENT OF PARKINSON’S DISEASE

The treatment of PD is purely symptomatic; none of the available pharmacological agents have clearly been shown to halt disease progression. Since the introduction of long-term L-DOPA treatment during the 60’s (Cotzias et al., 1967, Barbeau, 1969), which to a great extent replaced the previous use of anticholinergic drugs (Goetz, 2011), L-DOPA remains a cornerstone in the pharmacological treatment of PD (Mercuri and Bernardi, 2005). The majority of agents used in PD target dopaminergic transmission, therefore some relevant aspects of dopamine synthesis, release and metabolism will be covered next.

#### 1.3.1 Dopamine synthesis, release and metabolism

##### 1.3.1.1 Dopamine synthesis and release

All catecholamines, dopamine, noradrenaline and adrenaline, are produced from L-tyrosine, a naturally occurring dietary amino acid. L-Tyrosine is actively absorbed from the gasterointestinal tract to the circulation and over the blood brain barrier (BBB) via the large neutral amino acid (LNAA) transporter. L-Tyrosine is further transported into the dopaminergic neuron and hydroxylated to L-DOPA by tyrosine hydroxylase (TH) (see Figure 3). This is the rate-limiting step in catecholamine formation. TH activity is negatively regulated by presynaptic D2 receptors and positively regulated by an increased firing rate of dopaminergic neurons (Cooper et al., 2003). L-DOPA is further decarboxylated to dopamine by aromatic amino acid decarboxylase (AADC). AADC decarboxylases all naturally occurring amino acids and is widely distributed. In the CNS, the enzyme is found in various cells including noradrenergic, dopaminergic and serotonergic (Goldstein et al., 1972, Hökfelt et al., 1973). VMAT2 is localized in the vesicular membrane (Pickel et al., 1996) and actively transports cytosolic dopamine into vesicles. Inside vesicles, dopamine is protected from intraneuronal metabolism. The importance of this mechanism is illustrated by the complete depletion of catecholamines which occurs following administration of the VMAT-blocker reserpine. When an action potential from the soma reaches the synapse, the vesicular membrane is fused with the outer nerve cell membrane and the contents is released into the extracellular space via calcium-dependent exocytosis (Westfall and Westfall, 2006). Dopamine may however also be released in a non-exocytotic fashion involving NMDA-receptor activation (Grace, 1991) and reversed transport of dopamine via the DAT (Leviel, 2011). This type of release is not action potential dependent. Following release, dopamine activates postsynaptic dopamine receptors via synaptic or volume transmission. The DAT is localized at extrasynaptic sites (Pickel et al., 1996) and is, as previously mentioned, important for regulation of extracellular levels of dopamine.

##### 1.3.1.2 Dopamine metabolism

The cytosolic pool of dopamine is subject to metabolism by the enzyme MAO which is located in the outer mitochondrial membrane (Greenawalt and Schnaitman, 1970). The product of the MAO reaction towards dopamine is the aldehyde DOPAL which is
rapidly converted to the corresponding acid, DOPAC by ALDH. ALDH is found in the mitochondrial membrane or in a soluble cytosolic form (Marchitti et al., 2007). DOPAC is further metabolized by catechol-O-methyltransferase (COMT) to homovanillic acid (HVA) in the extracellular space (Kastner et al., 1994, Karhunen et al., 1995). Released dopamine is transported back to the dopaminergic neuron or metabolized to 3-metoxytyramine (3-MT) by COMT and subsequently to HVA by MAO and ALDH. Extracellular dopamine may also be taken up in postsynaptic structures via the organic cation transporter or a Na⁺-dependent transporter (Pelton et al., 1981, Semenoff and Kimelberg, 1985, Inazu et al., 1999, Westfall and Westfall, 2006) to be metabolized inside of these cells.

**Figure 3.** Schematic picture of dopamine synthesis, release and metabolism. The upper left compartment represents the periphery. L-DOPA, administered per orally to PD patients, is actively transported from the periphery to the CNS via the LNAA. L-DOPA is subject to extensive peripheral metabolism by AADC and COMT (see section 1.3.2). Abbreviations are given in the text. Modified from (Cooper et al., 2003).

### 1.3.1.3 MAO

The enzyme MAO deserves special attention in this thesis introduction. Besides an important role in dopamine metabolism, it is also involved in the oxidative deamination of noradrenaline, 5-hydroxytryptamine (5-HT) as well as the amines tryptamine and phenylethylamine (PEA). MAO was discovered to exist in two isoforms based on their preferential inhibition by clorgyline (MAO-A) (Johnston, 1968) and selegiline (MAO-B) (Knoll and Magyar, 1972). The isoforms of MAO differ in both tissue distribution and substrate specificity. MAO-A is abundantly expressed in the stomach, lungs and liver while MAO-B is preferentially found the liver (Berry et al., 1994a). In the CNS, there is a compartmentalization of the different isoforms of MAO to certain celltypes. MAO-A is mainly found in catecholaminergic neurons, i.e. in noradrenergic neurons of the locus coeruleus and in dopaminergic neurons of the SNpc, whereas MAO-B is localized in the 5-HT neurons of the raphe nucleus and in glial cells (Levitt et al., 1982, Westlund et al., 1985, Thorpe et al., 1987, Westlund et al., 1988, Saura Marti et al., 1990, Saura et al., 1992, Westlund et al., 1993, Jahng et al., 1997). MAO-A has higher
affinity for 5-HT \textit{in vitro} while MAO-B has higher affinity for PEA. Dopamine, noradrenaline, adrenaline are all considered to be mixed substrates for both isoforms (Waldmeier, 1987, Berry et al., 1994a). The \textit{in vivo} contribution of the specific MAO isoforms to dopamine metabolism, however depends on their abundance and compartmentalization, \textit{i.e.} which isoform that is present in the cells with access to cytosolic dopamine (Waldmeier, 1987). Numerous \textit{in vivo} pharmacological studies have confirmed a predominant role of MAO-A over MAO-B in striatal dopamine metabolism in rats (Kato et al., 1986, Butcher et al., 1990, Colzi et al., 1990, Wachtel and Abercrombie, 1994, Brannan et al., 1995, Lamensdorf et al., 1996, Fornai et al., 2000). This finding is consistent with the localization of MAO-A in dopaminergic neurons and with the idea that oxidative metabolism of dopamine mainly occurs within the dopaminergic neuron (Eisenhofer et al., 2004). However, in human \textit{ex vivo} studies dopamine mainly behaves as a MAO-B substrate (Glover et al., 1977, Garrick and Murphy, 1980, O'Carroll et al., 1983) and MAO-B inhibitors are routinely administered to PD patients as a means to reduce metabolism of dopamine. The reason for this apparent discrepancy is not completely resolved (Berry et al., 1994a, 1994b) but could relate to several circumstances. For example, while the compartmentalization of MAO-A and MAO-B seems to be preserved between species, humans are found to have higher MAO-B to MAO-A ratios in the striatum than rats (Fowler et al., 1987, Westlund et al., 1988, Saura et al., 1992). In addition, the degree of postsynaptic metabolism is suggested to be higher in humans than in rats (Stenström et al., 1987). Taken together, both of these species differences indicate that metabolism of dopamine to a larger extent depends on postsynaptic MAO-B in humans (Oreland et al., 1983). In addition, in PD patients as well as in animal models of PD, the reduced number of MAO-A containing dopaminergic terminals could further push the metabolism towards MAO-B. This possibility will be discussed in relation to the findings of Paper III (see section 4.2).

1.3.1.4 Dopamine synthesis and release in the PD state

In PD, reduced levels of dopamine are restored by supplementation with the precursor L-DOPA (Lloyd et al., 1975). The paradox in this situation is that the neurons which normally convert L-DOPA to dopamine degenerate. The intriguing question, which has been debated for many years, is where L-DOPA is decarboxylated and from where dopamine is subsequently released in the PD brain. The remaining dopaminergic neurons are likely responsible for a significant part of the dopamine release in the patient. However, in animal models of PD, where almost all dopaminergic neurons are destroyed, L-DOPA still produces a significant increase in extracellular dopamine (Abercrombie et al., 1990) indicating that other cell types may be involved in the production of dopamine from L-DOPA. Preclinical studies have demonstrated that the majority of striatal AADC disappear when dopaminergic neurons are lost but also that substantial amounts remain in other striatal cells (Hefti et al., 1980), including glial cells (Li et al., 1992, Nakamura et al., 2000), inter- or efferent neurons (Hefti et al., 1981, Melamed et al., 1981, Tashiro et al., 1989, Mura et al., 1995, Mura et al., 2000, Lopez-Real et al., 2003) and 5-HT terminals (Arai et al., 1996). Theoretically, L-DOPA can be converted to dopamine in all of these structures however only the 5-HT terminals express the VMAT needed for vesicular release of dopamine. Indeed,
substantial preclinical evidence suggests that the 5-HT terminals contribute to dopamine release in the dopamine denervated striatum (Tanaka et al., 1999, Navailles et al., 2010, Nevalainen et al., 2011). The finding that L-DOPA-induced dopamine output is nerve impulse-dependent (Miller and Abercrombie, 1999) and attenuated by reserpine pre-treatment (Kannari et al., 2000), further supports the role of the 5-HT-terminals in this process. The dynamics of dopamine release in the absence of dopamine neurons is however altered, as the regulatory effects of the presynaptic D2 receptors and the DAT are lost (Maeda et al., 1999, Miller and Abercrombie, 1999).

1.3.2 L-DOPA

The pharmacodynamic effects of L-DOPA are mediated by dopamine formed following decarboxylation (see Figure 3). L-DOPA can therefore be considered a prodrug. In the first clinical trials with L-DOPA a racemic mixture of D- and L-DOPA was used, the D-form is however not decarboxylated to dopamine and was additionally shown to induce hematologic side effects (Cotzias et al., 1967) and was therefore excluded. L-DOPA is taken per orally and is actively absorbed in the small intestine by the LNAA transporter. AADC is widely distributed in peripheral tissues such as the intestine and endothelial cells of the capillaries which allows for extracerebral formation of dopamine from the drug (Rahman et al., 1981). Indeed, inhibition of peripheral AADC was shown to increase central levels of catecholamines from L-DOPA and to increase the locomotor stimulant effect of the drug in animals (Bartholini et al., 1967, Butcher and Engel, 1969). Dopamine in itself has poor availability over the BBB (Bertler et al., 1966, Oldendorf, 1971) but can access peripheral vascular dopamine receptors and receptors in the area postrema where the BBB is leaky. Peripheral conversion of L-DOPA to dopamine causes many of the side effects of the drug such as vomiting, nausea and ortostatic hypotension, which are reduced by co-administration of L-DOPA with a peripheral AADC-inhibitor (PDI) such as carbidopa or benzerazide (Cotzias et al., 1969, Papavasiliou et al., 1972) (see Figure 3). The addition of a PDI improves the pharmacokinetics of L-DOPA by dramatically increasing the bioavailability and half-life, enabling a 75% reduction in the daily dose of L-DOPA needed to produce a clinical effect (Deleu et al., 2002). The plasma concentration of L-DOPA (+ PDI) reaches peak-values within an hour and the plasma half-life is short, approximately 1-2 hours (Nutt et al., 1985, Deleu et al., 2002). L-DOPA is transported over the BBB using the LNAA for which there is a competition with other dietary amino acids (Alexander et al., 1994). In the CNS, L-DOPA is decarboxylated to dopamine and the relief of parkinsonian symptoms is achieved by the subsequent activation of postsynaptic dopaminergic receptors. Patients typically require 400-500 mg L-DOPA/day which is administered in a preparation of 100/25 (L-DOPA and benserazide in mg respectively) taken four times daily.

1.3.2.1 Therapeutic effects and side effects of L-DOPA

L-DOPA is the single most effective agent to relieve the symptoms of PD (Goetz et al., 2005). It efficiently controls the majority of motor symptoms of PD (Cotzias et al., 1967), the exceptions being later onset motor symptoms such as freezing of gait and postural instability. L-DOPA improves quality of life, increases the time patients can manage on their own and, in addition, increases survival (Chen et al., 2006). Almost all
patients respond to L-DOPA treatment and initially enjoy a sustained improvement of motor function; this period is often referred to as the “honeymoon”. Patients are relatively unaware of fluctuations in the motor response in relation to the administration of a new dose of L-DOPA and the clinical response can outlast the fall in plasma levels of L-DOPA. This type of response which can be sustained even if an L-DOPA dose is missed is termed the long-duration response (LDR). The LDR is successively built up over days to weeks after the initiation of L-DOPA therapy and contributes substantially to the overall motor improvement produced by chronic treatment (Anderson and Nutt, 2011). The LDR was first identified by the gradual deterioration in motor function occurring over several days following L-DOPA withdrawal (Cotzias et al., 1967, Muenter and Tyce, 1971). Over the time course of treatment and as the disease progresses the pharmacokinetics and pharmacodynamics of L-DOPA are altered and a short duration response (SDR) to L-DOPA becomes apparent (Nutt and Holford, 1996, Obeso et al., 2000a, Deleu et al., 2002, Nutt, 2003, 2008, Olanow et al., 2009b, Anderson and Nutt, 2011). The SDR is a measure of the duration of benefit from one single dose of L-DOPA (Olanow et al., 2009b); it closely parallels plasma levels of L-DOPA and sets in within minutes following drug administration (Nutt et al., 1992, Contin et al., 1994). The SDR is measurable from the start of L-DOPA treatment but may be too subtle to be noticed by the patient and is then masked by the LDR (Olanow et al., 2009b, Anderson and Nutt, 2011). The emergence of an apparent SDR, however, marks the onset of motor fluctuations and dyskinesias which develop in as many as 70-80% of patients following long-term treatment (Ahlskog and Muenter, 2001). Motor fluctuations are characterized by an apparent improvement of motor function “ON” following each given dose of L-DOPA and an apparent decline in motor ability “OFF” between dosing intervals. This type of fluctuating response is termed wearing off phenomenon or end-of-dose deterioration. Another type of motor fluctuation which commonly appears later on is the ON/OFF phenomenon with rapid and unpredictable transitions between “ON” and “OFF” states without apparent correlation to L-DOPA levels. The most common form of dyskinesia occurs when plasma concentrations of L-DOPA peak and is therefore termed peak-dose dyskinesia. Peak-dose dyskinesia is abnormal involuntary, jerky movements with a dance-like pattern which are viewed as a sensitized response to therapy. Diphasic dyskinesias are characterized by ballistic movements of the legs which occur when L-DOPA levels are increasing or decreasing. Dyskinesias can also present as prolonged muscle spasms/dystonia (Obeso et al., 2000a). The degree of dyskinesia determines the impact it has on the quality of life; mild dyskinesias can even be preferred to being in an “OFF” state. Severe dyskinesia however has a dramatic negative influence on several aspects of daily life such as social interaction, mobility, and balance (Encarnacion and Hauser, 2008). The risk factors for developing motor fluctuations and dyskinesias are duration and severity of disease and dosage and duration of L-DOPA treatment (Schrag and Quinn, 2000). Thus, patients with an early disease onset (Quinn et al., 1987) and patients treated with high doses of L-DOPA (Fahn, 2005, Sharma et al., 2006, Sharma et al., 2008) are much more likely to develop motor complications. Motor fluctuations often precede the onset of dyskinesias and therefore also represent a risk factor (Encarnacion and Hauser, 2008). The mechanism behind the shift from a stable to a fluctuating response accompanied by dyskinesias remains unknown. The peripheral pharmacokinetics of the drug remains
unaltered during the course of treatment (Gancher et al., 1987, Nutt et al., 1992). Therefore changes in the central kinetics of L-DOPA, dopamine release and the postsynaptic response have been suggested (see section 1.3.4). Patients who experience motor fluctuations and peak-dose dyskinesias are not easily managed and represent a major clinical challenge, as both the motor benefit and dyskinesias seem to depend on L-DOPA dosage. Thus manipulations to reduce dyskinesias also reduce the symptomatic effect, indicating a narrowing of the therapeutic window. The existence of a therapeutic window in severely dyskinetic patients has even been questioned i.e. the threshold concentration for anti-parkinsonian effect and dyskinesia are suggested to be similar or identical (Nutt, 2008). The occurrence of motor complications as a side effect to L-DOPA treatment was observed in the early trials of the drug (Cotzias et al., 1969, Yahr et al., 1969), therefore several other treatment strategies evolved to prevent or reduce their incidence. A brief description of these, in chronological order of introduction, follows below. It should however be mentioned that none of the newer agents have replaced the use of L-DOPA, but may provide substantial benefit as initial monotherapy or adjunct treatment to reduce motor complications.

1.3.3 Other pharmacological agents for PD

1.3.3.1 Dopamine receptor agonists

Dopamine receptor agonists, generally called dopamine agonists, are routinely used as monotherapy and adjunct treatment to L-DOPA and provide symptom relief by directly activating dopamine receptors. The dopamine agonists used in PD-treatment differ in their pharmacokinetic and pharmacodynamic profile (Deleu et al., 2002, Nyholm, 2006) but mainly activate dopamine D2 receptors. Dopamine agonists have several potential advantages over L-DOPA; the longer half-life will provide increased duration of the therapeutic effect and the direct action at dopamine receptors eliminates the need of the enzymatic machinery. The ergot-derivate bromocriptine was first introduced as adjuvant treatment to L-DOPA in the 1970s (Calne et al., 1974) and was later followed by several other ergot derivates. The use of ergot derivates is however limited by their adverse event profile including cardiac valve fibrosis (Rascol et al., 2004) and has thus largely been replaced by the newer non-ergot derivates such as pramipexole and ropinirole. Dopamine agonists have been shown to induce significantly less dyskinesias as compared to L-DOPA (Rinne et al., 1998, Parkinsonstudygroup, 2000, Rascol et al., 2000, Bracco et al., 2004, Holloway et al., 2004, Oertel et al., 2006) and to efficiently reduce motor fluctuations by reducing “OFF” time (Lieberman et al., 1998, Pinter et al., 1999). Once L-DOPA treatment is initiated patients are at the same risk of developing dyskinesias but the onset is significantly delayed (Rascol et al., 2006). There have also been reports on a disease modifying i.e. neuroprotective effect of dopamine agonists (Parkinsonstudygroup, 2002, Whone et al., 2003) but there is yet no consensus on the matter (Ahlskog, 2003). Subcutaneous injections of apomorphine, a short acting D1 and D2 agonist with rapid onset is used to provide a quick rescue from disabling “OFF” states (Stibe et al., 1988). In similarity to L-DOPA administration, dopamine agonists induce nausea, vomiting and orthostatic hypotension. Dopamine agonists, however, have a higher propensity to induce somnolence, hallucinations and impulse control disorders as compared to L-DOPA (Olanow et al., 2009b). The benefit provided by
dopamine agonists with regards to motor fluctuations and dyskinesias make them an attractive first hand choice in PD-treatment, especially in young-onset PD patients.

1.3.3.2 MAO-B inhibitors

MAO-inhibitors increase brain levels of catecholamines by inhibition of their metabolism (see Figure 3). Non-selective, irreversible MAO-inhibitors were introduced as antidepressants in the 1950’s, but have been replaced by other agents due to their harmful cardiovascular side effects, the “cheese effect”. MAO-A is preferentially involved in the metabolism of dietary amines such as tyramine. Inhibition of the enzyme can cause accumulation of tyramine which displaces noradrenaline in the sympathetic nerve terminal thereby causing hypertensive crisis (Horwitz et al., 1964). Reversible inhibition of MAO-A or selective inhibition of MAO-B can be used to circumvent the “cheese effect”. Moclobemide, a reversible inhibitor of MAO-A, is currently used for treating depression but may also have beneficial effects in PD patients (Youdim and Weinstock, 2004). The irreversible MAO-B-inhibitors selegiline and rasagiline are routinely used in PD. Selegiline (L-deprenyl), developed by Knoll and colleagues (Knoll et al., 1965), was the first inhibitor to be evaluated for PD treatment (Birkmayer et al., 1975). Selegiline potentiates the symptomatic effect of L-DOPA (Birkmayer et al., 1975, Heinonen and Rinne, 1989), reduces the dose of L-DOPA needed for symptom relief (Larsen et al., 1999, Pålhagen et al., 2006) and reduces motor fluctuations (Ives et al., 2004). Selegiline was also found to have mild symptomatic effects and can be used as monotherapy early on in the disease (Parkinsonstudygroup, 1993). The clinical effectiveness of selegiline is mainly attributed to decreased metabolism of dopamine in the CNS (Riederer and Youdim, 1986). Other effects, such as stimulated release via PEA (Paterson et al., 1991) or the active metabolites l-metamphetamine and l-amphetamine (Reynolds et al., 1978, Karoum et al., 1982) as well as blocked re-uptake of dopamine (Lai et al., 1980, Azzaro and Demarest, 1982, Fagervall and Ross, 1986) have also been suggested to contribute. Rasagiline is another, more potent MAO-B inhibitor which has similar effects as selegiline. It is effective as monotherapy and reduces motor fluctuations as adjunct to L-DOPA (Stocchi et al., 2008).

MAO-B inhibitors have attracted much attention due to their possible neuroprotective effects. The potential for neuroprotection was spurred by the finding that MAO-B was involved in the neurotoxic effects of MPTP (Chiba et al., 1984) and that selegiline, via the inhibition of MAO-B, protected dopaminergic neurons from MPTP toxicity (Cohen et al., 1984, Heikkila et al., 1984). Both selegiline and rasagiline have been shown to slow down the progression of motor disability in clinical trials (Parkinsonstudygroup, 1993, Palhagen et al., 2006, Olanow et al., 2009a). Despite these promising results the issue of neuroprotection is not firmly established (Fox et al., 2011) and the mechanism by which MAO-B-inhibitors would mediate neuroprotection remains elusive (Tatton and Chalmers-Redman, 1996, Olanow et al., 2009b). Due to their potential neuroprotective effects and mild symptom relief MAO-B inhibitors provide an attractive treatment strategy for early PD and are also useful as adjuncts to reduce motor fluctuations in advanced stages of the disease.
1.3.3 COMT inhibitors

COMT inhibitors were introduced in the 1990s and used as adjunct treatment to the standard formulation of L-DOPA and a PD. There are two COMT inhibitors available, entacapone and tolcapone. The peripheral metabolism of L-DOPA is mainly carried out by AADC, following blockade of this enzyme the L-DOPA is readily metabolized by COMT. COMT inhibitors act by inhibiting peripheral conversion of L-DOPA to 3-O-methyldopa (3-OMD) (see Figure 3), thereby increasing its bioavailability, plasma half-life and delivery to the CNS (Kaakkola, 2000). The addition of entacapone to L-DOPA was shown to increase the plasma half life of L-DOPA to two and a half hours (Nutt et al., 1994). Tolcapone has been shown to penetrate the BBB in animals studies (Forsberg et al., 2003) and could thus potentially increase central levels of L-DOPA and reduce the conversion of dopamine to 3-MT (see Figure 3). Tolcapone has been associated with liver toxicity (Olanow, 2000) and therefore liver function has to be monitored during its use. Addition of COMT inhibitors to L-DOPA effectively increases ON-time thereby preventing motor fluctuations and wearing off but may increase the risk for expression of dyskinesias if the dose of L-DOPA is not reduced (Lees, 2008). The addition of entacapone to L-DOPA in early PD-patients was not shown to reduce the development of dyskinesias (Stocchi et al., 2010). COMT inhibitors are therefore mainly used as adjuncts to L-DOPA to reduce motor fluctuations.

1.3.4 Mechanisms underlying motor complications and their management

As previously mentioned L-DOPA provides superior symptomatic control but the treatment is complicated by the high degree of motor complications. A major challenge in PD research is thus to find strategies to prevent or reduce L-DOPA-induced side effects by identifying the mechanisms underlying their development and expression. Although central kinetics of L-DOPA appears to be crucial for the emergence of motor complications, the pathogenic mechanisms responsible for the development of motor complications remain elusive. The currently held view integrates both presynaptic and postsynaptic alterations in the nigrostriatal connection (Cenci and Lundblad, 2006, Olanow et al., 2006, Iravani and Jenner, 2011).

1.3.4.1 Presynaptic mechanisms underlying development of motor complications

Individuals treated with L-DOPA for many years who were misdiagnosed with PD never developed motor complications (Barbeau, 1969). Motor complications are however rapidly induced in MPTP-exposed, severely dopamine depleted, humans and monkeys treated with L-DOPA (Ballard et al., 1985, Pearce et al., 1995). Analogously, in experimental animals the development of dyskinesias was shown to depend on the extent of denervation (Schneider, 1989, Boyce et al., 1990b, Papa et al., 1994, Di Monte et al., 2000, Winkler et al., 2002). The integrity of the dopaminergic system therefore seems to be of paramount importance for the correct handling of exogenous L-DOPA, i.e. the synthesis and packing of dopamine in vesicles and subsequent physiological release of the transmitter (Olanow et al., 2006). As the dopaminergic neurons progressively decrease in number, the ability to buffer dopamine produced by
exogenous L-DOPA is thought to decline (Chase et al., 1988, Chase, 1998). As a consequence, dopamine release will largely be mediated by non-dopaminergic cells lacking the proper regulatory machinery for regulated release (Maeda et al., 1999, Miller and Abercrombie, 1999). The system becomes increasingly dependent on exogenously supplied L-DOPA and owing to L-DOPA’s short half-life the levels of dopamine will oscillate considerably between doses. These fluctuations are in sharp contrast to the relatively constant levels of dopamine in the normal striatum which is maintained by the tonic firing of SNpc neurons (Floresco et al., 2003, Grace, 2008). The constant activation of dopamine receptors is crucial for normal basal ganglia function (Obeso et al., 2000b) and intermittent pulsatile stimulation of dopamine receptors is thought to result in a cascade of maladaptive postsynaptic changes ultimately responsible for the expression of motor complications (Cenci and Lundblad, 2006, Olanow et al., 2006).

In addition, the degeneration of dopaminergic neurons has been shown to result in an excessive increase in extracellular dopamine following L-DOPA administration in dopamine-depleted animals as well as in PD patients (Abercrombie et al., 1990, Tedroff et al., 1996). It is not clear whether this excessive release is involved in the development or expression of dyskinesias but peak-dose dyskinesia is associated with high levels of striatal dopamine following L-DOPA administration in PD (de la Fuente-Fernandez et al., 2004, Pavese et al., 2006) and the same phenomenon has been observed in animals (Meissner et al., 2006, Lee et al., 2008, Lindgren et al., 2010).

1.3.4.2 Postsynaptic mechanisms underlying development of motor complications

There is compelling evidence for postsynaptic sensitization in dyskinetic animals, especially in the D1-receptor expressing MSNs of the direct pathway. Cellular markers associated with the MSNs in the direct pathway such as prodynorphin mRNA and the precursor pre-proenkephalin B are up-regulated (Cenci et al., 1998, Winkler et al., 2002, Henry et al., 2003, Aubert et al., 2007), D1 receptor signaling is increased (Picconi et al., 2003, Aubert et al., 2005, Santini et al., 2007, Westin et al., 2007) and the trafficking of the D1 receptor is altered (Guigoni et al., 2007, Berthet et al., 2009). These findings suggest that LID is associated with hyperactivity of the direct pathway MSNs with subsequent alterations in the firing pattern of the output structures of the basal ganglia and increased facilitation of movement (Cenci, 2007) (see Figure 1). Indeed, both GPi and SNr show abnormal firing patterns in dyskinetic patients and animals (Papa et al., 1999, Alonso-Frech et al., 2006, Meissner et al., 2006). Accordingly, GABA levels are increased in the SNr but not in the GPe in dyskinetic rats (Mela et al., 2007). The sensitized D1-signaling cascade indicates that activation of this receptor increases the risk for motor complications. In fact, dopamine D1 receptor antagonists have been shown to reduce dyskinesia (Westin et al., 2007). However, activation of the D1 receptor is of importance for the full symptomatic effect, therefore D1 receptor antagonists do not represent a viable strategy to reduce motor complications (Grondin et al., 1999).
Introduction

1.3.4.3 Management of motor complications

A therapeutic strategy in PD is to mimic the physiological stimulation of dopamine receptors, i.e., to provide continuous dopaminergic stimulation and thereby reduce the risk for motor complications (Chase et al., 1989, Olanow et al., 2006). Preclinical studies have shown that the half-life of the dopaminergic agent and mode of administration, continuous versus intermittent, is correlated to the risk for motor complications (Blanchet et al., 1995, Bibbiani et al., 2005a). Accordingly, patients treated with long-acting dopamine receptor agonists develop less motor complications as compared to patients treated with L-DOPA (Rinne et al., 1998, Bracco et al., 2004, Holloway et al., 2004, Oertel et al., 2006). In order to delay the development of dyskinesia, treatment is often initiated with a dopamine agonist or an MAO-B inhibitor, especially in younger patients (Olanow et al., 2009b). COMT inhibitors have been shown to reduce dyskinesias in animal studies (Smith et al., 2005, Marin et al., 2006a) but this has not been confirmed in patients (Stocchi et al., 2010), which could possibly relate to the fact that this dose regimen still allows levels of L-DOPA and dopamine to fluctuate (Kuoppamaki et al., 2009).

Once they appear motor fluctuations can be controlled by adding a dopamine agonist (Lieberman et al., 1998, Pinter et al., 1999), a COMT-inhibitor (Lees, 2008) or an MAO-B inhibitor (Stocchi et al., 2008), by subcutaneous injections or infusions of apomorphine (Pietz et al., 1998) or continuous infusion of L-DOPA (Nyholm and Aquilonius, 2004). Established dyskinesias are not easily managed as adjunctive treatment with dopamine agonists, COMT-inhibitors and MAO-B inhibitors may worsen dyskinesias and strategies to reduce dyskinesias typically worsen Parkinsonism (Encarnacion and Hauser, 2008). Infusion therapies with L-DOPA or apomorphine have however been reported to reduce the occurrence of pre-existing dyskinesias (Katzenschlager et al., 2005, Eggert et al., 2008). Amantadine was initially used as an antiviral agent and discovered to provide benefit against tremor and akinesia in PD-patients (Schwab et al., 1969). Amantadine is the only clinically used pharmacological agent with reported anti-dyskinetic effects (Verhagen Metman et al., 1998, Del Dotto et al., 2001). The antidyskinetic properties of amantadine may be attributed to antagonism of NMDA receptors which are implicated in striatal plasticity (see section 1.4.2). The use of amantadine is however limited by cognitive side effects. Clozapine is an atypical antipsychotic agent used to treat drug-induced psychosis in PD-patients; it has also been shown to provide anti-dyskinetic effects but is not routinely used (Pierelli et al., 1998, Durif et al., 2004).

As most PD patients eventually require L-DOPA throughout the course of their treatment, much effort is made to further unravel the mechanisms which underlie the development of LID and to identify novel treatments to improve motor complications. The available animal models to study LID and novel experimental approaches derived from these will subsequently be discussed.
1.4 L-DOPA-INDUCED DYSKINESIA (LID)

1.4.1 Animal models of LID

Animal models of PD have provided an invaluable tool in the development of therapeutics for treatment of PD symptoms such as L-DOPA (Carlsson et al., 1957). In addition, models of PD have contributed to the understanding of the pathogenic mechanisms involved in the development of motor complications. There are several different ways to mimic the dopamine deficiency in PD in order to evaluate the effects of anti-parkinsonian therapies (Duty and Jenner, 2011), whereas the study of motor complications has been successfully performed in two models based on a toxic insult to catecholaminergic neurons following administration of the selective neurotoxins 6-hydroxydopamine (6-OHDA) or MPTP. These models mimic the permanent loss of dopamine which causes the motor symptoms of PD and thereby confer face validity.

1.4.1.1 The 6-OHDA model

In the late sixties Ungerstedt showed that intracerebral injections of 6-OHDA into catecholamine rich regions of the rat brain caused depletion of dopamine and noradrenaline (Ungerstedt, 1968). 6-OHDA is structurally similar to dopamine, with the exception of the hydroxyl group, and is taken up into dopaminergic and noradrenergic neurons by their respective transporter to accumulate within the cytosol. The mechanism by which 6-OHDA destroys catecholaminergic neurons is not completely understood, but it involves oxidative stress induced by ROS formed from its autoxidation (Sachs and Jonsson, 1975) and direct inhibition of mitochondrial complexes (Glinka and Youdim, 1995). 6-OHDA is administered via intracerebral injections since it does not cross the BBB. The injection sites and amounts can be varied to reproduce different stages of the disease. The unilaterally lesioned or “hemiparkinsonian” model is preferred since bilateral injections of 6-OHDA negatively influences feeding-behavior (Dunnett et al., 1983). In the present thesis work the toxin was injected in the median forebrain bundle (MFB) along which all forebrain monoaminergic projections run, this procedure results in $\approx 95\%$ reduction of striatal dopamine and may be suggested to model a very late stage of PD. The dopaminergic imbalance between the lesioned and intact striatum is manifested in a rotational behavior and animals rotate away from the hemisphere with dominant activation (Ungerstedt, 1976). The direction of turning is classified with reference to the lesioned striatum i.e. ipsi- and contralateral rotation. Ipsilateral rotation predominates in the untreated animal and reflects the dominant activation of the intact striatum. Systemic administration of amphetamine will act exclusively on the intact side and increases ipsilateral turning intensity (Zetterström et al., 1986). Contralateral rotation, on the other hand, is induced by L-DOPA and dopamine agonists and is thought to reflect activation of supersensitive dopamine receptors (Ungerstedt, 1971) (see Figure 4). This functional asymmetry can be used to evaluate the effects of symptomatic treatments by measuring the elicited rotational activity (Schwarting and Huston, 1996). The animals also develop asymmetry in the use of the limbs which is seen as difficulties in adjusting posture and to move during spontaneous behaviors such as grooming, exploration and walking (Cenci et al., 2002). These deficiencies are used to pharmacologically evaluate anti-parkinsonian effects in the cylinder and rotarod tests.
The unilateral model of PD was further developed by the Cenci-group as a means to study LID (Cenci et al., 1998). Rats express abnormal involuntary movements (AIMs) following L-DOPA administration and these affect the orofacial region, the trunk of the body and the limbs. AIMs are characterized by their purposeless, repetitive pattern which interferes with physiological motor activities. Rodent AIMs bear resemblance to peak-dose dyskinesias observed in humans although they differ by their species specific motor repertoire (Cenci et al., 2002). The development/expression of AIMs is dependent on dopaminergic lesion extent (Winkler et al., 2002) and in similarity with patients the severity increases with the L-DOPA dose (Lindgren et al., 2007). The model has been additionally validated by screening drugs with low liability to induce dyskinesias and drugs known to elicit anti-dyskinetic effect in primates (Lundblad et al., 2002, Dekundy et al., 2007). The scoring of rodent AIMs thus represent a widely used preclinical screening model for putative anti-dyskinetic agents.

**Figure 4.** Rotational behavior in the unilateral 6-OHDA lesion model. 6-OHDA is injected in the right hemisphere, the motor system is crossed at the level of the medulla and the left side of the rodent body will therefore display motor deficits. The asymmetry can be observed as a rotational behavior and the rat will rotate away from the hemisphere with the dominant activation. In the untreated state, the rat rotates away from the left hemisphere and the rotation is directed towards the right lesioned striatum, ipsilateral rotation. Amphetamine stimulates dopamine release from presynaptic dopaminergic terminals in the intact striatum and further increase ipsilateral rotation. Administration of L-DOPA reverses the rotational behavior and animals rotate away from the lesioned side, rotation is thus directed towards the left intact hemisphere, contralateral rotation. Modified from (Schwarting and Huston, 1996).

1.4.1.2 The MTPT-model

The non-human primate MPTP model was developed following the observation that this toxin produced irreversible parkinsonism in humans (Langston et al., 1983). MPTP it is metabolized to MPP\(^+\) by MAO-B (Chiba et al., 1984), which is taken up into dopaminergic neurons (Javitch et al., 1985) where it interferes with the mitochondrial complex 1 (Nicklas et al., 1985). MPTP can be administered systemically and is able to penetrate the BBB. The toxin can also be used in some mice strains but has limited effects in rats. MPTP-treated monkeys exhibit motor features similar to PD patients i.e. bradykinesia, rigidity, tremor and postural instability as well as dyskinesias arising from chronic L-DOPA treatment (Boyce et al., 1990a, Langston et al., 2000). The MPTP-model is predictive for symptomatic and anti-dyskinetic agents and is commonly used as the last step before taking a drug into clinical trial (Duty and Jenner, 2011).
1.4.2 Novel experimental approaches to target LID

The intricate interplay between the dopaminergic system and other basal ganglia modulators such as glutamate, serotonin, noradrenaline and adenosine in the regulation of basal ganglia output has led to an emerging interest of their involvement in motor complications (Brotchie, 2005). Several strategies aimed at targeting non-dopaminergic systems as a means to provide symptomatic relief and/or reduce dyskinesias have been investigated (Fox et al., 2008) and the next section will therefore provide a brief overview of this area.

1.4.2.1 Glutamate

Increased glutamatergic transmission has been implicated in both the induction and expression of dyskinesias (Chase and Oh, 2000, Brotchie, 2005, Jenner, 2008). Glutamatergic neurons converge onto the MSNs and activate ionotropic NMDA and AMPA receptors as well as metabotropic glutamate receptors. Dopamine receptors modulate the gating and trafficking of glutamatergic NMDA and AMPA receptors via intracellular signaling cascades in the MSNs (see section 1.2.4.4). This functional interaction is affected by the pulsatile stimulation of dopamine receptors which occurs during chronic L-DOPA treatment (Chase and Oh, 2000) and various alterations in glutamate receptor function has been found in dyskinetic animals (Iravani and Jenner, 2011). The sensitized response of the D1signaling cascade and the close interaction of this receptor and glutamate receptors (Conn et al., 2005, Fiorentini et al., 2008) further supports the notion that strategies targeting glutamatergic transmission may be beneficial in the treatment of dyskinesis. Indeed, the NMDA receptor antagonist amantadine alleviates dyskinesias in PD patients (Verhagen Metman et al., 1998, Del Dotto et al., 2001) and several preclinical studies have indeed shown beneficial effects of various glutamate antagonists on dyskinesia (Blanchet et al., 1998, Bibbiani et al., 2005b, Mela et al., 2007, Rylander et al., 2009, Rylander et al., 2010a) (for review see (Iravani and Jenner, 2011)).

1.4.2.2 Noradrenaline

The noradrenergic system is involved in the pathophysiology PD as loss of noradrenergic neurons of the LC is commonly observed post mortem (Hornykiewicz and Kish, 1987, Zarow et al., 2003, Fornai et al., 2007, McMillan et al., 2011). The loss of noradrenergic neurons in PD is implicated in motor deficits, such as freezing of gait and postural instability, as well as in non-motor deficits, such as dementia, depression and impaired attention (Delaville et al., 2011).

Although sparsely innervated, there is anatomical evidence for a noradrenergic projection to the striatum (Lindvall and Björklund, 1974, Mason and Fibiger, 1979, Jones and Yang, 1985) and to the substantia nigra (Jones and Yang, 1985). Adrenergic receptors are highly expressed in the basal ganglia system, the $\alpha_{2A}$-, $\alpha_{2C}$- and $\beta_{1}$-receptors are found in the striatum (Nicholas et al., 1996, MacDonald et al., 1997), and $\alpha_{2C}$-receptors have been localized to striatal MSNs of both the direct and indirect pathway (Holmberg et al., 1999). In the SN both $\alpha_{1}$-receptors (Jones et al., 1985) and $\alpha_{2C}$-receptors (Lee et al., 1998) are expressed. Besides a modulation of the dopaminergic system at the somatodendritic and terminal level (Grenhoff et al., 1993,
Hertel et al., 1999, Gobert et al., 2004), noradrenaline may modulate postsynaptic signaling cascades in the MSNs via α2-receptor activation (Hara et al., 2010). Indeed, noradrenaline has been measured in the striatum in vivo (Cenci et al., 1992, Li et al., 1998, Dawson et al., 2000, Gobert et al., 2004) and striatal noradrenaline release was found to be under the control of α2 receptors and to increase following administration of the selective noradrenaline reuptake inhibitor reboxetine (Gobert et al., 2004).

Preclinical studies in animal models of PD suggest that the noradrenergic system may be implicated in LID. This conclusion is derived from the finding that α2-receptor antagonists may provide an antidyskinetic effect without interfering with the antiparkinsonian effect of L-DOPA (Gomez-Mancilla and Bedard, 1993, Henry et al., 1999, Grondin et al., 2000, Fox et al., 2001, Savola et al., 2003, Johnston et al., 2010). However, the mechanism by which α2-antagonists mediate an anti-dyskinetic action remains elusive but may involve attenuation of dopamine release from L-DOPA (Buck et al., 2010) and/or modulation of GABA release in the basal ganglia nuclei (Zhang and Ordway, 2003, Alachkar et al., 2006). It is unclear wheter this latter effect is mediated via antagonism of postsynaptic α2-receptor activation by the endogenous ligand or via blockade of presynaptic auto-inhibitory receptors resulting in increased terminal release of noradrenaline (Gobert et al., 2004). Despite some promising results in PD patients, idazoxan was not further investigated due to limiting side effects (Rascol et al., 2001).

1.4.2.3 Serotonin

The serotonergic system is involved in a complex regulation of the basal ganglia system and possibly in both the induction and expression of LID (Fox et al., 2009). The release of dopamine from the 5-HT terminals has been proposed to contribute to pulsatile stimulation of dopamine receptors and to the expression of LID (Carta et al., 2007). Additionally, dyskinetic animals and patients show maladaptive plasticity in 5-HT system with an increased sprouting of striatal 5-HT terminals (Rylander et al., 2010b). Serotonergic autoreceptor agonists have been shown to dampen the release of dopamine from L-DOPA (Kannari et al., 2001, Lindgren et al., 2010) and to reduce the expression of dyskinesias (Carta et al., 2007, Munoz et al., 2008, Lindgren et al., 2010). Consistent with these findings, saritozan, a partial 5-HT1A agonist, was shown to reduce dyskinesias in PD patients (Olanow et al., 2004b, Bara-Jimenez et al., 2005). In another study, saritozan was, however, not effective in this regard and higher doses of the drug were found to worsen Parkinsonism (Goetz et al., 2007).

1.4.2.4 Adenosine

A2A receptors, expressed in the indirect pathway MSNs, are functionally linked to D2-receptors and antagonize their activation. A2A antagonists may thus provide symptomatic relief by increasing the responsiveness of the D2-receptor upon activation and subsequently reduce the activity in the indirect pathway (Ferre et al., 1997, Fuxe et al., 2007). In animal models of PD, A2A antagonists have been shown to increase the locomotor stimulant effect of dopaminergic drugs (Koga et al., 2000, Lundblad et al., 2003) and to reduce the development of apomorphine-induced dyskinesias (Bibbiani et al., 2003). The potential anti-dyskinetic effect of A2A antagonists may involve D2 receptor independent mechanisms (Fuxe et al., 2007). Istradefylline is an A2A antagonist that has been evaluated as adjunct to L-DOPA in PD patients; it was shown...
to significantly reduce motor fluctuations but to slightly increase dyskinesias (Mizuno et al., 2010). Other clinical trials suggest that istradefylline may be useful to increase the symptomatic effect of a suboptimal dose of L-DOPA and thereby reduce the expression of dyskinesias (Bara-Jimenez et al., 2003).

1.5 DEUTERIUM-L-DOPA

1.5.1 Deuterium isotope effects

Deuterium or "heavy hydrogen" is a non-radioactive, naturally occurring, stable isotope of hydrogen which contains an additional neutron; it is termed D or $^2$H. The higher mass of the deuterium atom, as compared to that of hydrogen, dramatically increases its bond strength/stability to carbon (C) which requires more energy to be cleaved. The rate of a chemical reaction involving the cleavage of a C-H bond can thus be significantly reduced if hydrogen is exchanged by deuterium and this is defined as a primary isotope effect. The magnitude of the primary isotope effect is estimated by comparing the rate of the deuterium and hydrogen reaction, respectively. However, deuterium substitutions adjacent to the active cleavage site may also alter the rate of a chemical reaction, these secondary isotope effects are generally lower in magnitude (Foster, 1985). Deuterium has been widely used as a tracer in studies of drug pharmacokinetics and metabolism as well as in studies of endogenous biological processes (Foster, 1985, Koletzko et al., 1997, Kushner et al., 1999). Deuterium substitutions have also been evaluated as a strategy to reduce drug metabolism and hence improve pharmacokinetic and pharmacodynamic properties (Foster, 1985, Kushner et al., 1999). While none of the earlier candidates made their way into the clinic, the use of deuterium substituted drugs is presently gaining increasing interest (Sanderson, 2009).

1.5.2 In vitro isotope effects on dopamine metabolism and conversion to noradrenaline

Deuterium has also been used as a tool to study enzyme kinetics (Cleland, 2005). An isotope effect in an enzyme catalyzed reaction indicates that the bond which involves cleaving of the deuterium atom is the rate-limiting step. MAO catalyzes the first step in the oxidative deamination of the α-carbon in the dopamine molecule by the removal of one hydrogen and the amine group, which generates an aldehyde, DOPAL. The enzyme ALDH abstracts the remaining hydrogen atom which is replaced by a hydroxyl group to form the corresponding acid DOPAC (see Figure 5). Using deuterium labeled amines, dopamine, serotonin and tyramine, it was shown that the rate-limiting step in MAO-metabolism is the removal of the α-carbon hydrogen atom (Belleau et al., 1960, Yu et al., 1981, Yu et al., 1982, Yu et al., 1986, Yu, 1988). The magnitude of this primary isotope effect was found to be in the range of 2, indicating that the rate of oxidative deamination of dopamine by MAO is twice as slow when the α-carbon is deuteriated. Secondary isotope effects in the MAO catalyzed reaction were also observed following substitution of the second hydrogen at the α-carbon in the dopamine molecule (Yu et al., 1986) and following β-substitutions in the kynuramine molecule (Belleau and Moran, 1963).
Introduction

Figure 5. Oxidative metabolism of the α-carbon in the dopamine molecule is catalyzed in two steps by the sequential action of MAO and ALDH.

The conversion of dopamine to noradrenaline involves the cleavage of a C-H bond and the introduction of a hydroxyl group at the β-carbon, a reaction catalyzed by the enzyme dopamine β-hydroxylase (DβH) (see Figure 6). This reaction takes place inside of noradrenergic vesicles following transport of dopamine by the VMAT (Cooper et al., 2003). In vitro experiments have demonstrated that deuterium substitutions at β-carbon in the dopamine molecule may produce a large primary isotope effect on the rate of noradrenaline formation (Miller and Klinman, 1983). In addition, a secondary isotope effect on noradrenaline formation was found following α-carbon substitutions (Miller and Klinman, 1985). Taken together, deuterium substitutions in the dopamine molecule may significantly alter the rate of MAO and DβH activity via primary and secondary isotope effects.

Figure 6. Conversion of dopamine to noradrenaline via β-carbon hydroxylation.

1.5.3 Deuterium-L-DOPA

Against the given background, describing the limitations of current L-DOPA therapy and the apparent sensitivity of dopamine metabolism and noradrenaline formation to deuterium isotope effects, the present work aimed to investigate the potential utility of deuterium substitutions in the L-DOPA molecule as a means to improve the central kinetics and efficacy of the drug. There are five isoforms of deuterium-L-DOPA, produced by different combinations of α- and β-carbon substitutions (see Figure 7), which could display different metabolic profiles depending on the degree of primary and secondary isotope effects. As previously mentioned, in the decarboxylation process the carboxyl group is removed by the enzyme AADC and deuterium substitutions on the α- or β-carbons of the L-DOPA molecule would be retained in dopamine formed from it. We therefore hypothesized that dopamine formed from decarboxylation of deuterium-L-DOPA, carrying a deuterium atom at the α-carbon, would be more resistant to metabolic degradation by MAO and display an increased brain half-life. As a consequence, deuterium-L-DOPA might provide a more sustained stimulation of dopamine receptors and possibly allow lower doses of the drug to be used. Such properties would ultimately be advantageous in the treatment of Parkinson’s disease. Similarly, dopamine formed from deuterium-L-DOPA carrying deuterium atoms at the
β-carbon might display a reduced rate of noradrenaline formation. Therefore, the present thesis work aimed to evaluate the structure-activity relationship of different deuterium-L-DOPA isoforms \textit{in vivo} with regard to neurochemistry and motor behavior and to further investigate potential clinical benefits in an animal model of Parkinson’s disease.

\textbf{Figure 7.} Molecular structure of L-DOPA. Deuterium is introduced at the α- and/or β-carbons which are retained in the dopamine molecule following decarboxylation of L-DOPA. There are two hydrogens available for substitution on the β-carbon and one on the α-carbon. The nomenclature of the L-DOPA isoforms is derived from the position and number of deuterium substitutions.
2 SPECIFIC AIMS OF THE STUDY

The overall aim of the present study was to investigate if *in vitro* isotope effects on dopamine metabolism are significant following administration of deuterium-L-DOPA 
*in vivo*. The experiments were designed to specifically:

1. Investigate the structure-activity relationship of different isoforms of deuterium-L-DOPA on dopamine metabolism in the intact animal and motor behavior in the temporarily dopamine-depleted rat

2. Investigate if chronic administration of deuterium-L-DOPA might prove advantageous over standard L-DOPA in an animal model of LID

3. Compare the effects of deuterium-L-DOPA with the combination of standard L-DOPA and a clinically used MAO-B inhibitor on dopamine metabolism and motor behavior in an animal model of Parkinson´s disease
3 MATERIAL AND METHODS

3.1 ANIMALS

Male albino rats from the Wistar strain were used in all experiments with the exception of the experiments described in Paper II where female Sprague-Dawley rats were used. Upon arrival from the distributor, animals were acclimatized to the novel environment for five days before the start of an experiment. The animals were housed under temperature and humidity controlled conditions, on a 12 hour light/dark cycle. Standard laboratory rat chow and tap water was available ad libitum. All efforts were made to minimize the number of animals used and their suffering. Experiments were approved by, and conducted in accordance with, local animal ethics committees. In vivo microdialysis and locomotor activity experiments were approved by the Stockholm North Committee on Ethics of Animal Experimentation, ethical approval numbers: N340/02, N338/05, N 28/09 and N211/04. Behavioral experiments performed during chronic L-DOPA treatment were approved by the Malmö-Lund Committee on Ethics of Animal Experimentation, ethical approval numbers: M 249/05 and M 231/08. In vivo pharmacokinetic experiments were approved by the German Committee on Ethics of Animal Experimentation, ethical approval number: N 2443/06.

3.2 EXPERIMENTAL DESIGN (PAPER I, II, III AND IV)

In Paper I, four different isoforms of deuterium-L-DOPA were screened for isotope effects on striatal dopamine metabolism using in vivo microdialysis. The experiments were performed in two sets, each comparing two deuterium-L-DOPA isoforms to L-DOPA. The effect of the drugs was studied on dopamine and DOPAC output in intact animals. In addition, the effect of α,β,β-D3-L-DOPA and β,β-D2-L-DOPA was compared to L-DOPA for locomotor activity in reserpinized rats.

In Paper II, the acute and chronic effects of L-DOPA and α,β,β-D3-L-DOPA treatment were compared with regards to motor behavior in the 6-OHDA-lesioned rat. In the acute study, a dose-response curve was established using the L-DOPA-induced rotation test to quantify motor stimulation. A lower equipotent dose of α,β,β-D3-L-DOPA to L-DOPA was established and subsequently included in the chronic treatment design. Four treatment groups were thus studied; L-DOPA, α,β,β-D3-L-DOPA (both equivalent and equipotent to L-DOPA) and vehicle. Animals were administered the different treatments once daily for three weeks. The anti-parkinsonian effect was evaluated by the cylinder and the rotarod tests while dyskinesia was scored using the AIMS test.

In Paper III, the effects of α,β,β-D3-L-DOPA and L-DOPA alone or in combination with the MAO-B inhibitor selegiline were compared in 6-OHDA-lesioned animals. The animals were first evaluated for acute behavioral effects and, following a wash-out period, neurochemical effects were studied by means of in vivo microdialysis. Behavioral effects of the study drugs were evaluated by monitoring locomotor, rearing and rotation activity and neurochemical effects were studied by the simultaneous detection of striatal L-DOPA, dopamine, noradrenaline, DOPAC, 3-MT, HVA and 5-hydroxyindole acetic acid (5-HIAA).
In Paper IV, the effect of L-DOPA and α,β,β-D3-L-DOPA on striatal noradrenaline, dopamine and DOPAC were evaluated by means of in vivo microdialysis in intact rats.

3.3 DRUGS

3.3.1 Study drugs

Four different isoforms of deuterium L-DOPA, in which deuterium had been introduced at the α- or β-carbon of L-DOPA (see Figure 7), were synthesized at ChiroBlock GmbH, Germany and provided by BiRDS Pharma GmbH, Germany. The purity of these isotopic variants of L-DOPA was confirmed by the manufacturer using gas chromatography and mass spectroscopy. L-DOPA was purchased from Welding GmbH, Germany. L-DOPA and deuterium-L-DOPA were dissolved in different strengths of acidic solution (0.06-0.6 M HCl) depending on the concentration of the drug and whether or not it was co-administered with a PDI. Prior to injection the drug solution was pH balanced with the equimolar amount of NaOH.

Carbidopa (10 mg/kg; Paper I and IV; Welding GmbH, Germany) was dissolved following the same protocol as L-DOPA compounds and administered 30 minutes prior to their injection. Benserazide hydrochloride (7.5 mg/kg in Paper II and 12 mg/kg, Paper III; Sigma-Aldrich, Sweden) was dissolved in saline and co-administered with L-DOPA compounds. Selegiline (R(-)-Deprenyl hydrochloride, Sigma-Aldrich, Sweden) was dissolved in saline. Amphetamine (D-amphetamine, Sigma-Aldrich, Sweden) was diluted in saline.

3.3.2 Drugs used to induce experimental PD

Reserpine, a generous gift from AstraZeneca, Sweden, was dissolved in a minimal amount of glacial acetic acid and further diluted to its final volume in 5.5% sucrose solution. 6-OHDA-HCl (Sigma-Aldrich, Sweden) was dissolved in saline containing 0.02% ascorbic acid.

3.3.3 Drugs used for stereotaxic surgery

In Paper I, III and IV, animals were anaesthetized using a cocktail containing Hypnorm (fentanyl citrate 0.39 mg/kg and fluanisone 12.5 mg/kg, VetaPharma, United Kingdom) and midazolam 6.25 mg/kg (Hameln pharmaceuticals GmbH, Germany). In Paper III, rats were anaesthetized, by a mixture of fentanyl citrate (0.37 mg/kg, B. Braun, Germany) and medetomin-HCl (0.24 mg/kg, Orion Pharma, Finland). The local anaesthetic bupivacaine (Marcain 2.5 mg/ml, AstraZeneca, Sweden) was injected in to reduce pain. For post-operative analgesia the animals received an injection of buprenorphine (0.01 mg/kg, Temgesic, Schering-Plough, Belgium). Atropine (0.14 mg/kg, Merck NM, Sweden) was administered to reduce parasympathetic activity.

3.3.4 Other drugs

Tetrodotoxin (Sigma-Aldrich, Sweden), a blocker of voltage-gated sodium channels, was diluted in physiological perfusion solution (Apoteksbolaget, Sweden) and administered to the striatum via reversed dialysis in order to verify the action potential dependent nature of dopamine and noradrenaline peaks.
3.4 \textit{IN VIVO MICRODIALYSIS (PAPER I, III AND IV)}

3.4.1 Surgery and microdialysis

Briefly, concentrical dialysis probes, with an active dialysis length of 3.5 mm, were implanted under stereotaxic surgery. The coordinates used to target the striatum were (in mm relative to bregma and the dura mater) AP: +0.6, ML: -3.0, DV: -6.2 according to the atlas of Paxinos and Watson 4\textsuperscript{TH} or 6\textsuperscript{TH} edition. The probe was fixed to the skull with anchor screws and dental cement. Following surgery, the animals were housed individually. Dialysis experiments were conducted approximately 48 h after surgery in awake and freely moving animals. The probe was perfused by physiological perfusion solution (147 mM NaCl, 3.0 mM KCl, 1.3 mM CaCl$_2$, 1.0 MgCl$_2$ and 1.0 mM NaHPO$_4$, pH 7.4).

Apoteksbolaget, Sweden) at a steady flow rate of 2.5 µl/min which was controlled by a microinfusion pump (Harvard Apparatus, USA). Dialysis occurred through a semipermeable membrane (Filtral AN69, Hospal Industrie, France) and dialysate was collected in 15 (Paper I) or 30 minute (Paper III and IV) intervals.

3.4.2 Neurochemical analysis

Dialysate samples were automatically injected onto a C-18 column (Paper I and IV, Supelcosil 150*4.6 mm, 3 µM; Paper III, Kinetex 150*4.6 mm, 2.6 µM) for separation by high performance liquid chromatography (HPLC). The loading and injecting modes of the injector (Valco Instruments, USA) were controlled by the Totalchrom software (PerkinElmer, USA). The mobile phase was delivered to the column by a HPLC pump (Model 2150, Pharmacia LKB, Sweden or Model 2250, Bischoff Chromatography, Germany) at a flow rate of 0.7-0.8 ml/min. In paper I and IV the mobile phase consisted of a sodium acetate buffer (55 and 61 mM respectively) which was pH adjusted with glacial acetic acid to 4-5. In Paper III the mobile phase consisted of phosphate buffer (0.015 M) which was pH adjusted with phosphoric acid to ≈3. The mobile phases additionally contained methanol (10-13%), EDTA (0.01-0.03 mM) and heptanesulfonic or octanesulfonic acid. The online quantification of dopamine, noradrenaline and metabolites was achieved by electrochemical detection. Following separation, dialysis samples were subject to sequential oxidation and reduction in an analytical cell (ESA model 5011, Thermo scientific, USA) and the respective potentials (+ 400 mV and – 200 mV in Paper I and IV; + 250 mV and – 300 mV in Paper III) were controlled by a potentiostat (Esa Coulochem II or III, Thermo scientific, USA). Treatment was usually initiated following approximately 3-4 hours of dialysis when the baseline levels had stabilized (< 10 % variation).

3.4.3 Histological verification of probe placement

Following dialysis experiments, the rats were administered an overdose of sodium pentobarbital (≈120 mg/kg i.p., Apoteket AB, Sweden). In Paper I and IV the brain was immediately dissected and fixated in a solution containing 4% formaldehyde and 25% sucrose until further sectioning. In Paper III the animals were transcardially perfused and the tissue was processed for further immunohistochemical analysis (see section 3.5.1.2). All brains were cut on a microtome, sections showing tissue damage from the dialysis probe were mounted on superfrost slides, stained with neutral red and finally
dehydrated. The position of the dialysis probe was verified under light microscopy and compared to the rat brain atlas by Paxinos & Watson, 4\textsuperscript{TH} or 6\textsuperscript{TH} edition. Only animals showing probe damage in the striatum were included in the subsequent data analysis.

3.4.4 Data analysis and statistics

The experiments were analyzed in the TotalChrome software (PerkinElmer, United states) which generates peak area and height for each analyte and sample. The experimental data values were compared to those of a known analyte concentration and expressed as fmol/minute. In Paper II and IV basal concentrations of dopamine, DOPAC and noradrenaline (only Paper IV) were statistically compared between the treatment groups using one-way analysis of variance (ANOVA) and there were no significant differences between the groups. Therefore, data are presented and analyzed as the per cent change compared to baseline (the samples collected one hour preceding treatment). In Paper I, DOPAC/DA-ratios were calculated using the total output of DOPAC and dopamine, respectively. In Paper III, 6-OHDA lesioned animals were studied and extracellular concentrations of dopamine, noradrenaline, DOPAC, 3-MT and HVA were close to or below the detection limit of the assay at baseline. In fact, 3-MT levels were only detected in three animals and basal levels of noradrenaline were never detected. The effect of drug treatment on dopamine and metabolites was therefore presented in absolute values. Blank values, \textit{i.e.} concentrations below detection limit, were replaced by the lowest detectable value; dopamine 0.0027 fmol/min, DOPAC 0.053 fmol/min, 3-MT 0.0699 fmol/min and HVA 0.124 fmol/min. The existing basal values of dopamine, DOPAC and HVA were compared between treatment groups by means of one-way ANOVA and there were no significant differences between the groups. Basal levels of 5-HIAA were readily detectable and as no significant difference was found between treatment groups, data are presented and analyzed as the percent change compared to baseline (the samples collected one hour preceding treatment). Treatment effects over time were statistically compared by two-way ANOVA and followed by the Newman-Keuls post hoc test for multiple comparisons (Paper I) or the Fishers Least significant difference post hoc test (Paper III and IV) when appropriate, \textit{i.e.} when a significant time*treatment interaction was found in the ANOVA analysis. All statistical comparisons were calculated in the STATISTICA software (StatSoft, Inc. USA). The significance level was set to $\alpha=0.05$ in all tests.

3.5 THE RESERPINE MODEL OF PD (PAPER I)

Pharmacological treatment of PD is based on the seminal findings that L-DOPA was able to antagonize the akinetic effects induced by antipsychotic agent reserpine (Carlsson et al., 1957), which could be linked to restoration of central levels of dopamine (Carlsson et al., 1958). Reserpine inhibits vesicular storage of monoamines, dopamine, noradrenaline and 5-HT by inhibiting the VMAT (Schuldiner, 1994). Inhibition of vesicular storage causes depletion as the amines are catabolised in the cytoplasm; this phenomenon is observed as a rapid increase in metabolites following reserpine administration (Elverfors and Nissbrandt, 1991, Heeringa and Abercrombie, 1995). The dose of reserpine administered in the present study, 5 mg/kg, has been shown to cause a profound depletion of striatal dopamine and 5-HT which persists after
24 hours (Elverfors and Nissbrandt, 1991, Heeringa and Abercrombie, 1995). Reserpine-treated rats show the cardinal motor features of PD i.e. tremor, rigidity and hypokinesia (Colpaert, 1987). Systemic administration of anti-parkinsonian agents to reserpinized animals will reverse akinesia and increase locomotor activity. Quantification of locomotor activity thus provides a means to compare the motor stimulant effects of different drugs. Rats were housed on a 12 hr reversed light-dark cycle (lights off at 07:00). The night before experiments, rats were pre-treated with reserpine (5 mg/kg) or vehicle. Locomotor experiments were performed 10-12 hours later (see section 3.7.1).

3.6 THE 6-OHDA LESION MODEL OF PD (PAPER II AND III)

Some methodological aspects of the 6-OHDA-model are provided in the Introduction (see section 1.4.1.1). The rats were anaesthetized and mounted in a stereotaxic frame. The skin was cut and a hole was drilled in the skull bone. 6-OHDA-HCL (3.5 µg/µl free base 6-OHDA in 0.02% ascorbic acid, Sigma-Aldrich, Sweden) was injected at two sites to target the MFB 2.5 µl at (I) A-P -4.4, M-L -1.2, D-V -7.8, (tooth bar at – 2.4) and 2 µl at (II) A-P -4.0, M-L -0.8, D-V -8.0, (tooth bar at + 3.4) according to (Paxinos & Watson 6TH edition). The needle was left in place for 2.5 and 2 minutes, respectively, after the two injections.

3.6.1 Evaluation of 6-OHDA lesion extent

It is important to confirm similar lesion extent between treatment groups as the dopaminergic system is protective for the expression of dyskinesias (Winkler et al., 2002, Lundblad et al., 2004). In Paper II, lesions extent was behaviorally evaluated using amphetamine-induced rotation. Following completion of the behavioral experiments; the dopamine denervations was evaluated by tyrosine hydroxylase immunoreactivity or dopamine transporter binding. Only animals with ≥94% dopamine denervation were included in the subsequent statistical analysis. In Paper III, lesion success was evaluated by determination of basal concentration of dopamine in the lesioned striatum by means of dialysis. The cut off for inclusion in the study analysis was set to <0.04 fmol/µl (Lindgren et al., 2010). The brains were additionally evaluated for tyrosine hydroxylase immunoreactivity after completion of dialysis experiments.

3.6.1.1 Amphetamine-induced rotation (Paper II)

Systemic administration of amphetamine will stimulate dopamine release from the intact terminals and increase ipsilateral turning intensity (Zetterström et al., 1986). The animals were tested approximately one week after the lesion. The rotational response elicited by an injection of 2.5 mg/kg D-amphetamine (Sigma-Aldrich, Sweden) was quantified in an automated rotometer. The cut off rotational score was set to >5 ipsilateral rotations (relative to the lesion) per minute, indicating >90% of striatal dopamine denervation (Winkler et al., 2002).

3.6.1.2 Tyrosine hydroxylase immunohistochemistry (Paper II and III)

Animals were deeply anaesthetized with sodium pentobarbital (=120 mg/kg i.p. Apoteksbolaget AB, Sweden) and transcardially perfused with saline followed by ice cold 4% paraformadehyde (Sigma-Aldrich, Sweden). The brain was dissected and
placed in 4% paraformaldehyde for 2 hours and then transferred to 20 % sucrose (1-3 days). The brains were frozen on dry-ice and stored in -20°C until sectioning. Sections (40 µM Paper II; 30 µM Paper III) were cut on a freezing microtome and stored (cryoprotective solution at -20°C Paper I; 0.1 M PBS at 4°C Paper III) for further immunohistochemical analysis.

A detailed description of the immunohistochemical procedure can be found in the supplementary material accompanying Paper II and Paper III. Briefly, sections were rinsed and endogenous peroxidase activity was blocked by incubation with 3% H2O2 in methanol (Paper II). Unspecific antibody binding was reduced by incubation in 5% goat serum (Paper II) or 10% BSA (Paper III) for 1 hour. The tissue was incubated with the primary polyclonal antibody rabbit anti-tyrosine hydroxylase (Paper II; 1:1000, Pel-Freez Biologicals, United States, overnight) or (Paper III; 1:5000 AB152, Millipore, USA, 48 hours). Sections were further incubated with the secondary biotinylated antibody (Paper II; 1:200, goat anti-rabbit, Vector Lab, United States and Paper III; 1:400, sheep anti-rabbit, Vectastain Elite ABC, Vector Laboratories, United States) for 1 hour at room temperature. Following incubation, sections were rinsed and processed using standard avidin-biotin-horse radish peroxidase (1:1000 in 0.05 M PBS, Vectastain Elite ABC, Vector Laboratories, United States) assay for 1 hour at room temperature. The resulting peroxidase activity was detected employing 3’3’-diaminobenzidine (DAB-kit, Sigma, Sweden). The tissue was finally mounted on glass slides and photographed for subsequent image analysis.

3.6.1.3 Dopamine transporter radioligand binding (Paper II)

Animals were euthanized with sodium pentobarbital (≈120 mg/kg, Apoteksbolaget AB, Sweden) followed by decapitation. The brains were rapidly dissected and frozen on dry ice. Sections (14 µM) were cut and collected throughout the striatum, thaw-mounted on superfrost slides and stored at -20°C. Slides were pre-incubated in 50 mM Tris-HCl (pH 7.5) for 20 minutes. Incubation with the radioligand (125I) RTI-55 (2200 Ci/mmol, 50 pM, Perkin Elmer, Sweden) was performed for 1 hour in Tris-HCl (pH 7.5) with fluoxetine (10 µM Lilly, Sweden), to prevent binding to the serotonin transporter. To verify unspecific binding, one assay was performed with nomifensine (100 µM, Research Biomedicals International, Natick, United States). Sections were exposed to autoradiographic film (Kodak BioMax MR-1, Perkin Elmer, Sweden) (Boja et al., 1992) was performed for 1 hour in Tris-HCl (pH 7.5) with [14C] Microscale (Amersham, England) for 1 day. The films were manually processed with Kodak GBX-developer and fixer (Sigma-Aldrich, Sweden) and scanned for image analysis.

3.6.1.4 Image analysis

Digital images of the brain sections were converted to grey scale the Image J software (free download at http://rsbweb.nih.gov/ij/) and inverted. The mean grey value from the intact and the lesioned side were measured and the background value was subtracted from both. The reduction of signal on the lesioned side as compared to the intact was calculated in percent.
3.6.1.5 Data analysis and statistics

The treatment groups mean values for TH and DAT staining were compared by one-way ANOVA and there was no significant difference between treatment groups ($\alpha=0.05$). In Paper II, the mean reduction in DAT binding for all animals included in the chronic treatment study was $96.1\pm0.15$ and in Paper III, the mean loss of TH immunoreactivity in the lesioned striatum for all animals included in the study was $97.6\% \pm 0.7$.

3.7 ACUTE MOTOR BEHAVIOR (PAPER I, II AND III)

3.7.1 Locomotor activity (Paper I and III)

The experiments were performed in open field Plexiglas boxes (70x70 cm and 35 cm high, Kungsbacka Mät och Reglerteknik AB, Sweden) in which each side was equipped with two rows of photocells (8 cells per row, lower level at 3 cm and the upper level at 13 cm) either emitting or receiving infrared light, forming a two layer grid of infrared light beams. A locomotor event was registered when two consecutive beam breaks occurred in the lower layer, and summarized automatically every five minutes. In Paper I, locomotor activity was measured in reserpinized animals 10-130 minutes post administration of L-DOPA compounds. In Paper III, 6-OHDA-lesioned animals were continuously evaluated for locomotor activity during baseline (15 minutes), selegiline/vehicle administration (60 minutes) and up to 135 minutes post administration of L-DOPA or D3-L-DOPA.

3.7.2 Rearing activity (Paper III)

Rearing activity was manually scored from video recordings of the experiments performed in locomotor activity chambers (see above). The separate scoring of the left and right paw during rearing activity will give an indication of the lesion-induced asymmetry and its potential normalization following treatment. The number of rearings performed using the left paw for support was evaluated as percent of the total number of rearings performed during baseline (15 minutes), vehicle/selegiline treatment (60 minutes) and L-DOPA or D3-L-DOPA treatment (135 minutes).

3.7.3 L-DOPA-induced rotation (Paper II and III)

The phenomenology and terminology of rotation in the unilateral 6-OHDA-lesioned rat is described in the Introduction (see section 1.4.1.1 and Figure 4). In Paper II, a dose-response curve of different doses of L-DOPA and D3-L-DOPA was established. The total number of contralateral rotations performed 0-180 minutes post-administration was quantified in automated rotometry bowls. In Paper III, rotation was evaluated in locomotor activity chambers and the number of rotations made contra- or ipsilateral to the lesioned side were scored manually during baseline (15 minutes), selegiline/vehicle administration (60 minutes) and up to 135 minutes post administration of L-DOPA or D3-L-DOPA.

3.7.3.1 Methodological considerations

The use of L-DOPA-induced rotation to evaluate anti-parkinsonian efficacy during chronic treatment is complicated by the fact that the behavior sensitizes i.e. an acute subthreshold dose of L-DOPA may become increasingly effective to elicit rotation.
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following repeated administration (Schwarting and Huston, 1996, Henry et al., 1998, Lundblad et al., 2002). This sensitized response has been suggested to represent a dyskinetic motor pattern (Henry et al., 1998). Moreover, it is not correlated to the behavioral outcome of chronic L-DOPA-treatment in other tests of physiological motor function (Lundblad et al., 2002). The drug-induced rotation test may therefore not be sensitive enough to discriminate between anti-akinetic and dyskinetic effects of a putative therapy (Marin et al., 2006b). During the course of chronic treatment, the anti-parkinsonian effect of D3-L-DOPA and L-DOPA were therefore evaluated by the cylinder and rotarod tests in which performance is compromised by dyskinesias.

3.7.4 Data analysis and statistics

In Paper II, the dose-ratio for L-DOPA and D3-L-DOPA was graphically evaluated at 50% of the rotational response induced by the highest drug doses administered. Time*treatment interactions were statistically evaluated using two-way ANOVA followed by post hoc test when appropriate (Newman-Keuls multiple comparisons test in Paper I; Fishers Least significant difference post hoc test in Paper III). In Paper III, the significant time effect obtained from locomotor activity experiments was further evaluated by separate t-tests. All statistical comparisons were calculated in the STATISTICA software (StatSoft Inc., USA) and the significance level was set to α = 0.05.

3.8 MOTOR BEHAVIOR DURING CHRONIC TREATMENT (PAPER II)

3.8.1 The cylinder test

The cylinder test measures the spontaneous explorative behavior an animal performs in a novel environment. The animal explores the cylinder by standing on the hindlimbs, supporting itself with the forelimbs against the cylinder wall. The lesion creates a limb-use asymmetry in supporting wall contacts, which can be restored by L-DOPA-treatment (Lundblad et al., 2002). Rats were placed in a glass cylinder, without prior habituation, and videotaped for five minutes. Animals showing little tendency to explore were stimulated by quickly turning the lights on and off in the experiment room, by a mild shake of the cylinder or by quickly picking up the rat and place it back in the cylinder. The experiments were scored by a blinded observer, counting the number of supporting wall contacts made with the right and the left paw, and a limb-use asymmetry score was calculated. Dyskinetic animals with severely disrupted explorative behavior were excluded from the analysis, based on the observation that the animal mostly remained on the cylinder floor preoccupied with involuntary movements during the five minute session. The rats were tested in the cylinder test once before the start of chronic-treatment, and once during. In the session performed during chronic treatment, the animals were tested 140 minutes post drug administration.

3.8.2 The rotarod test

The rotarod test measures the ability of the animal to remain on a rotating rod at accelerating speed. Performance in this test is sensitive to dopaminergic lesioning and is improved after L-DOPA treatment (Lundblad et al., 2003). Each training session consisted of three separate trials on the rotarod (Rotamex 4/8, Columbus Instruments, United states). Animals were placed on the rod which spun at a rate of 4 rotations per
minute and then gradually accelerated to 44 rotations per minute over 90 seconds. When the rat fell off the rod the trial was over and the time recorded. To keep the rats alert, they were tapped on the tail several times during the trial. The rats were trained on the rotarod one session per day on three consecutive days to obtain stable baseline performance before the start of chronic treatment. During the chronic treatment period, baseline performance off L-DOPA (6 hours after the last L-DOPA injection) and performance on L-DOPA was evaluated once every week. After the last treatment week, the rats were kept on a drug-free interval for two days and then re-tested on the rotarod at 20 and 140 minutes post injection of L-DOPA compounds.

### 3.8.3 Scoring of abnormal involuntary movements (AIMs)

Dyskinesias were evaluated by scoring the animals for AIMs (Lundblad et al., 2002, Cenci and Lundblad, 2007), twice weekly during the course of chronic treatment. The animals were put in plastic cages and observed for one minute every 20 minutes up to 3 hours after drug administration. The rats were scored, by a blinded observer, for limb, axial and orolinguinal involuntary movements, (0= no dyskinesia, 1= dyskinesia <30 seconds, 2= dyskinesia >30 seconds, 3= continuous dyskinesia that could be interrupted by an external stimuli, 4= continuous dyskinesia not interruptible by an external stimuli). The external stimulus applied was a pen tap in the cage floor.

### 3.8.4 Data analysis and statistics

All data, except for AIMs, were analyzed by two-way ANOVA followed by Newman-Keuls test when appropriate i.e. a significant time*treatment interaction was found in the ANOVA analysis. AIMs data were analyzed by non-parametric statistics. Independent comparisons of single variables were performed with the Kruskal-Wallis ANOVA by Ranks followed by the Mann-Whitney U-test. Independent comparisons of multiple variables were performed with the Mann-Whitney U-test. Dependent comparisons were performed with the Friedman ANOVA followed by the sign-test. All statistical comparisons were calculated in the STATISTICA software (StatSoft, Inc. USA) and the significance level in all tests was set to \( \alpha = 0.05 \).

### 3.9 PERIPHERAL PHARMACOKINETICS OF L-DOPA AND \( \alpha,\beta,\beta \)-D3-L-DOPA (PAPER II)

The peripheral decarboxylase inhibitor carbidopa was injected 30 minutes before the injection of L-DOPA or D3-L-DOPA (50 mg/kg). Approximately 1 ml whole blood per animal and sampling time was collected from the retrobulbar venous plexus under light ether anesthesia. Blood samples were collected before injection of L-DOPA or D3-L-DOPA, and then at 2.5, 5, 10, 15, 30, 45, 60, 75, 90, 120, 150, 180, 240, 300 and 360 minutes post injection and processed to plasma. L-DOPA and D3-L-DOPA concentrations were determined in plasma samples using HPLC coupled to electrochemical detection. The lower detection limit of the assay was 20 ng/ml of L-DOPA or D3-L-DOPA. Missing data points, values below the detection limit of the assay, were replaced by the mean values from animals in the same treatment group at that time point. Data was analyzed using repeated measures ANOVA.
4 RESULTS AND DISCUSSION

4.1 STRUCTURE/ACTIVITY RELATIONSHIP OF DEUTERIUM SUBSTITUTIONS IN THE L-DOPA MOLECULE ON DOPAMINE OUTPUT AND METABOLISM

As previously discussed, bonds between carbon and deuterium are more stable as opposed to bonds between carbon and hydrogen and may therefore produce primary and secondary isotope effects on the rate of an enzymatic reaction (see section 1.5). The α-carbon-hydrogen bonds are removed during metabolism of dopamine to DOPAC and the β-carbon-hydrogen bond is abstracted during conversion to noradrenaline (see Figure 5 and 6). The combination of different carbon-deuterium substitutions in the L-DOPA molecule may thus produce different metabolic profiles. We first set out to evaluate structure-activity relationships of α- and β-substitutions on dopamine metabolism by screening the different isoforms of deuterium L-DOPA (see Figure 7). Using cerebral microdialysis, dopamine metabolism can be studied in vivo by monitoring extracellular levels of monoamines and metabolites. The effect of a high dose of the test compounds (50 mg/kg) were therefore evaluated on dopamine and DOPAC output in the striatum of intact animals.

![Figure 8](image-url)

Figure 8. Effects of carbidopa (10 mg/kg i.p) + vehicle, L-DOPA (50 mg/kg i.p.), β,β-D2-L-DOPA (50 mg/kg i.p.) or α,β,β-D3-L-DOPA (50 mg/kg i.p.) on A) striatal dopamine output, B) striatal DOPAC output and C) DOPAC/dopamine (DA) ratios. Data are expressed as per cent of baseline and presented as mean ± S.E.M. Arrows indicate the first and second injection, separated by 30 minutes.*=p<0.05, **=p<0.01, ***=p<0.001, α,β,β-D3-L-DOPA vs. L-DOPA, +<0.05, β,β-D2-L-DOPA vs. L-DOPA.

Interestingly, the first two isoforms evaluated, β,β-D2-L-DOPA and α,β,β-D3-L-DOPA, produced opposing effects on dopamine metabolism (see Figure 8). β,β-D2-L-DOPA gave rise to lower levels of dopamine and higher levels of DOPAC compared to L-DOPA, thus suggesting an increased turnover of dopamine. In accordance herewith, the calculated DOPAC/dopamine ratio, which constitute an estimate of dopamine turnover, were higher than those observed following L-DOPA-treatment. These results suggest that the β-carbon substitutions alone increase MAO-mediated enzymatic degradation of the substrate. The mechanism responsible for this effect is unknown but has also been observed in vitro (Yu et al., 1982). α,β,β-D3-L-DOPA on the other hand produced an increase in dopamine outflow of similar magnitude as L-DOPA, whereas the effect of α,β,β-D3-L-DOPA was dramatically prolonged, i.e.
extracellular levels of dopamine remained elevated for several hours. The levels of DOPAC were similar as those produced by L-DOPA while the DOPAC/dopamine ratio was significantly lower, indicating that the metabolism of dopamine formed from α,β,β-D3-L-DOPA was reduced. The effect elicited by α,β,β-D3-L-DOPA confirmed our hypothesis of a significant in vivo isotope effect on dopamine metabolism and underlined the importance of the α-carbon deuterium atom to produce such an effect. In fact, the rate limiting step in dopamine metabolism is the removal of an α-carbon hydrogen, evidenced by the significant primary isotope effect in this reaction demonstrated in vitro (Yu et al., 1982, Yu et al., 1986, Yu, 1988). Our findings thus extend previous in vitro observations while contrasting the effects observed following administration of the racemic mix of deuterium-substituted DOPA (D,L-α,β,β-D3-L-DOPA) in vivo (Dewar et al., 1985). The failure of the latter study to detect the differences observed herein are most likely due to the use of tissue homogenates to compare the effects of D,L-α,β,β-D3-L-DOPA and D-L-DOPA on striatal dopamine. Analysis of tissue levels of dopamine is a method that can not distinguish intra- and extracellular levels of dopamine and therefore differences in the dynamics between these compartments may not be detected.

As β,β-carbon substitutions alone seemed to slightly increase MAO activity, we hypothesized that an isoform of L-DOPA labeled with deuterium only at the α-carbon could produce an even more pronounced effect on dopamine metabolism as compared to α,β,β-D3-L-DOPA. Thus, to evaluate if β-substitutions counteracts the effects of the α-carbon substitution, the effects of α-D1-L-DOPA and α,β-D2-L-DOPA were subsequently studied using the same protocol. Surprisingly, α-D1-L-DOPA did not increase dopamine output significantly and the levels of DOPAC were significantly reduced as compared to those observed following L-DOPA administration. α,β-D2-L-DOPA produced intermediate effects that were similar to those produced by L-DOPA (see Figure 9). The lack of effect of α-D1-L-DOPA indicates that the single α-deuterium substitution negatively influences other processes which are related to the availability of dopamine in the terminal. One hypothetical explanation is that the decarboxylation of α-D1-L-DOPA was reduced. The β-carbon substitutions thus seem necessary to counteract this effect.

**Figure 9** Effects of carbidopa (10 mg/kg i.p) +L-DOPA (50 mg/kg i.p.), α-D1-L-DOPA (50 mg/kg i.p.) or α,β-D2-L-DOPA (50 mg/kg i.p.) on A) striatal dopamine output, B) striatal DOPAC output and C) DOPAC/DA ratios. Data are expressed as per cent of baseline and presented as mean ± S.E.M. Arrows indicate the first and second injection, separated by 30 minutes. *=p<0.05, **=p<0.01, ***=p<0.001, α-D1-L-DOPA vs. L-DOPA.
The altered effects of deuterium-substituted L-DOPA may potentially be related to differences in the uptake and elimination of the drug from the periphery. However, as evidenced by the plasma concentration measurements of L-DOPA and α,β,β-D3-L-DOPA (see Paper II, supplementary material) the deuterium-substitutions in α,β,β-D3-L-DOPA do not affect the peripheral pharmacokinetics of the drug.

In summary, the potent effect observed following administration of α,β,β-D3-L-DOPA suggests that the combination of one deuterium substitution on the α-carbon and two on the β-carbon produce the most favorable in vivo isotope effects on dopamine metabolism. Thus, despite the slight enhancement of dopamine metabolism observed following β-carbon substitutions alone they seem necessary to stabilize the α-carbon substitution for an efficient presynaptic handling of deuterium L-DOPA in vivo. As the β-carbon is not directly cleaved by MAO, it may also contribute to produce secondary isotope effects on dopamine metabolism (Belleau and Moran, 1963), i.e. MAO activity is reduced by the deuterium substitutions surrounding the active cleavage site. This combination of deuterium substitutions in the dopamine molecule were never investigated in vitro, but studies using deuterium labeled 5-HT show a similar magnitude of the isotope effect on MAO metabolism from α,α,β,β-5-HT as for α,α-5-HT (Yu et al., 1982). Our in vivo data suggest that the turnover of dopamine formed from α,β,β-D3-L-DOPA is reduced, which is observed as a prolonged neurochemical response following its administration.

The observed acute effects of α,β,β-D3-L-DOPA on dopamine metabolism might be advantageous in the treatment of Parkinson’s disease. The increased half-life of dopamine formed from α,β,β-D3-L-DOPA may serve to reduce pulsatile stimulation of dopamine receptors and to extend the motor benefit from each dose of the drug, thereby enabling a prolongation of the dosing interval or a reduction in the dose of L-DOPA. Altogether, a reduced L-DOPA load and more sustained stimulation of dopamine receptors could improve L-DOPA treatment by reducing the risk for motor fluctuations and dyskinesias. Therefore, α,β,β-D3-L-DOPA was further investigated for neurochemical and behavioral effects in the 6-OHDA model of PD. Additionally, the effects of this isoform with regard to noradrenaline formation were compared to L-DOPA.

4.2 NEUROCHEMICAL EFFECTS OF α,β,β-D3-L-DOPA (PAPER I, III, IV)
4.2.1 Effects on noradrenaline formation

As L-DOPA is the precursor of both dopamine and noradrenaline, L-DOPA administration may significantly increase the output of noradrenaline. Dopamine is transported into vesicles via the VMAT and is there converted to noradrenaline by the enzyme DβH which introduces a hydroxyl group at the β-carbon (see Figure 6). As the rate of DβH to produce noradrenaline from dopamine is subject to both primary and secondary isotope effects in vitro (Miller and Klinman, 1983, 1985), formation of noradrenaline from α,β,β-D3-L-DOPA may be significantly reduced as compared to L-DOPA. Therefore, in the next round of experiments we investigated the effects of low dose (5 mg/kg) of α,β,β-D3-L-DOPA and L-DOPA on striatal noradrenaline and dopamine output in intact animals. Interestingly, L-DOPA administration significantly
increased striatal output of noradrenaline while $\alpha,\beta,\beta$-D3-L-DOPA administration left basal levels of noradrenaline unaltered (see Figure 10 A).

![Figure 10: Effects of carbidopa (10 mg/kg i.p.)+L-DOPA (5 mg/kg i.p.) or $\alpha,\beta,\beta$-D3-L-DOPA (5 mg/kg i.p) on A) striatal noradrenaline output, B) striatal dopamine output and C) striatal DOPAC output. Data are expressed as per cent of baseline and presented as mean ± S.E.M. Arrows indicate the first and second injection, separated by 30 minutes *p<0.05, **p<0.01 $\alpha,\beta,\beta$-D3-L-DOPA versus L-DOPA; *p<0.05, **p<0.01, ***p<0.001 L-DOPA versus vehicle; *p<0.05, **p<0.01 and ***p<0.001 D3-L-DOPA versus vehicle.

In a recent microdialysis study L-DOPA (50 mg/kg) was found to increase striatal noradrenaline output (Bianco et al., 2008). The present results confirm these findings and show that also a lower dose of L-DOPA significantly increases striatal noradrenaline release. The opposing effects of $\alpha,\beta,\beta$-D3-L-DOPA and L-DOPA on noradrenaline output are in all probability the result of delayed $\beta$-hydroxylation of deuterium dopamine by D$\beta$H, indicating a significant in vivo isotope effect in this reaction. This could also imply that the release of dopamine may be increased from noradrenergic terminals following administration of $\alpha,\beta,\beta$-D3-L-DOPA. The reduced output of noradrenaline from $\alpha,\beta,\beta$-D3-L-DOPA may prove to be of clinical importance (see section 5).

4.2.2 Effects on dopamine output

4.2.2.1 Intact animals

Similar to the results observed after administration of high doses of L-DOPA and $\alpha,\beta,\beta$-D3-L-DOPA (Figure 8), a low dose (5 mg/kg) of $\alpha,\beta,\beta$-D3-L-DOPA produced a prolonged elevation of dopamine outflow as compared to L-DOPA (Figure 10 B). In fact, even though L-DOPA produced a slight increase in extracellular levels of dopamine over baseline; this effect was not significant when compared to vehicle administration. Previous studies investigating the effects of L-DOPA on extracellular dopamine, in rats, have shown inconsistent results. While some have reported decreased or unaltered dopamine output following administration of 50 mg/kg of L-DOPA (Abercrombie et al., 1990, Wachtel and Abercrombie, 1994, Miller and Abercrombie, 1999, Kannari et al., 2000) other studies, including the present, report increases using the same dose (Kaakkola and Wurtman, 1993, Fornai et al., 2000, Bianco et al., 2008) or even lower doses of the drug (Orosz and Bennett, 1992, Maeda et al., 1999, Lindgren et al., 2010). This discrepancy has elegantly been shown to relate
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to the fact that peripheral AADC inhibitors may enter the CNS and dose-dependently inhibit the activity of striatal AADC (Jonkers et al., 2001). Studies showing more pronounced effects of L-DOPA on dopamine output have consistently administered lower doses of benserazide or the less potent inhibitor carbidopa.

The enhanced effects of α,β,β-D3-L-DOPA on extracellular levels of dopamine indicate that the central kinetics of deuterium dopamine is altered. As in vivo microdialysis measures extracellular levels of dopamine, which represent the sum of release and clearance by re-uptake, metabolism and diffusion, from all cells surrounding the probe, it is not possible to dissect the exact mechanism by which α,β,β-D3-L-DOPA produces this effect. However, as an altered activity of MAO towards deuterium dopamine is the most likely explanation for our findings, the theoretical mechanisms which underlie the patterns of extracellular dopamine observed can at least be partly understood.

In general, dopamine release may occur via exocytotic vesicular release and via carrier-mediated reversed transport of the cytosolic pool of dopamine (see section 1.3.1.1). There is experimental evidence showing that both types of release may occur following L-DOPA administration in both intact and lesioned animals (Koshimura et al., 1992, Sarre et al., 1992, Mizoguchi et al., 1993, Sarre et al., 1994, Miller and Abercrombie, 1999, Kannari et al., 2000). Dopamine released following L-DOPA administration may to a significant extent be controlled by the activity of MAO, which regulates the cytosolic concentration of the transmitter by an efficient metabolism of newly synthesized dopamine. Inhibition of MAO would thus increase the cytosolic pool of dopamine which stimulates carrier-mediated release (Levi and Raiteri, 1993) and additionally may increase the vesicular fraction of dopamine available for exocytotic release, as demonstrated in vitro (Buu, 1989). Thus, reduced intracellular clearance of dopamine formed from α,β,β-D3-L-DOPA by MAO can be predicted to increase both the cytosolic and vesicular pool of dopamine available for release, a that may explain the prolonged elevation of dopamine output observed following administration of α,β,β-α,β,β-D3-L-DOPA. This conclusion also derives support from studies showing that inhibitors of MAO-A, the exclusive isoform present in the dopaminergic neurons, increase L-DOPA-induced dopamine output in the intact striatum (Wachtel and Abercrombie, 1994, Brannan et al., 1995, Finberg et al., 1995).

In addition to presynaptic mechanisms, the clearance of released deuterium dopamine by postsynaptic MAO-containing cells may be affected. Administration of a MAO-B inhibitor may allow for an indirect estimation of postsynaptic metabolism, as this isoform is localized at “extra-dopaminergic” sites. Under basal conditions MAO-B inhibition has no effect on dopamine levels in rats (Butcher et al., 1990, Colzi et al., 1990, Wachtel and Abercrombie, 1994, Brannan et al., 1995, Lamensdorf et al., 1996, Fornai et al., 2000). However, following L-DOPA administration MAO-B inhibition significantly increases L-DOPA-induced dopamine output (Wachtel and Abercrombie, 1994, Brannan et al., 1995). These findings illustrate the role of postsynaptic compartments in the regulation of extracellular dopamine, which may become
increasingly important under conditions of high substrate availability, such as following L-DOPA administration.

In summary, α,β,β-D3-L-DOPA elevates extracellular levels of dopamine more efficiently than L-DOPA, an effect that in all likelihood may be attributed to reduced activity of MAO towards deuterium dopamine. The increase in extracellular levels of dopamine may reflect an altered presynaptic handling of deuterium dopamine inside of the MAO-A containing dopaminergic terminals, resulting in an enhanced release, as well as reduced clearance of released deuterium dopamine at MAO-A and -B containing postsynaptic sites (see Figure 3).

4.2.2.2 6-OHDA-lesioned animals

As discussed in the introduction the dynamics of dopamine formation and release are altered in the dopamine depleted brain (see section 1.3.1.4). For example, the metabolism of dopamine is shifted from the dopaminergic neuron to other striatal compartments. Surprisingly, neither MAO-A nor -B inhibition was found to affect striatal dopamine output following L-DOPA administration to 6-OHDA lesioned animals (Wachtel and Abercrombie, 1994). This finding indicates that the ability to alter dopamine output from L-DOPA by modulation of MAO activity is lost following severe degeneration on dopaminergic neurons, a conclusion which contrast the behavioral effects produced by α,β,β-D3-L-DOPA in this model (see section 4.3). Therefore, the neurochemical effects of α,β,β-D3-L-DOPA were subsequently investigated in the dopamine-depleted striatum. α,β,β-D3-L-DOPA administration was shown to produce an increased dopamine output in comparison with L-DOPA administration (see Figure 11A), indicating that the effects of the deuterium substitution on the dynamics of dopamine release and clearance remain in the almost complete absence of dopaminergic neurons. The temporal pattern of dopamine output was, however, altered as compared to that observed in intact animals (Figures 8 and 10). While the duration of dopamine output following α,β,β-D3-L-DOPA administration was not as pronounced in the lesioned animals, there was a significant effect on the magnitude of the increase as compared to L-DOPA. In similarity with the experiments performed in the intact animals, the effects of α,β,β-D3-L-DOPA may here be attributed both to increased release from the decarboxylating cell due to decreased MAO-metabolism and/or decreased metabolism of deuteriated dopamine following its release.

There is compelling experimental evidence showing that the release of dopamine from L-DOPA is carried out by 5-HT neurons in dopamine-depleted animals (Tanaka et al., 1999, Navaillies et al., 2010, Nevalainen et al., 2011). The role of presynaptic MAO in 5-HT terminals to metabolize L-DOPA-induced dopamine is however unclear (Tanaka et al., 1999) and only sparse localization of MAO is found in the terminal areas of the 5-HT system (Levitt et al., 1982, Westlund et al., 1988, Jahng et al., 1997, Arai et al., 2002). If the 5-HT terminals do contribute to dopamine metabolism, it is probably mediated by MAO-A, since only MAO-A inhibition affects striatal metabolism of 5-HT (Kato et al., 1986, Butcher et al., 1990, Stanley et al., 2007).
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Figure 11. Neurochemical effects in the 6-OHDA lesioned striatum. Vehicle (1 ml/kg, s.c.) or selegiline (1 mg/kg, s.c.) were administered first, one hour later L-DOPA or α,β,β-D3-L-DOPA (3 mg/kg s.c., co-administered with benzerazide 12 mg/kg) was injected. Arrows indicate time of injection. A. Dopamine. B. DOPAC. C. 3-MT. Data is presented as mean ±SEM. *p<0.05, **p<0.01, ***p<0.001 vehicle/ α,β,β-D3-L-DOPA versus vehicle/L-DOPA; +p<0.05, +p<0.01, +++p<0.001 selegiline/L-DOPA versus vehicle/L-DOPA; ◊p<0.05 vehicle/D3-L-DOPA versus selegiline/L-DOPA; ¤p<0.05, ¤¤p<0.01, ¤¤¤p<0.001 vehicle/α,β,β-D3-L-DOPA versus selegiline/α,β,β-D3-L-DOPA.

In the same experiments, the effects of α,β,β-D3-L-DOPA and L-DOPA alone as well as in combination with the clinically used MAO-B inhibitor selegiline (see section 1.3.3.2) on dopamine metabolism were compared. α,β,β-D3-L-DOPA and the selegiline/L-DOPA combination produced similar effects on dopamine output and these effects were significantly higher than in the group receiving L-DOPA alone. Given the presumption that 5-HT terminals release dopamine and lack MAO-B, we suggest that the effects produced by selegiline/L-DOPA administration was the result of a less efficient metabolism of released dopamine at MAO-B containing postsynaptic sites, i.e. astrocyte processes surrounding the synapses (Levitt et al., 1982, Westlund et al., 1988) and postsynaptic neurons (Finberg, personal communication). Moreover, selegiline pre-treatment did not potentiate the effects of α,β,β-D3-L-DOPA, a finding which suggests that the increased output of dopamine generated by α,β,β-D3-L-DOPA predominantly reflects a decreased metabolism of released dopamine and that this effect is mediated mostly via MAO-B. Against this background it can furthermore be concluded that the presynaptic contribution to the elevated dopamine levels observed following administration of α,β,β-D3-L-DOPA in dopamine-depleted rats should be rather small, which may explain the temporal difference between the effects of α,β,β-D3-L-DOPA in intact versus lesioned animals.

It should be noted that MAO-B inhibition has previously been shown not to affect dopamine levels following L-DOPA administration in the lesioned rat striatum (Wachtel and Abercrombie, 1994, Finberg et al., 1995). This discrepancy may be attributed to differences in the extent of the lesions or to the fact that the sensitivity of the assays used has been improved since the publication of the two previous studies.

In summary, α,β,β-D3-L-DOPA produced an increase in dopamine output that was higher than the effect of L-DOPA in the lesioned striatum. This effect closely resembled that of L-DOPA in combination with selegiline. It is proposed that the increased concentration of extracellular dopamine mainly depends on reduced metabolism of released deuterium-dopamine at MAO-B containing postsynaptic sites.
### 4.2.3 Effects on dopamine metabolism

#### 4.2.3.1 DOPAC

Our data consistently show increased levels of dopamine following α,β,β-D3-L-DOPA administration as compared to non-modified L-DOPA (Figure 8, 10 and 11), which is attributed to an altered activity of MAO towards deuterium dopamine. The effects of α,β,β-D3-L-DOPA on the MAO metabolite DOPAC were however less prominent. Since previous studies have shown that inhibition of MAO-A markedly reduces extracellular DOPAC levels in L-DOPA-treated animals (Wachtel and Abercrombie, 1994, Brannan et al., 1995, Finberg et al., 1995) it would be expected that the elevated dopamine levels following α,β,β-D3-L-DOPA should be associated with a coincident decrease in extracellular DOPAC levels. When compared with effects of L-DOPA administration, reduced DOPAC levels were only observed following administration of a low dose of α,β,β-D3-L-DOPA in the intact striatum (see Figure 10 C). α,β,β-D3-L-DOPA is, however, not likely to inhibit MAO per se, but rather to produce a delay in the metabolism of dopamine formed. Extracellular levels of DOPAC represent a measure of intracellular MAO metabolism of dopamine (Butcher et al., 1988, Zetterström et al., 1988, Eisenhofer et al., 2004). DOPAC levels in the extracellular space range from 160-400 fmol/μl while those of dopamine are in the range of 2 fmol/μl. Small changes in the intraneuronal pool of dopamine, which result in increased extracellular levels of the transmitter, may therefore not be reflected in the extracellular level of DOPAC. Thus, extracellular levels of DOPAC may not actually reflect the altered dynamics of deuterium dopamine metabolism. In addition, DOPAC is not a direct product of MAO metabolism (see Figure 5). In contrast, the direct product, DOPAL, may provide a better indication of MAO activity. DOPAL is an unstable intermediate which makes it difficult to quantify. However, DOPAL has successfully been measured in two microdialysis studies (Colzi et al., 1996, Fornai et al., 2000). Fornai (2000) showed that DOPAL levels are dramatically increased following L-DOPA administration. To further gain insight into the details of dopamine metabolism following α,β,β-D3-L-DOPA administration it would therefore be of considerable interest to compare the effects of L-DOPA and α,β,β-D3-L-DOPA on DOPAL formation.

The finding that DOPAC levels were readily elevated by L-DOPA also in the lesioned striatum (Figure 11 B) and that this increase was insensitive to MAO-B inhibition, suggests that DOPAC mainly originates from MAO-A activity, although the site of DOPAC formation remains unknown. The significant increase in dopamine levels following MAO-B inhibition and lack of reduction in L-DOPA-induced DOPAC levels could however readily be explained by the small contribution that this fraction of extracellular dopamine would have on the total formation of DOPAC in the lesioned striatum.

#### 4.2.3.2 3-MT

3-MT has been proposed to provide a better index of changes in extracellular dopamine as compared to DOPAC (Brown et al., 1991, Kuczenski and Segal, 1992) as it is exclusively formed from dopamine metabolism by COMT in the extracellular space.
3-MT levels were monitored in the lesioned striatum (see Figure 11 C) and were found to increase following α,β,β-D3-L-DOPA and selegiline/L-L-DOPA treatment as compared to administration of L-DOPA alone. This may be the direct consequence of the increased elevation of extracellular levels of dopamine levels seen following these two treatments. Alternatively, since deamination of 3-MT to HVA is catalyzed by MAO, the increased levels of 3-MT may possibly reflect a decreased rate of this reaction (see Figure 3). Interestingly, 3-MT has been shown to act as an agonist on the trace amine-associated receptor 1 as well as dopamine receptors and intracerebral infusion of 3-MT has been shown to stimulate locomotor activity (Nakazato and Akiyama, 2002, Sotnikova et al., 2010). The possible role of 3-MT in the mediation of behavioral effects produced by L-DOPA remains to be clarified, but may have contributed to motor activation in the present study, as all treatment groups with increased 3-MT levels, as compared to L-DOPA, also displayed increased motor activation (see section 4.3.1).

In conclusion, our data show that α,β,β-D3-L-DOPA provides a means to alter the dynamics of dopamine handling by MAO as well as DβH without direct enzyme inhibition, i.e. dopamine and noradrenaline output may be altered by both primary and secondary isotope effects on dopamine metabolism in vivo. These deuterium isotope effects, that are produced following administration of α,β,β-D3-L-DOPA, may also translate into an altered behavioral profile as compared to that of L-DOPA, which subsequently will be discussed.

4.3 BEHAVIORAL EFFECTS OF α,β,β-D3-L-DOPA IN ANIMAL MODELS OF PARKINSON’S DISEASE (PAPER I, II AND III)

As previously concluded, the increased half-life of dopamine produced from α,β,β-D3-L-DOPA may provide substantial benefits in the treatment of PD. Therefore, the next series of experiments were performed in well established animal models of PD and were designed to estimate the acute behavioral potency of α,β,β-D3-L-DOPA as well as its effects on parkinsonian-like motor dysfunction and dyskinesia during chronic treatment.

4.3.1 Acute behavioral potency of α,β,β-D3-L-DOPA

In the reserpine model of PD, locomotor activity was automatically quantified following administration of L-DOPA, α,β,β-D3-L-DOPA and β,β-D2-L-DOPA (200 mg/kg). While both L-DOPA and α,β,β-D3-L-DOPA reversed the reserpine-induced akinesia, the effect elicited by α,β,β-D3-L-DOPA was of significantly larger magnitude as compared to that of L-DOPA (see Figure 12). The ββ-D2-L-DOPA isoform, which had been shown to display an increased turnover of dopamine in dialysis experiments (see Figure 8), was unable to stimulate locomotor activity. The reversal of reserpine-induced akinesia by L-DOPA is known to reflect a significant dopamine formation and postsynaptic receptor activation. Taken together, our data suggest that the increased locomotor activity produced by α,β,β-D3-L-DOPA predominantly or even exclusively depends on an increased dopamine output.
**Figure 12** Effects of vehicle (in non-reserpinized rats), carbidopa (10 mg/kg i.p) + vehicle, L-DOPA (200 mg/kg i.p), β,β-D2-L-DOPA (200 mg/kg i.p) or α,β,β-D3-L-DOPA (200 mg/kg i.p) or α,β,β-D3-L-DOPA (200 mg/kg i.p) on locomotor activity in reserpinized rats. Rats were placed in the locomotor activity boxes 10 minutes after the second injection. Data are expressed as mean ± S.E.M. ***=p<0.001, *p=<0.05 α,β,β-D3-L-DOPA versus L-DOPA.

α,β,β-D3-L-DOPA and L-DOPA were subsequently compared in the 6-OHDA-lesioned rat (Paper II and III). The unilateral 6-OHDA lesion induces an asymmetry in the body which, among other motor abnormalities, can be observed as a rotational behavior (see section 1.4.1.1). We used L-DOPA-induced rotation to establish a dose-response curve of the acute effect of L-DOPA and α,β,β-D3-L-DOPA (D3-L-DOPA), as the intensity of rotation may reflect quantitative aspects of dopaminergic transmission (Ungerstedt, 1976, Schwarting and Huston, 1996). In Paper II, the total number of contralateral rotations performed up to 3 hours following administration of different doses of L-DOPA and D3-L-DOPA (3-8 mg/kg) was measured. The dose-response curve for D3-L-DOPA was shifted to the left, which indicates an increased potency (see Figure 13). The equipotent dose of α,β,β-D3-L-DOPA to L-DOPA was graphically determined at 50% of the rotational response (EC50) induced by the highest drug doses administered and found to correspond to ≈60% of a given L-DOPA dose.

**Figure 13.** Dose-effect curve for the acute behavioral response to α,β,β-D3-L-DOPA and L-DOPA as measured by contralateral rotation (0-180 minutes post-injection). L-DOPA (3.2, 6.4 and 8 mg/kg) and D3-L-DOPA (3.2, 4.8 and 6.4 mg/kg) were administered s.c (n=5-6/dose). The line represents 50% of the effect mediated by the highest doses, the equipotent dose of α,β,β-D3-L-DOPA to L-DOPA was graphically determined at this point. Data are presented as mean ±SEM.

In Paper III, the effect of the same dose (8 mg/kg) of D3-L-DOPA and L-DOPA were compared, alone as well as in combination with the clinically used MAO-B inhibitor selegiline (1 mg/kg). All treatments were first evaluated for acute behavioral effects and subsequently, following a wash-out period, neurochemical effects were studied in the lesioned striatum. The behavioral experiments were designed to simultaneously
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monitor locomotor activity, L-DOPA-induced rotation and rearing activity. There was a trend towards increased locomotor activity in D3-L-DOPA-treated animals as well as in selegiline pretreated animals receiving L-DOPA or D3-L-DOPA, as compared to animals receiving L-DOPA alone. There were, however, no statistically significant differences between the groups (see Figure 14 A). A majority of the animals responded with ipsilateral rotation following L-DOPA and D3-L-DOPA treatment. Total rotational activity, independent of direction, was summarized to quantify behavioral activation in this study. D3-L-DOPA produced an increased rotational activity as compared to L-DOPA again confirming D3-L-DOPA’s increased potency (see Figure 14 B). Moreover, selegiline pre-treatment was found to potentiate the effect of L-DOPA, which is in line with its clinical effect in L-DOPA-treated PD patients (see section 1.3.3.2). Interestingly, the behavioral effect produced by D3-L-DOPA and selegiline/L-DOPA was of similar magnitude, which indirectly suggests that D3-L-DOPA may provide equal clinical benefit as the combination of selegiline and L-DOPA.

![Figure 14](image-url)

Figure 14 Behavioral effects of L-DOPA and D3-L-DOPA alone and in combination with selegiline. Selegiline 1 mg/kg or vehicle was injected one hour preceding the administration of L-DOPA or D3-L-DOPA (8 mg/kg s.c.). Data are presented as mean ± SEM. A. Locomotor activity. Baseline (total activity performed 15 minutes preceding L-DOPA or D3-L-DOPA administration) is compared to treated (total activity performed for 120 minutes post-injection). *p<0.05 and **p<0.01 compared to baseline. B. L-DOPA-induced rotation. Arrows indicate time of injection. *p<0.05 D3-L-DOPA versus L-DOPA; +p<0.05, ++p<0.01 Selegiline/L-DOPA versus L-DOPA; p<0.05 D3-L-DOPA versus Selegiline/D3-L-DOPA. C. Rearing activity. The number of rearings performed using the left paw for support is presented as percent of the total number of rearings performed during baseline (15 minutes), vehicle/selegiline treatment (60 minutes) and L-DOPA or D3-L-DOPA treatment (135 minutes). The line at 50% represents an equal use of both paws. **p<0.01 compared to baseline, ***p<0.001 compared to baseline and +p<0.05 compared to L-DOPA.
Limb use asymmetry was evaluated by scoring left and right paw supporting wall contacts during rearing activity. Unilateral lesions produce an asymmetric use of the forelimbs and an equal use of both limbs indicates that the asymmetry has been restored. All treatments were shown to significantly increase the use of the left parkinsonian limb for support during rearing activity (Figure 14 C). This finding indicates that, although the ipsilateral rotational response might be interpreted as a predominant activation of the intact striatum, dopaminergic transmission in the lesioned side was of sufficient magnitude to restore the lesion-induced asymmetry.

In summary, D3-L-DOPA was shown to display an increased behavioral potency as compared to L-DOPA, evidenced by an increased locomotor activity in reserpinized animals and increased rotation activity in the 6-OHDA-lesioned animal. Although the study designs do not permit a direct correlation, the present behavioral data support the neurochemical observations of both primary and secondary isotope effects on dopamine metabolism following administration of D3-L-DOPA.

4.3.2 Effects of D3-L-DOPA during chronic treatment (Paper II)

The increased behavioral potency of D3-L-DOPA observed in parkinsonian animals indicates that the dose of the drug may be significantly reduced without a loss of the symptomatic effect, a property which may decrease the risk for motor complications. In order to test this hypothesis, a chronic treatment study was performed in 6-OHDA-lesioned animals receiving daily injections of D3-L-DOPA and L-DOPA for three weeks. Four treatment groups were included: L-DOPA (8 mg/kg), D3-L-DOPA (8 mg/kg), vehicle and an additional group treated with the equipotent dose of D3-L-DOPA i.e. ≈60% of the L-DOPA dose (5 mg/kg), as determined from the acute dose-response curve (see Figure 13). The anti-akinetic effects of the different treatments were evaluated using the cylinder and rotarod tests and the liability to induce dyskinesias was evaluated by the AIMs test.

4.3.2.1 Effects of D3-L-DOPA and L-DOPA on parkinsonian-like motor dysfunction

In the cylinder test the animal explores the cylinder by standing on the hindlimbs, supporting itself with the forelimbs against the cylinder wall. The rats were tested for baseline performance before the start of chronic treatment and the lesion was shown to reduce the use of the left limb to 25 % of all supporting contacts (see Figure 15 A). Treatment with L-DOPA did not improve performance relative to baseline values in the same animals, but there was a significant difference between the L-DOPA-treated and vehicle-treated animals confirming the anti-parkinsonian effect of L-DOPA previously observed in this test (Lundblad et al., 2002, Stefanova et al., 2004). Both the equipotent and equivalent dose of D3-L-DOPA produced a more pronounced anti-parkinsonian effect as compared to L-DOPA. This finding clearly supports the notion of an increased potency of the anti-parkinsonian effect produced by D3-L-DOPA. The rotarod test was intended to test the duration of the anti-akinetic response of L-DOPA and D3-L-DOPA as motor performance is measured at two different time-points, 20 and 140 min after drug administration. However, performance in the rotarod test was negatively compromised by dyskinesias during the course of the chronic treatment, an effect that was most pronounced in the groups treated with higher doses of the L-DOPA.
compounds. Animals treated with the lower equipotent dose of D3-L-DOPA, remained on the rotarod for a longer time than vehicle-treated animals, indicating an anti-parkinsonian effect of the drug in this test. Moreover, animals treated with the equivalent dose D3-L-DOPA showed improved performance at 140 minutes post injection. Although non-significant, these trends point in favor of the D3-L-DOPA-treated groups (see Figure 15B).

**Figure 15.** Anti-parkinsonian effects of D3-L-DOPA (5 and 8 mg/kg) and L-DOPA (8 mg/kg) during chronic treatment. **A.** The cylinder test. The rats were tested for baseline performance before the start of chronic-treatment (untreated) and during chronic treatment (treated). Within comparisons (untreated vs. treated), *p<0.05; **p<0.01; ***p<0.001. Comparison between the groups (treated vs. treated), +p<0.05; ++p<0.01; +++p<0.001 vs. vehicle; ¤p<0.05 vs. D3-L-DOPA 8 mg/kg. **B.** The rotarod test. Animals were evaluated on the rotarod after a two-day drug free interval. Each trial consists of 90 s at accelerating speed; performance is presented as seconds on the rotarod and compared to vehicle-treated animals. The rats were tested 20 and 140 minutes post-injection of L-DOPA and D3-L-DOPA. Data are presented as mean ±SEM.

**4.3.2.2 Effects of D3-L-DOPA and L-DOPA on dyskinesia**

Animals were scored for AIMs twice weekly during the course of the three-week treatment regimen. Treatment with the higher doses of L-DOPA and D3-L-DOPA produced a pronounced expression of AIMs from the first scoring session and throughout all the remaining sessions during the three-week experiment. Dyskinesia did not differ between animals treated with the equivalent doses of D3-L-DOPA and L-DOPA (see Figure 16). In contrast, animals treated with the equipotent dose of D3-L-DOPA expressed lower AIMs scores in all test sessions and, in addition, lower cumulative AIMs scores for the entire treatment period. Dyskinesia scores in animals treated with the high doses appeared to approach a ceiling effect, since a dose of L-DOPA more than two times higher, previously was shown to result in comparable AIMs scores (Lindgren et al., 2007). Nevertheless, animals treated chronically with L-DOPA expressed significantly higher AIMs in the last session as compared to the first; whereas no such effect was observed in either group treated with D3-L-DOPA (see Figure 16). Taken together, these findings indicate that the development of dyskinesia may have been slightly reduced during D3-L-DOPA treatment.
Figure 16. Effects of D3-L-DOPA and L-DOPA on development/expression AIMs. Data are presented as the cumulative AIMs score. Scoring was performed twice weekly for three weeks, resulting in a total of 6 sessions. Animals were scored every 20 minutes for 180 minutes after drug administration (9 observations/animal/session). The maximal score for each of the three subtypes of AIMs was 4. **A.** Median cumulative AIMs score for each session. Maximal theoretical score for each session was 108 (4*3*9). *= p<0.05 D3-L-DOPA 5mg/kg vs. L-DOPA 8 mg/kg; += p<0.05 D3-L-DOPA 5 mg/kg vs. D3-L-DOPA 8 mg/kg. **B.** Median cumulative AIMs score for the entire treatment period. Maximal theoretical score was 648 (4*3*9*6). *= p<0.05 D3-L-DOPA 5 vs. L-DOPA 8 mg/kg.

A highly significant conclusion of this study is that deuterium substitutions in the L-DOPA molecule allows for a significant dose reduction without loss of anti-parkinsonian efficacy and a reduced incidence of dyskinesia. As previously discussed, the risk for motor complications is positively correlated with L-DOPA dosage in PD patients (Schrag and Quinn, 2000, Sharma et al., 2006, Sharma et al., 2008). Similarly, the clinical benefit from L-DOPA treatment, as measured by the unified Parkinson’s disease rating scale, has previously been shown to increase dose-dependently (Fahn et al., 2004). Moreover, the therapeutic effect and side effects of a single dose of L-DOPA are not easily dissociated in dyskinetic patients; therefore a reduction of the L-DOPA dose to control dyskinesia may compromise the therapeutic benefit. Our preclinical studies propose that D3-L-DOPA has a wider therapeutic window than L-DOPA. In the clinical setting D3-L-DOPA would thus allow for adequate control of the parkinsonian symptoms at an overall lower dosage which should be associated with lower risk for motor complications.
5 SUMMARY AND CONCLUDING REMARKS

L-DOPA remains as the gold standard symptomatic treatment for PD, but long-term treatment with L-DOPA is complicated by the gradual emergence of motor complications. Risk factors for motor complications include disease severity, dosage of L-DOPA and duration of treatment (Schrag and Quinn, 2000, Fahn, 2005, Sharma et al., 2006, Sharma et al., 2008). While the mechanism underlying the emergence of motor complications remains largely unknown, non-physiological, pulsatile stimulation of postsynaptic dopamine receptors resulting from L-DOPA therapy is a prevailing hypothesis (Chase, 1998, Olanow et al., 2006). In the present thesis we investigated the potential utility of deuterium substitutions in the L-DOPA molecule to improve treatment of PD. Deuterium-L-DOPA was expected to yield dopamine more resistant to enzymatic degradation as deuterium forms a stronger bond with carbon.

Among the deuteriated isoforms evaluated, α,β,β-D3-L-DOPA(D3-L-DOPA) was shown to significantly prolong the elevated striatal dopamine output as compared to the corresponding effect of L-DOPA. However, while L-DOPA also increased striatal output of noradrenaline, D3-L-DOPA left the striatal output of noradrenaline unaltered. In 6-OHDA-lesioned animals D3-L-DOPA was shown to produce an increased dopamine output as compared to L-DOPA administration. While the MAO-B inhibitor selegiline potentiated the effect of L-DOPA on striatal dopamine output, it did not potentiate D3-L-DOPA’s effect which closely resembled that of the selegiline/L-DOPA combination. An acute challenge with D3-L-DOPA was subsequently shown to produce a substantially larger motor activation than L-DOPA, both in the reserpine and the 6-OHDA-lesioned animal models of PD. In addition, the behavioral effect produced by D3-L-DOPA was found to be of similar magnitude as the combination of selegiline and L-DOPA. Chronic treatment with a significantly lower dose of D3-L-DOPA, i.e. 60% of the administered L-DOPA dose, was found to produce similar anti-akinetic effect while the expression of dyskinetic movements was markedly reduced. In comparison with an equivalent dose of L-DOPA, D3-L-DOPA produced a larger anti-akinetic effect but yet a similar expression of dyskinesia. Moreover, both doses of D3-L-DOPA were associated with a seemingly reduced development of dyskinesia.

Taken together, our data demonstrate an increased potency of D3-L-DOPA in comparison with L-DOPA and a widening of the therapeutic window. The neurochemical effect of D3-L-DOPA was studied under two conditions, i.e. in rats with an intact dopaminergic system or in rats with near complete dopamine denervation. The PD patient presents with motor symptoms when striatal tissue levels of dopamine have decreased by approximately 80% (Bernheimer et al., 1973), and the degenerative process slowly progresses over the years (Agid, 1991). The 6-OHDA rodent model studied herein, which is associated with ≈95% striatal dopamine denervation, might therefore be regarded as a model of late stage PD. The striatal output of dopamine following administration of D3-L-DOPA in the intact animals remained elevated for a longer period of time than the corresponding effect of L-DOPA, whereas this temporal difference was less pronounced in the lesioned striatum. Thus, the temporal difference in extracellular dopamine levels formed from L-DOPA and D3-L-DOPA is largely
related to D3-L-DOPA’s effect on the dynamics of dopamine release and clearance in the presence of dopaminergic terminals. It is therefore possible that D3-L-DOPA might produce an even more prolonged increase in striatal dopamine output in PD patients with less severe neurodegeneration, i.e. in early stage PD patients. Therefore, it is of considerable interest that the increased half-life of dopamine formed from D3-L-DOPA may protect against a subsequent development of dyskinesia in two ways. First, reduced fluctuations of dopamine levels should prevent pulsatile stimulation of dopamine receptors. Second, a prolonged half-life would allow an increased dosing interval, which in effect means a reduction of the total L-DOPA load. Indeed, at 60% of the corresponding L-DOPA dose, D3-L-DOPA produced equal anti-parkinsonian effect and dyskinesia was markedly reduced. In the late stage PD patient, adequate symptomatic treatment with D3-L-DOPA may therefore be associated with lower risk for motor complications.

Primary and secondary isotope effects in the metabolism of dopamine formed from D3-L-DOPA may have several mechanistic consequences depending on the level of integrity of the dopaminergic system, and the potential benefits with D3-L-DOPA can be derived from two convergent, but inter-dependent, mechanisms; an increased half-life of dopamine formed from each dose of D3-L-DOPA and an increased potency. While the increased duration to a large extent may depend on the degree of integrity of presynaptic dopaminergic terminals, the increased potency is probably related to a combination of both pre- and postsynaptic mechanisms involving MAO-metabolism. Consequently, it may well be that in early stage PD patients, with less severe neurodegeneration, the dose of D3-L-DOPA, which would be required to produce sufficient symptomatic effect, may be even lower than the 60% observed in our model of late stage PD.

Although, MAO-A expression has been reported to be higher in rats than in humans (Fowler et al., 1987, Westlund et al., 1988, Saura et al., 1992), our data clearly demonstrate a role for postsynaptic MAO-B metabolism of dopamine formed from L-DOPA in the lesioned rat. In man, postsynaptic MAO-B dependent dopamine metabolism is reported to be of even greater magnitude (Stenström et al., 1987), which largely may explain the clinical efficacy of MAO-B inhibitors in PD patients (Oreland et al., 1983). Our data showing that D3-L-DOPA increased extracellular levels of striatal dopamine to a similar extent as the combination of selegiline and L-DOPA and that selegiline was unable to potentiate the effects of D3-L-DOPA, indicate that the increased dopamine output in all three treatment groups may be attributed to decreased postsynaptic MAO-B dependent dopamine metabolism. Given the relatively less significant MAO-B dependent postsynaptic metabolism of dopamine in rats as compared to humans, treatment with D3-L-DOPA may be expected to be more efficient in PD patients than indicated by our experiments in lesioned rats. In addition, D3-L-DOPA offers the potential advantage over MAO-B-inhibitors to reduce presynaptic MAO-A metabolism, since MAO-A is the exclusive MAO isoform expressed in dopaminergic neurons across species. This effect may in turn augment synaptic levels of dopamine released from remaining terminals.
The reduced output of noradrenaline from D3-L-DOPA observed herein, may be attributed to decreased DβH activity towards the β-carbon (Miller and Klinman, 1983). Previous studies have suggested that a concomitant degeneration of central noradrenergic neurons may contribute to both the pathophysiology and symptomatology of PD, although severe degeneration of e.g. LC neurons has also been observed in Alzheimer’s disease which lacks the neurological core symptoms of PD (Hornykiewicz and Kish, 1987, Zarow et al., 2003, Fornai et al., 2007, Delaville et al., 2011, McMillan et al., 2011). Interestingly, supplementation of noradrenaline with the synthetic precursor L-threo-3, 4-dihydroxyphenylserine (L-DOPS) has been reported to improve certain motor aspects in L-DOPA-treated PD patients (Ogawa et al., 1985, Tohgi et al., 1990). These data may thus suggest that augmentation of noradrenergic transmission might be useful in PD. It should be noted, however, that no consensus exists as regards the putative role of noradrenaline formed from L-DOPA for its therapeutic effect as well as its side effects in PD patients. Our data in intact animals clearly show that noradrenaline can be formed in the striatum following L-DOPA administration. However, the 6-OHDA used to lesion the dopaminergic system in our experiments also lesions noradrenergic neurons (Fulceri et al., 2007, Barnum et al., 2012). Accordingly, L-DOPA did not increase noradrenaline output in lesioned animals and thus it seems highly unlikely that the behavioral differences observed between rats treated with L-DOPA and D3-L-DOPA, i.e. with regard to the anti-parkinsonian effect and occurrence dyskinesia, are indeed related to noradrenaline. The well-established, anti-dyskinetic effect of α2 antagonists in animal models may theoretically be related to blockade of either pre- or postsynaptic α2 receptors. As blockade of presynaptic autoreceptors will increase striatal noradrenaline release (Gobert et al., 2004) the antidyskinetic effect might be related to enhanced noradrenergic neurotransmission, but the effect may alternatively be due to blockade of postsynaptic α2 receptors. Significantly, a recent experimental study performed in 6-OHDA-lesioned rats showed that infusion of noradrenaline into the striatum triggered the onset of dyskinesia in L-DOPA-treated animals (Buck and Ferger, 2009). This finding indicates that a putative formation of noradrenaline from L-DOPA in the striatum might, if anything, contribute to enhance the expression of dyskinesia by activation of postsynaptic receptors. Consequently, the reduced formation of noradrenaline from D3-L-DOPA may well even serve to dampen the expression of LID.

The most important conclusion from the present set of preclinical experiments is that deuterium substitutions in the L-DOPA molecule allows for a significant dose reduction without loss of anti-parkinsonian efficacy and a reduced incidence of dyskinesia. In short, D3-L-DOPA appears clearly more potent and exhibits a wider therapeutic window than L-DOPA, which implies that the anti-parkinsonian effect and the risk for motor complications may be dissociated. In principle, our findings thus argue against the current assumption that drugs that increase dopaminergic transmission inevitably also increase the expression of dyskinesia (Encarnacion and Hauser, 2008, Nutt, 2008). The present results suggest that in the clinical setting treatment with D3-L-DOPA may offer several potential advantages over available pharmacotherapies used in PD, including conventional L-DOPA. A prolonged anti-parkinsonian effect of each dose may contribute to delay both the gradual development and expression of motor
fluctuations and dyskinesias. The higher potency of D3-L-DOPA should allow for adequate control of parkinsonian symptoms at an overall lower dosage and would thereby contribute to an additional reduction of the risk for motor complications. Finally, the lower dose of D3-L-DOPA needed for therapeutic effect may also reduce the expression of already established dyskinesia. In all simplicity, the similar effect produced by D3-L-DOPA and the combination of selegiline and L-DOPA suggest a potential clinical advantage of monotherapy with D3-L-DOPA in the treatment of PD.
Acknowledgements

6 ACKNOWLEDGEMENTS

This thesis was focused on the making and breaking of deuterium-carbon atom bonds. The bonding which led to the present work was however not restricted deuterium atoms; I have had the good fortune to form a couple of stable bonds with all the fantastic people surrounding me over these years. This work would not have been possible without you. I especially would like to thank:

My supervisor, Björn Schilström, for taking me in as your PhD student and giving me this opportunity. You have shared your scientific enthusiasm and knowledge and always kept your door open for discussions on all matters of science and life in general. You are a true inspiration and friend! Thank you for your endless support, patience and for believing in me.

My co-supervisor, Torgny Svensson, for your great scientific mind, your enthusiasm and encouragement. I also wish to thank you for always taking the time to help me to polish my writing, for all your support over the years and for your wonderful sense of humor.

All the, past, and present people working in the “Svensson corridor”:

Aki falken you have such an amazing crazy loving personality and I just want to say “merci”… Calle we kind of grew up together as PhD-students and I really look forward to an invitation to your dissertation party®. I will miss you roomie! Åsa for all the fun times we spent together and for always caring, Pixi, you rock! Thank you for your friendship and for your help with tricky regression analysis questions. Monica for your calm perspective, for good times in the south of France and for always taking time to help me with any matter, Carina for your sweet personality and for all the “singstar” battles, Jens for being such a great guy, Anna for teaching me everything there is to know about microdialysis and for your patience with all my questions, Olivia for being a friend and for fantastic “fika” cakes, Kristin for all the help with Paper III and for being a positive spirit, Kent for good times at conferences and parties, Anders for being you, Daniella for your glamour and for fun nights out, Lotta for being so kind and caring, Ann-Cathrine for nice conversations and laughs, Vladimir for bringing your Russian sweets to the “fika”, Shimako for your friendly ways, Carolina for your cool personality, Annie for being a good roommate and Adrian for nice chats.


To our collaborators: Giesbert Alken and Frank Schneider at BiRDS Pharma GmBH for all the inspiring meetings we have had. Angela Cenci-Nilsson for sharing your
knowledge about the AIMs test and Daniella Rylander for being such a nice person and travel companion.

To the head of the Department of Physiology and Pharmacology, Stefan Eriksson, for creating a good working atmosphere.

To all the present and former staff at the Department of Physiology and Pharmacology. For making my everyday life at work run smoothly: Renée Andersson (thank you for recovering my lost thesis documents from the server), Micke Elm and Eva-Britt Näsström. For help with various administrative matters: Eva Gipperth, Camilla Fors-Holmberg, Monica Pace-Sjöberg, Ulla Wester, Ulla Lindgren, Freddie Hellström, Ylva Haraldsdotter and Sophia Petterson. For help with the teaching activity Inger Johansson, Louise Bovin, Liselotte Lundblad and Peter Wolf. Finally, for help with my animals Per-Arne Åberg.


Till min familj:

Tack till min nya norrländska familj som välkomnat mig med öppen famn. Brith-Inger och Arne ni har kämpat mot cancer och vunnit, ni är så starka! Ann-Louise och Ted ni är världens goaste, jag ser fram emot att få träffa familjens lilla nytillskott när han/hon kommer 😊.

Tack till min bro Hjalmar och Nina för att ni är är mig kära. Tack till min gossige gudson Albin för att du ger perspektiv på tillvaron och tycker att faster ska kolla på "färet shaun" med dig istället för att skriva avhandling, sist men inte minst lilla charmtrollet Elsa som får mitt hjärta att smälta.

De bästa tanterna som finns, Mormor för att du alltid bryr dig om mig och är intresserad av det jag gör, du är underbar! Farmor jag älskar dig för att du är så rättfram och utan skrupler och för att du ber till gud för mig när det behövs.


7 REFERENCES


Ahlskog JE, Muenter MD (Frequency of levodopa-related dyskinesias and motor fluctuations as estimated from the cumulative literature. Mov Disord 16:448-458.2001).


References


Carta M, Carlsson T, Kirik D, Björklund A (Dopamine released from 5-HT terminals is the cause of L-DOPA-induced dyskinesia in parkinsonian rats. Brain 130:1819-1833.2007).


Cenci MA, Lee CS, Björklund A (L-DOPA-induced dyskinesia in the rat is associated with striatal overexpression of prodynorphin- and glutamic acid decarboxylase mRNA. Eur J Neurosci 10:2694-2706.1998).


References

Fahn S (Does levodopa slow or hasten the rate of progression of Parkinson's disease? J Neurol 252 Suppl 4:IV37-IV42.2005).


Gerfen CR (The neostriatal mosaic: multiple levels of compartmental organization. Trends Neurosci 15:133-139.1992).


Kannari K, Yamato H, Shen H, Tomiyama M, Suda T, Matsunaga M (Activation of 5-HT(1A) but not 5-HT(1B) receptors attenuates an increase in extracellular dopamine derived from exogenously administered L-DOPA in the striatum with nigrostriatal denervation. J Neurochem 76:1346-1353.2001).


Mura A, Jackson D, Manley MS, Young SJ, Groves PM (Aromatic L-amino acid decarboxylase immunoreactive cells in the rat striatum: a possible site for the conversion of exogenous L-DOPA to dopamine. Brain Res 704:51-60,1995).
References


Orosz D, Bennett JP (Simultaneous microdialysis in striatum and substantia nigra suggests that the nigra is a major site of action of L-dihydroxyphenylalanine in the "hemiparkinsonian" rat. Exp Neurol 115:388-393.1992).


Parkinsonstudgygroup (Dopamine transporter brain imaging to assess the effects of pramipexole vs levodopa on Parkinson disease progression. JAMA 287:1653-1661.2002).

Paterson IA, Juorio AV, Berry MD, Zhu MY (Inhibition of monoamine oxidase-B by (-)-deprenyl potentiates neuronal responses to dopamine agonists but does not inhibit dopamine catabolism in the rat striatum. J Pharmacol Exp Ther 258:1019-1026.1991).


Pearce RK, Jackson M, Smith L, Jenner P, Marsden CD (Chronic L-DOPA administration induces dyskinesias in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated common marmoset (Callithrix jacchus). Mov Disord 10:731-740.1995).


References


Sotnikova TD, Beaulieu JM, Espinoza S, Masri B, Zhang X, Salahpour A, Barak LS, Caron MG, Gainetdinov RR (The dopamine metabolite 3-methoxytyramine is a neuromodulator. PLoS One 5:e13452.2010).
Sulzer D (Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. Trends Neurosci 30:244-250.2007).
Tepper JM, Lee CR (GABAergic control of substantia nigra dopaminergic neurons. Prog Brain Res 160:189-208.2007).
References


Wickens JR (Synaptic plasticity in the basal ganglia. Behav Brain Res 199:119-128.2009).


