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

Ottosson, Daniella

2010

Link to publication

Citation for published version (APA):
Rylander, D. (2010). Involvement of non-dopaminergic systems in L-DOPA-induced dyskinesia Department of Experimental Medical Science, Lund University

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IN Volvement of Non-Dopaminergic Systems in L-Dopa-Induced Dyskinesia

by

Daniella Rylander

Academic dissertation
With the approval of the Faculty of Medicine at Lund University
this thesis will be defended on September 17, 2010
at 1 pm in Segerfalksalen, Wallenbergs Neuroscience Center, Lund, Sweden

Faculty opponent
Prof. Michaela Morelli
Department of Toxicology, Unit of Pharmacology and Pharmacognosy,
University of Calgliari, Calgliari, Italy
Involvement of non-dopaminergic systems in L-DOPA-induced dyskinesia

Abstract

Parkinson's disease and L-DOPA-induced dyskinesia (LID) does not merely involve the dopamine (DA) system but also include non-dopaminergic systems such as glutamate and serotonin (5-HT).

An aberrant glutamatergic transmission at the corticostriatal synapse, has been linked to LID. Pharmacological agents to glutamate receptors at this synapse, (of which some are already clinically tested), could prevent the aberrant signalling and the consecutive development of LID. In this thesis, a rat model of the disease was used for comparing the following substances for their anti-dyskinetic effects: 1) antagonist for L-type calcium channels, 2) antagonist of the NR2B subtype selective NMDA receptor, 3) agonist of the presynaptic mGluR2/3, 4) antagonist of postsynaptic mGluR1, or 5) mGluR5 receptor. Animals were treated chronically with L-DOPA alone or in combination with any of the antagonist/agonist. The L-type calcium channel antagonist iradipine, was shown inefficient in reducing LID. But notably, when iradipine was given at an earlier time-point (i.e. at the time of DA denervation) it could prevent pathological alterations in morphology in striatal neurons (induced by a DA depletion), and reduce the development of LID. Therefore, iradipine could in a prophylactic way reduce the development of dyskinesia but only when given at an early stage of the disease.

When comparing the different glutamate targets (given after the DA-denervation), results showed that the modulation of mGluR5 was superior to all other receptors/channel in relieving LID without compromising the therapeutic effect of L-DOPA. Promoted by these results, another mGluR5 antagonist fenobam, that has been clinically tested, was tested in both rats and monkeys model of LID. A more efficient alleviation of LID was achieved with a maximum effect of 70%. Thereby, fenobam represents the most effective "anti-glutamatergic" drug so far tested in experimental Parkinson's models and acts similarly in rat and monkey model of LID.

The second part of the thesis evaluates the 5-HT system. In the DA depleted striatum, L-DOPA is primarily taken up and converted to DA, in the residual striatal 5-HT terminals. However, these do not have an autoregulatory machinery for DA release, and can cause excessive DA release as a risk factor for dyskinesia. Here, a new mechanism of 5-HT maladaptive plasticity induced by chronic L-DOPA treatment, was revealed. Analysis of rat and monkey models, and post-mortem tissue from patients, consistently showed a positive association between striatal 5-HT fibre density and the severity of LID. This growth-promoting effect was paralleled by a greater stimulus-evoked DA release in dyskinetic animals compared to saline controls. Taken together, a maladaptive plasticity of 5-HT fibres in the striatum, should be seen as a susceptibility factor for the development of LID. Moreover, it could provide a biomarker for LID.

Key words: Parkinson's disease, dyskinesia, AIM, 6-OHDA, MPTP, DA, 5-HT, metabotropic glutamate receptor, sprouting, spine pruning, autoradiography, electron microscopy.

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Date: 6th October 2010
IN VolvEMENT OF NON-DOPAMINERGIC SYSTEMS IN L-DOPA-INDUCED DYSKINESIA

by

DANIELLA RYLANDER

Academic dissertation
With the approval of the Faculty of Medicine at Lund University
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Faculty opponent
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University of Calgliari, Calgliari, Italy

Lund University
Faculty of Medicine
Cover

An artistic illustration of sprouting serotonin fibres in the human striatum. Cover artwork by Tina and Desire Apelgren.
"The most beautiful thing we can experience is the mysterious. It is the source of all true art and science."

Albert Einstein
To my parents and to Simon
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Jag har i min avhandling undersökt två system: glutamat och serotonin, som båda tros spela viktig roll vid uppkomsten av biverkningarna. Med hjälp av antagonister till receptorer på celler kan man inhibera cellerna och desras system. Sådana antagonister, till t.ex. glutamatreceptorer, skulle tillsammans med L-DOPA kunna utgöra en bättre behandling för patienter med betydligt mindre eller helt utan dyskinesier.

I en beprövad rättmodell för Parkinsons förloras dopamincellerna i hjärnan efter en injektion av ett cellspecifikt gift. I sådan rättmodell har jag i avhandlingens första del, jämfört effekterna av olika antagonister till glutamatsystemet. Djuren behandlades under ett par veckor med L-DOPA tillsammans med en av de olika antagonisterna. Råttornas grad av dyskinesi samt den positiva (terapeutiska) effekten av L-DOPA mättes med beteendetester. Resultaten visade att djur som behandlades med L-DOPA tillsammans med en antagonist till receptorn metabotrof glutamat receptor 5 (mGluR5), hade mindre dyskinesi än de djur som behandlats med andra antagonist eller endast L-DOPA. För att föra denna upptäckt närmare klinisk relevans provades även en kliniskt testad mGluR5-antagonist fenobam. Fenobam gav tillsammans med L-DOPA en förlängd terapeutisk effekt med upp till 70% minskade dyskinesier i både rätt- och apmodell av sjukdomen. Även en annan antagonist till glutamatsystemet som specifikt blockerar s.k. kalciumkanaler kunde minska på dyskinesierna i rätta, men endast om antagonisten gavs i ett tidigt stadium av den Parkinsonliknande skadan. Detta eftersom antagonisten då kunde förhindra skadliga cellförändringarna i hjärnan.

I den andra delen av avhandlingen har jag undersökt serotonin systemet. Parkinsons sjukdom orsakar även en mindre förlust av nervceller som producerar serotonin. I en omfattande studie på rätta och apa likasom analys av hjärnvävnad från avlidna Parkinsonpatienter, visade vi för första gången att dyskinesi är kopplat till en högre densitet av serotonincells utskott. L-DOPA-behandling orsakade en tillväxt och förgrenning av dessa utskott i hjärnan. En sådan tillväxt kan gynna dyskinesier genom att leda till en förhöjd och skadlig frisättning av dopamin i hjärnan efter en L-DOPA administration. Även om hög serotoninhalt i hjärnan kan vara godartad vid t.ex. depression, visar min avhandling att den vanligaste och mest effektiva behandlingen för Parkinsons sjukdom (L-DOPA) leder till en ökad tillväxt av serotonincells och i sin tur utgör en stor riskfaktor för dyskinesi.
Sammantaget visar denna avhandling potentiella framtida mediciner som tillsammans med L-DOPA kan ge en bättre behandling för Parkinsonspatienter. Både fenobam (mGluR5-antagonisten) och isradipin (som blockerar kalciumkanaler) är dessutom redan testade i människa. Medan isradipine endast borde verka när den ges ett tidigt skede av sjukdomen verkar fenobam ha en positiv effekt även när Parkinsons fortskrider. Vidare visar denna avhandling på en ny patologisk förändring av serotoninsystemet efter kronisk L-DOPA-behandling. Denna förändring kan ses som en viktig riskfaktor för komplikationerna och bör beaktas vid behandlingen.

Förhoppningsvis kan vi i framtiden hitta lämpligare behandlingar så att fler Parkinsonspatienter kan få ett bra liv trots Parkinson, eller som för en drabbad uttryckte det ”ett bättre liv trots Parkinsons”.
Parkinson's disease and L-DOPA-induced dyskinesia (LID) does not only involve the dopamine (DA) system but also include non-dopaminergic systems such as glutamate and serotonin (5-HT).

An aberrant glutamate transmission at the corticostriatal synapse has been linked to LID. Pharmacological agents to glutamate receptors at this synapse, (of which some are already clinically tested), could prevent the aberrant signalling and the consecutive development of LID. In addition to the glutamate receptors, certain types of calcium channels (L-type-calcium channels) are involved in the glutamate transmission, being involved in the mechanism of spine pruning on the striatal neurons that occur after a DA depletion.

In this thesis, the 6-OHDA rat model was used for evaluating and comparing the following substances for their effects on akinesia or dyskinesia as well as LID-associated molecular and morphological alterations: 1) antagonist for L-type calcium channels, 2) antagonist of the NR2B subtype selective NMDA receptors, 3) agonist of the autoreceptor mGluR2/3, 4) antagonist of mGluR1 or 5) antagonist of mGluR5. Animals were treated chronically with clinical relevant dose of L-DOPA alone or in combination with any of the antagonist/agonist.

Results showed that the antagonist of L-type calcium channels isradipine was inefficient in reducing LID. However, when isradipine was given at an earlier time-point (i.e., at the time of DA depletion), it could prevent the morphological alterations of spine pruning and reduce the development of LID. Therefore, isradipine could in a prophylactic way reduce the development of dyskinesia but only at an early stage of the disease (when morphological alteration has not yet occur).

When comparing the different glutamate antagonists/agonist (after DA denervation), results showed that modulation of the target mGluR5 was superior to all other targets in alleviating LID without compromising the anti-parkinsonian effect of L-DOPA. Moreover, it normalised striatal nuclear changes that are associated to LID. Prompted by these results, another mGluR5 antagonist fenobam, that has been clinically tested, was evaluated in both rats and monkey model. In this study a more efficient alleviation of LID was achieved with a maximum effect of 70%. Furthermore fenobam prolonged the anti-akinetic effect of L-DOPA. Thereby fenobam represent the most effective “anti-glutamatergic” drug tested in experimental PD models so far and acts similarly in rat and primate models of LID.

The second half of the thesis evaluates the 5-HT system in LID. In the DA-depleted striatum, L-DOPA is primarily taken up and converted to DA, in residual 5-HT terminals. These however, do not have an autoregulatory machinery for DA release and could cause excessive DA release that triggers dyskinesia. This thesis has revealed a new mechanism of 5-HT maladaptive plasticity that is induced by chronic L-DOPA treatment in the striatum and possibly other brain areas as well. Analysis of rat and
monkey models of LID and post-mortem tissue from PD patients consistently showed a positive association between striatal 5-HT fibre density and the development of LID. The association was only true for dyskinesia induced by L-DOPA treatment, in contrast to dyskinesia induced with direct DA agonist. In dyskinetic animals, an L-DOPA-induced sprouting effect was demonstrated with increased number of 5-HT positive axon varicosities in the striatum compared to saline controls. The growth-promoting effect was paralleled with a greater stimulus-evoked DA release.

Altogether, 5-HT fibre innervation to the striatum could be seen as a susceptibility factor for developing LID.
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>AADC</td>
<td>Aromatic-L-amino acid dopadecarboxylase</td>
</tr>
<tr>
<td>AIM</td>
<td>Abnormal involuntary movements</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>D1</td>
<td>Dopamine receptor 1</td>
</tr>
<tr>
<td>D2</td>
<td>Dopamine receptor 2</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine uptake transporter</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>3,4-dihydroxyphenylalanine</td>
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<td>LID</td>
<td>L-DOPA-induced dyskinesia</td>
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<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>LTD</td>
<td>Long term depression</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus pallidus, external segment</td>
</tr>
<tr>
<td>GPi</td>
<td>Globus pallidus, internal segment</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-4-proprionoxypiperidine</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSN</td>
<td>Medium spiny neuron</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-human primate</td>
</tr>
<tr>
<td>NMEDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>pERK1/2</td>
<td>phosphorylated Extracellular signal-related kinase</td>
</tr>
<tr>
<td>pMSK-1</td>
<td>phosphorylated Mitogen activated signal-related kinase 1</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PDyn (PPE-B)</td>
<td>Prodynorphin</td>
</tr>
<tr>
<td>PPE-A</td>
<td>Preproenkephalin</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin uptake transporter</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNpr</td>
<td>Substantia nigra pars reticulata</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic nuclei</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VMAT2</td>
<td>Vesicular monoamine transporter 2</td>
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ORIGINAL PAPERS AND MANUSCRIPT


PAPERS OUTSIDE THE THESIS


Review article

INTRODUCTION

BASAL GANGLIA

The basal ganglia are an interconnected group of sub-cortical nuclei that are involved in the regulation of motor control and reward-motivated behaviours. The most widely accepted view of basal ganglia function has emerged from observations of humans afflicted with neurodegenerative diseases such as Parkinson's or Huntington's disease. Thus, it appears to play an important role in the subtle regulation of voluntary movements via two different modulatory pathways, the indirect and the direct pathway. Glutamatergic input form the cerebral cortex and from the thalamus converge into the basal ganglia nuclei in a topographical order where the signal is modified and sent via the ventral thalamus back to the cortex in a feedback loop. According to the Albin-DeLong theory (Alexander et al., 1986; DeLong, 1990), GABAergic projection neurons, from the primary input nucleus, striatum (that in humans is divided to caudate nucleus and putamen), project to the output nuclei, the substantia nigra pars reticulata (SNr) and the internal globus pallidus (GPi; entopenducular nucleus in non-primates) (Fig.1). This direct pathway provides an excitatory control of the thalamus and enhancement of movement-initiated signals. In contrast, the indirect projections from the striatum pass through the external globus pallidus (GPe) and subthalamic nuclei (STN), providing an inhibitory control of the thalamus and the consecutive movement (Fig. 1A) (Obeso et al., 2000b). GABAergic projection neurons of the striatum, referred to as medium spiny neurons (MSN), create two distinct pathways: the direct pathway, utilising substance P and expressing the opioid prodynorphin (PDyn), or the indirect pathway, expressing enkephalin (Gerfen et al., 1990). Both pathways are under modulatory control by the dopaminergic neurons that innervate basal ganglia nuclei. In addition, other neurotransmitters innervate the basal ganglia, e.g. noradrenaline, acetylcholine (Brotchie, 2005), adenosine (Richardson et al., 1997) and importantly for this thesis, serotonin (5-HT). In particular the striatum and output regions of the basal ganglia receive a dense serotonergic input (Hornykiewicz and Kish, 1987; Lavoie and Parent, 1990).

Although this model of basal ganglia organisation is now well established and has opened up for electrophysiological treatment, as in deep brain stimulation, it is still very simplified (Obeso et al., 2000b). Thus it can not explain why some stimulation or lesioning of STN or GPi (targets for deep brain stimulation) does not for example alleviate the overstimulation of glutamate output as predicted (Blanchet et al., 1994).
Dopamine (DA) plays a central role in motor and psychiatric functions and its loss leads to numerous neurological and psychiatric disorders. It is produced in the A8-A10 cell groups of the substantia nigra pars compacta (SNpc) and the adjacent ventral tegmental area. Here L-DOPA is decarboxylated from tyrosine by the rate-limiting enzyme tyrosine hydroxylase (Fitzpatrick, 1991) and then converted into DA by L-amino acid decarboxylase (AADC) enzyme. Dopamine is then transported to synaptic vesicles through the vesicular monoamine transporter 2 (VMAT2) (Guillot and Miller, 2009). The high affinity plasma membrane transporter (DAT) is involved in determine the intensity and duration of DA signalling and is located in the perisynaptic sites of the DA releasing terminals. Several pharmacological agents and neurotoxins, including the psychostimulants cocaine and amphetamine, act on this transporter (Torres et al., 2003).

Nigrostriatal axon terminals synapse onto the spines or dendrites of the GABAergic MSN of the striatum and modulate glutamate and GABA signalling. Two main types of DA receptors are expressed on the MSNs or on the DA terminals, i.e. D1 or D2 receptor. Activation of D1-like receptors (D1 and D5) on the MSNs lead to increased levels of cytosolic cAMP through the G-protein G_{olf} that is positively coupled to adenylyl cyclase. Increased cAMP by D1 receptor activation leads to phosphorylation of various intracellular targets (See Fig.1A)(Gerfen et al., 1990). The D1-receptor positively interacts with calcium channels and glutamate receptors to promote increased responsiveness of MSN to sustained glutamate release (Surmeier et al., 2007). In contrast to the net excitatory effect of the D1 signalling pathway, the D2 receptors exert an inhibitory effect on MSN. D2-like receptors (D2, D3 and D4 receptors) are coupled to G_{i/o} protein, which is negatively coupled to adenylyl cyclase and the downstream cAMP/PKA pathway (Neve et al., 2004). These receptors are located either presynaptically, on DA releasing terminal to suppress calcium channel activity, or postsynaptically on MSN where they diminish glutamate release and negatively modulate ion channels (Surmeier et al., 2007). In addition these receptors form functional complexes with other receptors, such as adenosine and metabotropic glutamate receptors 5 (Schwarzschild et al., 2006).

Together dopaminergic and non-dopaminergic modulation provide a balance between the direct and indirect pathways to delicately modulate voluntary movement, cognitive function and reward mechanisms. Any disturbances in the balance of output signals have severe consequences with motor dysfunctions such as those seen in Huntington's or Parkinson's disease.
PARKINSON'S DISEASE

As one of today's most common and well understood movement disorders, Parkinson's disease (PD) still demonstrates the characteristic posture and movement deficits as when it was first described 1817 by James Parkinson's:

"...involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported with a propensity to bend the trunk forwards, and to pass from a walking to a running pace, the senses and intellects being uninjured."

Today the disease affects approximately 1% of people over the age of 50 with its characteristic motor symptoms, muscle rigidity, tremor, postural imbalance and akinesia (Olanow, 2004). Although described almost two centuries ago, the underlying pathological explanation for the disease was not revealed until 1957 by the Swedish Nobel prize winner, Arvid Carlsson. Now it is known that the basic symptoms behind PD are due to a profound loss of nigrostriatal DA (Hornykiewicz, 1963).

Parkinsonian motor symptoms arise when 60-80% of the striatal DA innervation is lost (Marsden, 1990), causing an imbalance between the different basal ganglia pathways. Recalling the basal ganglia pathways (Fig.1) a loss of DA will cause an inhibition of the direct pathway and an enhancement of the indirect pathway (See Fig.1B) (DeLong, 1990). The net effect of this is less glutamate input to cortex and thalamus and suppression of movement, i.e. akinesia (Obeso et al., 2000b).

The pathogenic mechanism behind the DA cell loss is believed to involve oxidative stress, mitochondrial dysfunction, excitotoxicity and inflammation all of which could be triggered by any environmental or genetic predisposition (or by trauma to the head as for Muhammad Ali) (Kumar, 2005). Other than increasing age and to a lesser extent gender (men are more affected than women) (Korell, 2005), the disease aetiology is largely unknown. Environmental risk factors include exposure to pesticides and metals, viruses, well-water drinking and rural living. Some disease-causing mutations have been identified, e.g. the α-synuclein gene and parkin gene associated with the sporadic or juvenile form (Kumar, 2005).

Today the knowledge of the PD pathology has extended to the involvement of non-DA neurotransmitters such as noradrenaline, acetylcholine and interestingly for this thesis 5-HT (Bernheimer et al., 1961; Hornykiewicz and Kish, 1987; Scatton et al., 1983). Serotonin is, for example, believed to contribute the non-motor symptoms such as cognitive decline, sleep abnormalities and depression, features that dominate the later stage of the disease (Fox et al., 2008; Guttman et al., 2007).

Therapies for Parkinson's disease
As of today, no intervention to arrest, reverse or slow down the disease progression exist, although cigarette smoking, coffee consumption and the use of nonsteroidal anti-inflammatory drugs (NSAIDs) are proposed to protect against its development (Korell, 2005). However, several treatment interventions, pharmacological and surgical are available for symptomatic relief. Here in Lund, there have been clinical trials aiming at
replacing the lost DA cell with transplantation of fetal DA-producing cells into the striatum. Both in animal models and clinical studies such transplantations have shown major improvement in many motor deficits (Lindvall et al., 1994; Winkler et al., 1999). However problems such as large inter-individual variability in the clinical outcome, and the occurrence of post-operative dyskinesias, i.e. OFF L-DOPA limits this approach. Many patients are symptomatically relieved by surgical intervention with either deep brain stimulation or surgical lesioning of e.g. GPe or STN (See Fig.1). But unfortunately, surgical therapy is costly and invasive and not suitable for patients with psychological deficits or at an advanced age (Baba, 2005)

In the progress of the medical management of PD there has been a large effort for improving DA therapies as well as identifying non-DA drugs. Among the latter, anticholinergic drugs (that have actually been used decades before the introduction of L-DOPA), adenosine A2A antagonists (Fuxe et al., 2001) and NMDA antagonists (mainly as adjunct to L-DOPA pharmacotherapy) are used in clinics (Schwarzschild et al., 2006).

Since the early 1960s, DA replacement therapies have been extensively used with predictable effects (and side-effects). Dopamine agonists (mainly activating D2 and D3 receptors) are currently used drugs as are inhibitors of the DA metabolism, such as monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). Probably reflected by a longer duration of action, the DA agonists produce a low incidence of motor complications (Nutt et al., 2000). In line with this we have in a recent study showed an improvement of dyskinesia with a deuterium substituted L-DOPA molecule providing a more stable DA stimulation (Malmlöf, 2010).

So far none of the synthetic DA agonist have surpassed the efficacy of L-DOPA, which remains a cornerstone of anti-parkinsonian therapy. Despite its effective control of motor symptoms, L-DOPA causes high levels of motor complications, particularly involuntary movements, i.e. dyskinesia.
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Fig. 1. Schematic overview of basal ganglia pathways. In the physiological state A, glutamate input from the motor and premotor cortex projects in the direct pathway via striatum to GPi/SNpr. Alternatively it project in the indirect pathway to GPe and STN. Grey arrows indicate excitatory projections (glutamate) and black inhibitory (GABA). In the physiological state, nigrostriatal DA regulates the two different pathways. However in the parkinsonian state (B) the loss of nigrostriatal DA causes a pronounced activation of indirect pathway (note the thickness of arrows illustrating a higher degree of functional activity) that causes an excessive inhibition of movement. In L-DOPA-induced dyskinesia on the other hand (C) the non-physiological input of DA from L-DOPA instead causes overactivity of the direct pathway. Abbreviations: GPe and i: globus pallidus external and internal respectively, put: putamen, SNc: substantia nigra pars compacta and SNpr: substantia nigra pars reticulata, STN: subthalamic nuclei.

L-DOPA-INDUCED DYSKINESIA

L-DOPA (3,4 dihydroxy-L-phenylalanine) is the direct precursor of DA and constitutes the single most effective anti-parkinsonian drug that moreover prolongs the life span of PD patients (Marsden, 1990). During the first years of treatment L-DOPA efficiently relieves the PD motor symptoms (the so called honeymoon period). Nevertheless patients often experience a short lasting improvement (ON response) of L-DOPA after 5-10 years and/or develop motor complications such as dyskinesias (Obeso et al., 2000a). These affects as much as 90 % of the patients after 10 years of treatment (Ahlskog and Muenter, 2001) and can even be induced to a lesser degree by DA receptor agonists (Fabbrini et al., 2007). The non-physiological movements interfere with goal-oriented activities and can be socially embarrassing for the patient (Rascol et al., 1998). Therefore it limits the dose of symptomatic therapy and complicates the treatment in advance PD.

Even though the majority of patients experience dyskinesia after years of L-DOPA therapy, some individuals will, however, remain free from complications. Moreover, there is a large individual variability in the time of onset and the severity of dyskinesias (Linazasoro, 2005). Young patients are particularly vulnerable, as are advanced parkinsonian patients that are given a high dosage of L-DOPA (Manson and Schrag, 2006; Sossi et al., 2006).

Dyskinesia normally appears when L-DOPA reaches its peak plasma level in the brain referring to "peak-dose" dyskinesia. This is characterised as chorea of the limbs, trunk or head often associated with dystonia. Notable, dyskinesias can also be
induced when L-DOPA levels are low, increasing or decreasing, i.e. biphasic dyskinesia (Giron and Koller, 1996). Whereas chorea-like dyskinesia are thought to generally involve overactivity of the direct pathway, dystonic-like dyskinesia has been tentatively attributed to involve the indirect pathway of the basal ganglia (Bezard et al., 2001a; Mitchell et al., 1992). Dyskinesia could be seen as movement-releasing state (Fig.1), that is notably pathological in character. Dyskinesia is therefore not merely an exaggeration of physiological movement.

Striatal plasticity in L-DOPA-induced dyskinesia
L-DOPA-induced dyskinesia, which I will refer to as "LID", can be seen as an aberrant form of striatal plasticity where L-DOPA-derived DA signalling causes long-lasting molecular and behavioural changes similar to those seen in long-term memory formation. This is particularly evident on glutamatergic synapses where the memory storage mechanism of long-term potentiation (LTP) is malfunctioning in animals with dyskinesia (Picconi et al., 2003). Maladaptive plasticity in LID involves both presynaptic changes involving the regulation of L-DOPA-derived DA release and metabolism in the brain and postsynaptic changes on the MSN (Cenci and Lundblad, 2006) (Fig.2). Accordingly, dyskinesia is improved by treatments that either stabilize extracellular DA levels or dampen the supersensitive response of MSN. The relative contribution of pre- versus postsynaptic mechanisms may differ among dyskinetic subjects, making them more or less responsive to different treatment options.

The progress of DA neurodegeneration causes a main trigger for the development of LID together with a transient rise and fall in DA levels after repeated administration of L-DOPA (Chase, 1998) (Fig.2). The nigrostriatal DA cell loss forces L-DOPA to be taken up by non-DA terminals, i.e. 5-HT terminals that also contain AADC and thus can convert L-DOPA to DA. However, since these lack autoregulatory machinery for the release, the DA release becomes excessive and lead to large intermittent surges after L-DOPA administrations (Fig. 2,3). In this way 5-HT have come to play an important role in LID (Fig. 2) of which I will focus on in this thesis. Actually, dyskinesia has been linked to increased extracellular levels of DA in both animal models (Lindgren et al., 2010; Meissner et al., 2006) as well as in the clinics, (de la Fuente-Fernandez et al., 2004; Pavese et al., 2006).

The postsynaptic plasticity (white boxes in Fig.2) includes upregulation of transcription factors and plasticity genes in the striatal MSN (Cenci and Lundblad, 2006). These changes are most pronounced in the direct pathway, i.e. the prodynorphin-positive MSNs where a D1 receptor function has been suggested to be potentiated or even upregulated (Aubert et al., 2005; Konradi et al., 2004). Several targets of striatal nuclear signalling have been discovered such as phosphorylation of extracellular signal-related kinases (pERK) 1 and 2 (Westin et al., 2007), GABA synthesizing enzyme GAD67 mRNA (Cenci et al., 1998) and upregulation of the transcription factors FosB/ΔfosB (Andersson et al., 1999). These changes are induced both after a DA denervation and after chronic DA-agent treatment in a region specific manner as shown in both rat and monkey models of PD (Andersson et al., 1999; Doucet et al., 1996). The induction of ΔFosB in turn leads to an increased transcription of opioid peptides like
enkephalin and dynorphin in the D1 and D2 positive neurons respectively (Andersson et al., 1999; Quik et al., 2002). The expression in the lateral striatum has shown to tightly correlate with LID (Bezard et al., 2001a; Cenci et al., 1998). Since the activation of D1 receptors on the MSNs has an important role in regulating the glutamate transmission, a severe DA loss leads to alterations in glutamatergic signalling (Fig. 2) (Chase and Oh, 2000). These glutamatergic changes could negatively affect the development of LID.

Fig 2. Non-DA systems affected by DA denervation and L-DOPA treatment condition in the susceptibility to LID. Boxes in light grey illustrate presynaptic alterations that are occurring in the LID basal ganglia. Large DA surges, DA denervation and pulsatile L-DOPA treatment in turn cause postsynaptic changes (white boxes), especially on the D1-receptor positive MSNs, which includes upregulation of pERK1/2 signalling pathway and consecutive upregulation of ΔFosB and pDyn transcription. This in turn leads to altered activity pattern of output structures (recall from Fig. 1). On to these alterations, the non-DA systems: glutamate and serotonin (dark grey boxes) are implicated, either in contributing to the supersensitive responses on the corticostriatal synapse (glutamate) or providing the location for dysregulated DA efflux (serotonin).
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GLUTAMATE SYSTEM IN L-DOPA-INDUCED DYSKINESIA

The role of glutamate neurotransmission in the development and expression of dyskinesia is supported by elevated extracellular glutamate levels in the striatum of dyskinetic rats (Robelet et al., 2004), and excessive cortical activation in dyskinetic PD patients during the execution of simple movements (Rascol et al., 1998). Glutamate plays an important role in physiological motor skill learning, reward mechanisms and cognitive performance through participation in the two "classical" forms of long-term synaptic plasticity, i.e. long-term potentiation (LTP) and long-term depression (LTD) (Calabresi et al., 2007). This plasticity allows for enhanced or decreased network connection during important or reluctant environmental events in the healthy brain. This leads to addiction and habit formation. In pathological conditions however, this regulation is probably disrupted giving rise to purposeless, stereotyped movements, "a pathological habit" such as in LID (Conn et al., 2005). Parkinsonian motor symptoms are often attributed to an excessive glutamatergic activity in subthalamo-pallidal projections (see Fig.1), whereas LID has been linked to abnormal glutamate transmission in the striatum (See Fig. 2 and 3C) (Cenci and Lindgren, 2007; Chase et al., 2000).

Glutamate receptors and potential therapeutic targets

Multiple types of glutamate receptors and/or subunits exert modulated activity in DA-denervation and LID. Hence, there are potentially many targets for an “anti-glutamatergic” therapy of LID. Inhibiting or activating specific glutamate receptors could control either glutamate release or postsynaptic signalling and compensate for the aberrant plasticity. So far the specific functional properties of different targets are poorly understood.

Two categories of glutamate receptors are located in the basal ganglia: ionotropic and metabotropic. The ionotropic receptors N-methyl d-aspartate (NMDA) receptor and \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor are widely expressed in the brain and play pivotal roles in regulation of synaptic functions in the central nervous system. NMDA receptors have been linked to the pathology of the basal ganglia such as PD and LID with altered subunit expression (Dunah et al., 2000; Fiorentini et al., 2006; Ganguly and Keefe, 2001; Hallett et al., 2005). In fact, the only pharmacological agent so far designated as “efficacious” for the treatment of dyskinesia (although with side-effects) is amantadine, which exerts weak non-competitive antagonism of the NMDA receptor (Blanchet, 2003; Kornhuber et al., 1994; Metman et al., 1999). Antagonists of the AMPA receptor have shown some alleviating effects in animal models (Bibbiani et al., 2005; Konitsiotis et al., 2000) but clinical studies have halted due to lack of efficacy (Fox et al., 2008). In order to achieve more tolerable and efficient anti-dyskinetic treatment, a more selective targeting of glutamate receptor is preferable.

To specifically target subtypes of NMDA receptors could be one solution to this. The assemblies of NR1 and NR2A-D subunits form functional channels with differing physiological and pharmacological properties (Lau and Zukin, 2007). NMDA
receptors with NR2B subunits are particularly abundant in the striatum where they interact with DA receptors in mediating synaptic transmission and plasticity with long-term changes in the efficacy of glutamate transmission (LTP/LTD) (Gubellini et al., 2004) (Fig.3). Thus, this subunit is believed to coincide with both PD and dyskinesia, showing altered phosphorylation and trafficking from synaptic to extrasynaptic membranes (Calon et al., 2003; Dunah et al., 2000; Gardoni et al., 2006). Yet, studies using a NR2B subunit selective antagonist have opposing effects on dyskinesia, with some studies reporting a worsening and others alleviation of dyskinesia (Blanchet et al., 1999; Hadj Tahar et al., 2004; Nash et al., 2004; Nutt et al., 2008). A selective NR2B antagonist had not been tested in a rat model of LID before paper II in this thesis.

The metabotropic glutamate receptors (mGluRs) have received much attention as potential targets in the treatment PD and LID because of their modulatory role of glutamate transmission (Conn et al., 2005; Rouse et al., 2000). Group 1 mGluRs, includes mGluR1 and 5 and are highly enriched in the perisynaptic and post-synaptic membrane of striatal MSN as well as all interneurons where they are in a key position to modulate normal and abnormal striatal signalling (Lujan et al., 1997; Pisani et al., 2001a; Tallaksen-Greene et al., 1998; Testa et al., 1994). Subtype selective antagonists or genetic ablation of mGluR group 1 lead to blockade of LTP and LTD supporting the requirement of these receptors for these synaptic events (Gubellini et al., 2004). These G-protein coupled receptors are positively linked to phospholipase C (Gq coupled) to potentiate calcium channels, increase intracellular calcium and induce gene expression via the ERK1/2 pathway (Choe and Wang, 2002) (Fig. 3). In the MSN specifically, mGluR5 has positive functional interactions with the NR2 subunits of NMDA receptors as well as the adenosine A2A receptor (Guo et al., 2004; Heidinger et al., 2002; Mao and Wang, 2002; Schwarzschild et al., 2006) by a chain of anchoring proteins (including HOMER, Shank and PSD-95) that are clustering the receptors to the synaptic territory (Tu et al., 1999). Thus activation of mGluR5 potentiates NMDA currents and induces overactivity of the striatopallidal pathway with upregulation of PPE-A (Parelkar and Wang, 2003). Moreover mGluR5 has interaction with DA receptors (Conn et al., 2005) and mGluR5 antagonists 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and 2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) are able to compensate for a DA loss by normalizing the overactive neuronal activities in the STN and SNpr (Breysse et al., 2003). In line with in vitro studies, in vivo studies found anti-parkinsonian effects of mGluR5 blockade (Aguirre et al., 2005; Armentero et al., 2006; Breysse et al., 2003; Dekundy et al., 2006; Spoooren et al., 2000).

Dyskinetic rats and monkeys show enhanced striatal expression of mGluR5 (Konradi et al., 2004; Ouattara et al., 2009; Samadi et al., 2008b) suggesting a role for this receptor in the movement complication. In line with this, antagonism of mGluR5 prevents the development and attenuates already established expression of dyskinesia and its related molecular responses (Dekundy et al., 2006; Levandis et al., 2008; Mela et al., 2007; Morin et al., 2010; Yamamoto and Soghomonian, 2009). When we started our laboratory work for paper III, the anti-dyskinetic effect of mGluR5 antagonist had yet to be described in monkeys.
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In contrast to group I mGluRs (mGluR2 and 3) and III mGluRs (including mGluR4,6,7,8) are negatively coupled to adenylyl cyclase and are often expressed presynaptically on striatal neurons and interneurons where they modulate NMDA receptors and decrease transmitter release (Corti et al., 2002; Testa et al., 1994). Selective agonists of group II mGluRs potently decrease excitatory transmission at corticostriatal synapses, and also in a larger magnitude after DA denervation (Picconi et al., 2002). Thereby, mGluR2/3 seem to represent a drug target of potential interest for the treatment of dyskinesia, yet it has not been tested for anti-dyskinetic effect. Interestingly, agonists to group II mGluR, have also shown to increase striatal levels of DA (Gubellini et al., 2004) and provide neuroprotective and anti-parkinsonian effect in a rat PD model (Dawson et al., 2000).

L-type calcium channel at the corticostriatal synapse

L-type channels in striatal neuronal dendrites and spines are composed by one of two α-subunits: Ca_{1.2} or Ca_{1.3}. The channels are anchored near the glutamatergic synapses to mediate glutamate signalling (See Fig.3). L-type calcium channel are essential for glutamate-mediated CREB phosphorylation and c-fos gene induction in MSNs (Rajadhyaksha et al 1999), making them an interesting target when trying to inhibit an overactive glutamate signalling, such as that in LID (Chase et al., 2000). In fact, they are involved in neuroplasticity and neurotoxicity and L-type calcium channel blockers possess neuroprotective effects in MPTP animal models of PD (Kupsch et al., 1995). Interestingly, SNpc DA neurons have an unusual reliance on these channels to drive their intrinsic basal activity and this reliance seem to render them vulnerable to degeneration (Chan et al., 2007).

Interestingly, L-type calcium channels play an important role in controlling axonal guidance and synaptic contacts. The channels are linked to atrophy of dendritic spines and loss of corticostriatal synapses onto the striatopallidal neurons that occur in the parkinsonian brain (Day et al., 2006; McNeill et al., 1988). Accordingly, genetic or pharmacological blockade of L-type calcium channels with isradipine disrupt the synaptic pruning and the changes of glutamatergic synapses' structure, size and contacts that occur after DA depletion (Day et al., 2006). So far this is only speculated to be involved in the maladaptive plasticity of LID (Bezard et al., 2001a). In this thesis we hypothesise that the loss of spines and synaptic connectivity might participate in the development of LID altering information flow through the striatum and basal ganglia.
Fig. 3. Glutamate and DA neurotransmission at the corticostriatal synapse. Two types of MSN, the D1 expressing of the direct pathway (A) and the D2-expressing of the indirect (B) are proposed by mainstream view of the basal ganglia (Albin et al., 1989). Onto these glutamatergic corticostriatal terminals synapse to induce striatal signalling pathways leading to transcription of prodynorphin (pDyn) or preproenkephalin (PPE), respectively. In the DA depleted striatum (C) the serotonin (5-HT) terminals compensate for the uptake (via serotonin transporter (SERT)) and conversion of L-DOPA and release of DA (red dots.) SERT is expressed on 5-HT terminals away from the synaptic area terminating 5-HT signal by removal from the synaptic cleft. The unregulated DA release from these along with an overactive glutamate transmission (black dots) is thought to contribute to the development of dyskinesia via D1-signalling pathway. Potential antidyskinetic targets are illustrated in bolded text. Modified from Cenci 2007.

SEROTONIN SYSTEM IN L-DOPA-INDUCED DYSKINESIA

Serotonin, 5-hydroxytryptamine (5-HT) is produced in the raphe pontis, i.e. median raphe (B7) and dorsal raphe nuclei (B8), and signals to multiple brain areas to exert its well-known regulation of mood and emotion and to centres regulating food intake, sleep and circadian rhythms. Serotonin plays a role in brain development and regulates further motor function and pain sensitivity in the adult (Martinowich and Lu, 2008; Mattson et al., 2004). The system is vulnerable to neurodegeneration and the levels of 5-HT are greatly reduced in aged subjects as well as in neurodegenerative diseases such as PD (Halliday et al., 1990; van Luijtelaar et al., 1992). It has a remarkable capacity for compensatory sprouting after lesion of 5-HT system or after a non-5-HT system lesion (Liu et al., 2004; Maeda et al., 2005; Radja et al., 1993), a process that is under the control of brain-derived neurotrophic factor (BDNF).

There is growing evidence of synergism between 5-HT and BDNF both in the genetic susceptibility to affective disorders and in the response to antidepressant
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treatment (Henningsson et al., 2009; Martinowich and Lu, 2008). Possibly this dynamic duo also affect the plasticity of LID.

Serotonin and BDNF, a "dynamic duo"

Brain derived neurotrophic factor is the most abundant neurotrophin in the central nervous system, being expressed in all brain regions with the highest expression in hippocampus and cortex (Hofer et al., 1990). It has many effects on the 5-HT system: enhancing the activity of 5-HT synthesizing enzyme, 5-HT uptake and modifying the firing pattern of 5-HT neurons (Martinowich and Lu, 2008). In addition to its growth promoting effect on the 5-HT fibres, it has a stimulatory effect on transmitter release from 5-HT terminals (Goggi et al., 2002; Mamounas et al., 2000). Conversely, the 5-HT stimulation upregulates the expression of BDNF via a 5-HT receptor signalling-cascade (Martinowich and Lu, 2008).

In the basal ganglia, BDNF is abundantly synthesised in e.g. nigrostriatal and corticostriatal neurons (of layer II and V) and is retro- or anterogradely transported to the striatum (Altar and DiStefano, 1998). Through binding to its main receptor, the receptor tyrosine kinase (TrkB), it promotes neuronal survival, migration, phenotypic differentiation, axonal- and dendritic growth and synapse formation during development (Mattson et al., 2004).

Multiple indicia support a role for BDNF in dyskinesia. Chronic treatment with high doses of L-DOPA causes upregulation of BDNF in corticostriatal pathways that in turn is involved in behavioural sensitization (Guillin et al., 2001). Interestingly, a common single nucleotide polymorphism in the pro-domain of human BDNF gene, (valine to methionine substitution, Val66Met), has been identified to influence time of onset for PD or LID. In the adult brain the main function of BDNF involves strengthening the synaptic plasticity related to learning and memory by enhancing LTP at the synapses (Martinowich and Lu, 2008), a mechanism that in turn is involved in LID. In addition, BDNF protects from DA degeneration and counteracts depression and anxiety. The contribution of BDNF and 5-HT interaction to the motor and non-motor aspects of PD is a new, important area of investigation.

The role of serotonin in L-DOPA-induced dyskinesia

Biochemical evidence suggests an abnormal 5-HT transmission in the basal ganglia of PD patients and a neurodegenerative loss of 5-HT striatal projections, although to a variable extent (Bernheimer et al., 1961; Halliday et al., 1990; Kish et al., 2008). Thus, it is likely that this system contributes to the underlying mechanism of the disorder or treatment complications (Nicholson and Brotchie, 2002). In fact raphe-striatal terminals do play a major role in the pharmacotherapy of PD and LID. In the DA depleted striatum, the lack of DA terminals forces L-DOPA to instead be taken up by 5-HT terminals through the 5-HT transporter (SERT) (Kannari et al., 2006), as these neurons also express AADC and VMAT2 (Arai et al., 1995). Dopamine can then be stored in synaptic vesicles by VMAT2 and released upon stimulation together with 5-HT (Arai et al., 1995; Tanaka et al., 1999). Nevertheless, 5-HT axon terminals lack the autoregulatory feedback mechanism of DA terminals since neither DAT nor DA
autoreceptors are expressed (Arai et al., 1995; Tanaka et al., 1999). Consequently, the DA release becomes aberrant and leads to excessive swings in extracellular DA levels that are linked to dyskinesia (de la Fuente-Fernandez et al., 2004; Lindgren et al., 2010; Pavese et al., 2006; Navailles et al., 2010).

Supporting the role of 5-HT in LID, lesioning the 5-HT system reduce L-DOPA induced DA release in the striatum (Tanaka et al., 1999). Intrastralial transplantation of raphe neurons exacerbates LID (Carlsson et al., 2007) and oppositely 5-HT specific lesion dramatically reduces dyskinesia (Carta et al., 2007). Interestingly, blocking the fine-tuning of synaptic release by 5-HT autoreceptor agonists also decrease the L-DOPA derived DA release in addition to the 5-HT (Arai et al., 1995; Lindgren et al., 2010). Therefore these receptors provide potential therapeutic targets.

Among all the neurotransmitters, 5-HT has the highest number of receptors with at least 14 distinct members of receptors in addition to SERT (Torres et al., 2003). Selective 5-HT reuptake inhibitors (SSRIs) that bind to SERT are widely used therapies for depression and anxiety (Martinowich and Lu, 2008). The 5-HT receptors are predominantly G-protein coupled and among the most studied: 5-HT1 receptor is coupled to G\textsubscript{\alpha}1/G\textsubscript{\alpha}0 protein (Rouse et al., 2000). The 5-HT1A autoreceptor is located somatodendritically on the raphe level or on raphe terminals where it inhibits neurotransmitter release. Similarly to 5-HT1A, the 5-HT1B receptor is also expressed on the raphe-terminals in addition to non-raphe neurons such as GABAergic output neurons (Nicholson and Brotchie, 2002). Agonists to the autoreceptors have analgesic, anti-depressant and anti-anxiety like properties and serves as a potential target for treating alcoholism (Sari, 2004). Most importantly, agonists reduce the activity of 5-HT release and attenuate the DA release from the 5-HT terminals (Kannari et al., 2001) with an associated improvement of LID (Carta et al., 2007; Lindgren et al., 2010). Moreover, L-DOPA pharmacotherapy induces enhanced striatal expression of 5-HT1A/B receptor mRNA (Frechilla et al., 2001; Zhang et al., 2008). It therefore seems like the extra-raphe mechanisms of 5-HT system play a role in LID and chronic L-DOPA treatment.

In addition to this pre-synaptic mechanism, agonists can also bind to post-synaptic receptors, especially at higher doses. At this location, 5-HT1A and B agonists are believed to alleviate also D1 agonist-induced dyskinesia (Carta et al., 2007; Dupre et al., 2008; Jaunarajs et al., 2009). Unfortunately, postsynaptic activation of specifically the 5-HT1A receptor, has been linked to "serotonin syndrome" with flat body posture etc. (Goodwin et al., 1986). This could contribute to the appearance of hypokinesia and dystonia seen in monkey models of PD (Iravani et al., 2006) and provide a serious concern in clinical application. A synergistic effect between 5-HT1A and B agonists, at sub-threshold doses, might provide a better solution for therapeutic effect (Carta et al., 2008).

Given the negative role of 5-HT projections in the development of LID and the plasticity character of the 5-HT system, it seems possible that the 5-HT projections in parkinsonian subjects may vary individually, affecting the susceptibility to develop dyskinesia. Yet, the involvement of 5-HT fibre density in patients and non-human primates with dyskinesia is unknown. Moreover, it is not known whether this system
could be subjected to L-DOPA-induced plasticity. These questions will be evaluated in this thesis.

ANIMAL MODELS OF PARKINSON’S DISEASE

In order to explore pathophysiological hypotheses and test new therapies animal models are essential. A parkinsonian state in an animal either mimics the progressive nigral cell death, e.g. with overexpression of α-synuclein, and/or mimic the motor deficits, e.g. with pharmacological manipulation of DA system or toxins that causes persistent DA denervation. The most commonly used animal models are the 6-hydroxydopamine (6-OHDA) rat or the 1-methyl-4-phenyl-1,2,5,6 tetrahydropyridine (MPTP) monkey. These models imitate the DA deficiency rather than the process of PD neurodegeneration and are suitable for research of restorative or symptomatic screening for potential new pharmacotherapies in the late stage of the disease, (when the dyskinesias are most severe).

The 6-OHDA rat model
Since its first description by Ungerstedt 1968 (Ungerstedt, 1968) the 6-OHDA rat has been widely used as a tool for studying parkinsonian symptoms, the molecular mechanism behind the disease, pharmacotherapies and LID. The toxin is directly injected into the brain, where it is taken up by DAT to catecholaminergic (predominantly DA) neurons. The toxin accumulates in the cytosol where it inhibits mitochondrial complexes, forms free radicals and induces oxidative stress that will cause instant neurodegeneration. This animal model can produce partial or complete DA denervation of striatum, depending on where the toxin is injected and in what amount, and can be used for testing symptomatic drugs as well as restorative treatments (Bjorklund, 1991). For mimicking an early/mid stage of the disease the toxin is injected directly into the striatum in the terminal field of the DA pathway causing a slower progression of degeneration, whereas for mimicking a more severe and late stage of the disease injections are positioned onto the medial forebrain bundle, in close vicinity to the nigral cell bodies. This lesion causes a severe state of DA depletion in the striatum >97%, and severe cell loss in the SNpc.

The lesion is normally made unilateral in order to preserve the animals' ability to feed and maintain themselves without supportive care. Far from being a disadvantage, the unilateral lesion causes a functional asymmetry due to the DA imbalance that has long been used to unravel rotational behaviour after administration of a DA-agonists (Ungerstedt, 1976). Furthermore the hemispheric DA imbalance causes more complex behaviour as bias in forepaw use (Schallert et al., 2000) as well as stepping and sensory neglect (Marin et al., 2006), that can be used to study anti-parkinsonian treatment. More importantly for this thesis, the animal model has been developed, in our lab, as an experimental paradigm for studying LID sharing similar molecular, cellular and pharmacological mechanisms with LID in patients (Cenci, 2007; Cenci et al., 1998). Chronic treatment with a low dose of L-DOPA elicits abnormal involuntary movements...
(AIMs) of several body regions in the hemiparkinsonian rat. These reproduce the time course of "peak-dose" dyskinesia in PD and can then be alleviated by most anti-dyskinetic treatment used in the clinics (Dekundy et al., 2007; Lundblad et al., 2002).

**MPTP model in non-human primates**

The neurotoxin MPTP, discovered in 1983 by humans who self-administered contaminated heroin, causes parkinsonism (Langston and Ballard, 1983) indistinguishable from idiopathic PD (Lau, 2005). This has led to development of animal models, predominantly in non-human primates, particularly useful in studying dyskinesia (Bezard et al., 2001b). Although more costly, time-consuming, ethically problematic and available in a limited number of laboratories as compared to the 6-OHDA model described above, the MPTP model in non-human primates (NHP) shares much of the movement pattern seen in patients and is necessary for the development of potential clinical treatment (Cenci and Lundblad, 2007). The toxin is often given systemically (as in paper III and IV) but can also be administered by unilateral intracarotid infusion to induce a hemiparkinsonian state (Lau, 2005). Following a systemic injection, MPTP is readily absorbed into the brain where it is converted in the astrocytes into MPP+, 1-methyl-4-phenylpyridinium, the active toxic metabolite. This is then taken up by nigrostriatal DA nerve terminals, due to its affinity for DAT, to cause degeneration to a variable degree. Long-term exposure to small doses of toxins tends to cause the most neurodegeneration (Lau, 2005), as in animals included in paper III and IV.

Similar to PD patients, the MPTP lesioned primates respond to L-DOPA pharmacotherapy and include all problems in L-DOPA pharmacotherapy such as wearing-off, peak-dose dyskinesia and psychiatric complications (Bezard et al., 2001b). The behaviour can be assessed using either automated activity measurements in the cage, through infrared-based motion detectors or video based observations (Petzinger, 2005).
In this thesis rat and monkey models of PD and post-mortem material from PD patients was used to evaluate the role of the non-DA systems: glutamate and 5-HT in LID.
AIMS OF THE THESIS

This thesis has appraised two non-DA systems; the glutamate and the 5-HT system, regarding their contributory roles in the development and expression of LID. The studies herein aimed at evaluating behavioural and neurohistochemical effects of different pharmacological agents, targeting glutamate transmission in the search for a potential anti-dyskinetic drug. Moreover, this thesis aimed at evaluating further the role of the 5-HT system in LID. The specific aims of the separate papers were as follows:

· To evaluate the importance of L-type calcium channels and their regulation of lesion-induced spine pruning in LID.

· To compare anti-dyskinetic and anti-parkinsonian effects of compounds targeting glutamatergic transmission, also considering their effects on LID-associated molecular changes.

· To evaluate a mGluR5 antagonist under clinical development, in its anti-dyskinetic and anti-akinetic efficiency in rat and monkey models of PD.

· To evaluate the possible plasticity of the 5-HT system after dyskinesiogenic L-DOPA treatment in rat and monkey models of PD as well as in PD patients.

· To compare L-DOPA- and D1 agonist-induced dyskinesia in the rat, with respect to their behavioural profile and response to anti-dyskinetic glutamate and 5-HT treatments.
EXPERIMENTAL METHODS AND MATERIALS

SUBJECTS

In all papers, female Sprague-Dawley rats (Harlan, Netherlands) were used. These weighed 225-250 gram at the beginning of the experiments and were housed in a 12 hours dark-light cycle with food and water ad libitum. All the procedures were approved by the Malmö-Lund ethical committee on animal research.

In two papers (III and IV) behavioural data or neurohistochemical analysis of non-human primates were included. This was in collaboration with the research group of E. Bezard. Male or female rhesus monkeys (Macaca mulatta, Xierxin, Beijing, PR of China; mean weight = 5.3 ± 0.8 kg; mean age = 5 ± 1 years) as in (Aubert et al., 2005), were housed in individual primate cages under controlled conditions of humidity, temperature and light. Food and water were available ad libitum and animal care supervised by veterinarians skilled in the healthcare and maintenance of non-human primates. Experiments were carried out in accordance with European Communities Council Directive of 24 November 1986 (86/609/EEC) for care of laboratory animals in an AAALAC-accredited facility. Procedures were approved by the Institute of Laboratory Animal Science ethical committee.

Human basal ganglia tissue for paper IV was provided by the Queen Square Brain Bank, London, UK. Tissue was obtained from 5 neurologically healthy individuals and 22 pathologically verified idiopathic PD cases, which were divided in two groups based on the presence (n=12) or the absence (n=10) of LID in their clinical records. The three experimental groups were matched for age at death, post-mortem delay and tissue pH. In addition, the dyskinetic and non-dyskinetic PD cases were matched for age at disease onset, disease duration, and duration of dopaminergic pharmacotherapy. The cumulative and maximum L-DOPA doses were larger in the dyskinetic PD group than in the non-dyskinetic one.

6-OHDA LESION IN THE RAT

A hemiparkinsonian state was induced in the rat by unilateral injections of 6-OHDA in the ascending medial forebrain bundle according to our well-established methods (Westin et al., 2007). Rats were anesthetized with a mixture of Fentanyl® and Dormitor® (20:1, Apoteksbolaget AB, Sweden) and mounted on a stereotaxic frame (Kopf Instruments, Tujunga, USA). A total of 7.5 and 6 µg free-base 6-OHDA (6-Hydroxydopamine hydrochloride, Sigma Aldrich, Sweden, dissolved in 0.02% ascorbate-saline) were injected at the following coordinates (in mm, relative to bregma and dural surface): first injection: A = -4.4, L = -1.2, V = -7.8, tooth bar = -2.4 (2.5 µl); second injection: A = -4.0, L = -0.8, V = -8.0, tooth bar = +3.4 (2.0 µl). The injection
velocity was 1 µl per minute and the needle was kept in place for additional two minutes after the injection to allow diffusion of the toxin into the tissue. At the end of the surgery animals were given Temgesic® (0,167 mg/kg, Apoteksbolaget AB, Sweden) as analgesic treatment. The 6-OHDA solutions were prepared daily, kept in the dark on ice to avoid oxidation and were changed every 2-3 hours.

**MPTP LESION IN MONKEY**

All monkey experiments were performed in Beijing, China by our collaborators (Group of E. Bezard). Briefly, monkeys were intoxicated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) hydrochloride (0.2 mg/kg i.v. for 15 days) according to our standard method (Bezard et al., 2001b). Such a regimen of intoxication leads to near complete nigrostriatal denervation once a parkinsonian syndrome has fully developed (Guigoni et al., 2005). After inducing a stable, bilateral parkinsonian syndrome (constant disability scores over two consecutive weeks after at least 8 weeks post-MPTP), the monkeys were selected to be kept without treatment or to receive L-DOPA. Animals received daily oral administration of L-DOPA (Modopar®, L-DOPA/carbidopa, ratio 4:1) for 8 weeks at a tailored dose producing full reversal of parkinsonian symptoms (ranging from 15 to 20 mg/kg/day) and developed stable and moderate-severe LID as in (Bezard et al., 2003).

**BEHAVIOURAL TESTS IN THE RAT**

In a general rat experiment, 6-OHDA-lesioned rats were evaluated on their development of dyskinesia and akinesia using abnormal involuntary movement scale and rotarod or rotation tests described below. After the behavioural tests, animals were killed and brains processed for any morphological or biochemical analysis (Fig. 3).
Experimental methods and materials

Fig. 4. Time-line of a general rat experiment. After a unilateral 6-OHDA-lesion into medial forebrain bundle (MFB) animals were tested on amphetamine-induced rotation to evaluate the success rate of the lesions. Two weeks after this test, animals were daily treated with a clinical relevant dose of L-DOPA in a chronic experiment of three to four weeks. During this time the severity of dyskinesia were measured in repetitive AIMs tests. At the end of the experiment animals were killed and brains processed for histochemical or morphological analysis (IHC: immunohistochemistry, ISH: in situ hybridization, EM: electron microscopy).

Abnormal involuntary movements

The rodent AIM scale has been characterised and validated by our group (See (Cenci and Lundblad, 2007) and constitutes the first method to specifically assess the dyskinetic motor complications. The severity of LID is evaluated using a validated rat abnormal involuntary movement (AIM) scale, where axial, limb and orolinguial AIMs collectively represent the rodent equivalent of peak-dose/on phase dyskinesia in PD (Cenci and Lundblad, 2007). The procedure is described extensively in Cenci and Lundblad (Cenci and Lundblad, 2007). Briefly, rats were put in individual transparent cages before administration of L-DOPA, with or without the potential anti-dyskinetic substance (or the D1-agonist in paper V) to habituate to the environment and minimize stress. After the injections they were observed for one minute every 20 minutes during the time that the effect of L-DOPA persisted, i.e. 3 hours (or until dyskinetic movement ceased). Four subtypes of dyskinesia were rated: locomotor activity i.e. animal’s turning toward the side contralateral to the lesion; limb dyskinesia defined as circular and jerky movements of the forelimb and forepaw on the side contralateral to the lesion; axial dyskinesia, i.e. twisting of the upper body towards the side contralateral to the lesion and orolinguial dyskinesia illustrated as empty chewing movements, twitching of facial muscles and tongue protrusion. For descriptive photographs see Figure 5. Each subtype of dyskinesia was scored on a severity scale from 0 to 4, where 0 = no dyskinesia during observation time, 1 = occasional display of the AIM subtype, 2 = frequent appearance of this AIM subtype during more than half of each observation period, 3 = continuous appearance that can be disrupted by external stimuli and 4 = continuous appearance that will not be disrupted by any external stimuli. Since locomotve activity has shown not to provide a specific measure of dyskinesia (Dekundy et al., 2007; Lundblad et al., 2002) this subtype was handled separately or excluded from the collective AIMs score. In addition to the
Experimental methods and materials

severity of the dyskinetic movements the amplitude of AIMs was taken into account to further expand the dynamic range of total dyskinesia score (Cenci and Lundblad, 2007). Briefly, the amplitude was scored from 0 to 4 based on the lateral deviation of an animals' neck and upper trunk for axial scores, the extent of limb translocation and involvement of proximal muscles for limb scores and the involvement of jaw muscles and tongue protrusions for orolingual scores.

Rats presenting with a severity grade of 3-4 on at least one AIM subtype during a majority of the testing sessions were defined as dyskinetic, and rats with severity scores below 2 (i.e. 0-1) were defined as non-dyskinetic.

Fig. 5. An illustrative photograph of the different AIM subtypes in the 6-OHDA lesioned rat. In A is illustrated locomotive AIM defined as circular locomotion towards the contralateral side to the lesion. In B is shown the axial AIMs, which represent a more dystonic feature, upper body and neck twisting towards contralateral side to the lesion. In this animal the torsion is severe and causes the animal to loose balance. Orolingual AIMs in C are openings and closing of the jaws and tongue protrusion towards the contralateral side of the lesion (arrow). Limb AIMs in D comprise purposeless translocation of parkinsonian forelimb (left in this model). This is highlighted in black circle. Reprinted from Winkler et al (Winkler et al., 2002) with permission from Elsevier.
**Rotarod test**

Treatment effects on general motor performance in rats were evaluated using the rotarod test. This test reveals motor impairments in 6-OHDA lesioned rats that are significantly improved by L-DOPA treatment (Dekundy et al., 2006; Lundblad et al., 2003). Rats were pre-trained on the rotarod (Rotamex 4/8 Columbus Instruments, OH, USA) with an accelerating speed (4-44 turns/min during 90 s). This was done until they reached a stable baseline, 1-2 weeks before initiating drug treatments. A weekly training consisted in two separate trials at three consecutive days. The animals were tapped on the tail several times on each session for them to maintain alertness. During the experiment, L-DOPA or saline treated rats (with or without cotreatment) were tested on the rod once a week at 20, 40 or 60 min post-injection using the same acceleration mode as in the training phase. The time during which a rat remained on the rod, expressed in seconds, was used as a measure of rotarod performance. The average performance “on-drug” in every treatment arm was expressed as a percentage of baseline performance (“off-drug”) in each animal (Lundblad et al., 2003), and this parameter was used for statistical comparisons.

**Rotations**

As mentioned above, the unilateral DA lesion causes a functional asymmetry that can be seen as rotational behaviour after administration of a direct (e.g. L-DOPA) or indirect DA-agonist (e.g. amphetamine). L-DOPA or DA receptor agonists will cause greater stimulation of supersensitive DA receptors in the denervated striatum and cause rotation away from the lesion, i.e. contralateral. In contrast a DA releasing agent such as amphetamine, will create a greater DA release in the intact striatum that will make the animal turn towards the lesioned side, i.e. ipsilateral (Marin et al., 2006). Ipsilateral rotation is often used for assessing the DA denervation, e.g. amphetamine-rotations that is used for selecting successful lesions before an experiment. Contralateral rotation on the other hand, is widely used to assess the pharmacological potential of a candidate drug for PD (Marin et al., 2006). Moreover, rotations induced by chronic L-DOPA treatment have been associated with a progressive shortening of duration representing a valid model for the "wearing off" phenomena in patients (Papa et al., 1994). In contrast a sensitisation of rotations induced by L-DOPA have been proposed to share similarities with LID responding to anti-dyskinetic drugs (Marin et al., 2006). However, rotometry cannot discriminate between dyskinetic and anti-akinetic effects of anti-parkinsonian treatments since also low dyskinesiogenic substances provoke a full rotational response (Lindgren et al., 2007; Lundblad et al., 2002). Moreover a stable rotational response requires higher L-DOPA dose than that provoking dyskinesias.

In this thesis rotational behaviour was used to assess the success of the 6-OHDA lesions with amphetamine or to assess the general motor activation of L-DOPA (paper III). Rats were measured for their rotational behaviour using an automated rotometer (San Diego Instruments, San Diego CA). Two weeks post surgery, an amphetamine-induced rotation test (2.5 mg/kg D-amphetamine i.p. for 90 min recording) was applied to select rats with > 5 full turns/min ipsilateral to the lesion, corresponding to > 90% striatal DA depletion (Winkler et al., 2002). In paper III rats were administered...
Experimental methods and materials

with L-DOPA alone or in combination with the tested drug and monitored for 200 min. Clock-wise and counter clock-wise rotations (360° turns) were summed to provide a measure of overall motor activation. Results were expressed as number of rotations per five-minute interval.

BEHAVIOURAL TESTS IN THE MONKEY

Assessments of parkinsonism (PD scores), locomotor activity and dyskinesia (LID scores) were carried out by our collaborators in Beijing, China as in (Berton et al., 2009; Bezard et al., 2003; Gold et al., 2007; Munoz et al., 2008). In all assessment the animals were observed for 350 min. In PD motor scale, a PD score of 0 corresponds to a normal monkey and a score above 6 indicates severe parkinsonism. The severity of dyskinesia was rated using the NHP Dyskinesia Disability Scale (0, dyskinesia absent; 1, mild, fleeting and rare dyskinetic postures and movements; 2, moderate, more prominent abnormal movements, but not interfering notably with normal behaviour; 3, substantial, frequent and, at times, continuous dyskinesia intruding on the normal repertoire of activity; 4, severe, almost continuous dyskinetic activity, disabling to the animal and replacing normal behaviour). Locomotor activity was concomitantly measured every 5 min with infrared activity monitors.

DRUGS

In all papers rats were treated with 6–10 mg/kg L-DOPA methyl ester together with the peripheral DOPA-decarboxylase inhibitor, benserazide-HCl (15 mg/kg/day, both from Sigma-Aldrich, Sweden), which were dissolved in physiological saline and injected (s.c. or i.p.) at the volume of 1 ml/kg. L-DOPA was administered once daily in all experiments except for paper IV where it was given twice daily. In this paper, aiming at establishing a dose-response effect of 5-HT sprouting, an additional L-DOPA dose was used, 25-50 mg/kg/ injection (Guillin et al., 2001).

For paper I continuous release isradipine pellets (0.05, 0.1 and 0.2 mg/kg/day, all of which are clinically comparable doses (Surmeier, 2007) or inert control pellets (Innovative Research of America, Sarasota, USA) were implanted into the intercapsular space on the same day as 6-OHDA-injection or 7 weeks post-lesion. In the absence of a specific Cav1.3 antagonist, dihydropyridines offer the best therapeutic options being superior selective to L-type calcium channels than other blockers and approved for human use. Among these isradipine is more potent in blocking Cav1.3 at the depolarised membrane potentials (Surmeier, 2007).

In paper II the following compounds were coadministered with L-DOPA:

• MTEP, [(2-methyl-1,3-thiazol-4-yl) ethynylpyridine], a non-competitive mGluR5 antagonist (Busse et al., 2004) from Merz pharmaceuticals GmbH (Frankfurt, Germany). MTEP is a highly selective mGluR5 antagonist with
much fewer off-target effects than any related mGluR5 antagonist compound (Cosford et al., 2003) and that has shown anti-dyskinetic effect (Dekundy et al., 2006; Mela et al., 2007) with the same dose as used here.

- **EMQMCM**, [(3-ethyl-2-methyl-quinolin-6-yl)-(4-methoxy-cyclohexyl)-methanone methane sulfonate], a non-competitive mGluR1 antagonist (Lavreysen et al., 2002), Merz pharmaceuticals GmbH (Frankfurt, Germany). EMQMCM elective and highly potent non-competitive antagonist of mGluR1 (Lavreysen et al., 2002; Mabire et al., 2005).

- **LY379268**, [1R,4R,5S,6R-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate], a group II mGluR (2/3) agonist (Monn et al., 1999), Ascent Scientific (North Somerset, United Kingdom). LY 379268 is a potent, receptor selective, and systemically active mGluR2/3 agonist (Monn et al, 1999).

- Two selective NR2B antagonists were used in the acute and chronic experiment (Ro631908 and Ro256980, respectively) (Nikam and Meltzer, 2002; Zhou et al., 1999). The compounds had equal selectivity and potency (Mutel et al., 1998; Zhou et al., 1999) but were dissolved in different vehicles (saline and sterile water, respectively), based on the recommendations provided by the manufacturers.
  - **Ro25-6981**, [(±)-R*, S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)1-piperidine propanol], Sequoia Research Products Ltd. (Pangbourne, UK) and
  - **Ro631981**, (Zhou, et al 1999) [1-[2-(4-Hydroxyphenoxy)ethyl]-4-[((4-methylphenyl)methyl]-4-piperidinol hydrochloride], both of which are NR2B selective NMDA receptor antagonists (Nikam and Meltzer, 2002), Tocris Cookson Ltd (Bristol, UK).

- Isradipine, [4-(4-Benzofurazanyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid methyl 1-methylethyl ester], a L-type calcium channel antagonist (Hof et al., 1984). The drug was obtained from Sigma Aldrich, Sweden, (acute experiment) or provided by Boehringer Ingelheim Pharma GmbH & Co, KG, (München Germany, chronic experiment). In the chronic study, isradipine was given subcutaneously at a therapeutic dose in continuous–release pellets, which were implanted 1 day before treatment start.

In general, doses of the substances (see Table 1) were chosen based on indications of behavioural efficacy and *in vivo* receptor occupancy (where this information was available) in the literature. A detailed report on the choices of compounds and doses used is provided in Supplemental Material of paper II. If there were no dose-dependent effects in the acute study we chose the most frequently reported effective dose of the substance for the chronic study. Except for isradipine, substances were administered i.p. simultaneously with L-DOPA (MTEP or EMQMCM) or 10 min prior to L-DOPA injection (LY379268, Ro256981 and Ro631981) at the doses and vehicles as described
Experimental methods and materials

In experiment 1, isradipine in the acute experiment was administered 30 min before L-DOPA injection.

**In paper III,** fenobam was used as an mGluR5 antagonist. This substance has been under development already in the 1970s as non-benzodiazepine anxiolytic compound with potentially good safety profile (Pecknold et al., 1982) and is a potent, non-competitive mGluR5 antagonist (Porter et al., 2005). Since, MTEP has no translational potential this mGluR5 antagonist could be amenable to clinical use and conduct a comprehensive preclinical validation of the mGluR5 target.

Monkeys received daily oral administration of L-DOPA (Modopar®, L-DOPA/carbidopa, ratio 4:1) for 8 weeks at a tailored dose producing full reversal of parkinsonian symptoms (ranging from 15 to 20 mg/kg/day) (Berton et al., 2009; Bezar et al., 2003; Gold et al., 2007; Munoz et al., 2008). Since the pharmacokinetic properties of fenobam (N-(3-chlorophenyl)-N’-(4,5-dihydro-1-methyl-4-oxo-1H-imidazole-2-yl) urea (Pecknold et al., 1982) in rhesus monkeys were unknown, a pharmacokinetic study was conducted, comparing different vehicles of administration, 0.3 % Tween-80/saline (2 ml/kg) (Porter et al., 2005) or in DMSO/PEG 300 (1:9, v/v). For the acute experiment fenobam (Synexis inc. Durham, NC) was given in DMSO/PEG vehicle at 0, 5, 10 and 20 mg/kg, simultaneously with L-DOPA. In the chronic study, the drug was instead dissolved in Tween vehicle at the dose of 10 mg/kg.

In rat, fenobam was dissolved in DMSO/PEG for both the acute and chronic experiment and given per os (2 ml/kg) simultaneously with L-DOPA at the dose of 30 and 100 mg/kg (where only 30 mg/kg was used for the chronic experiment).

**In paper V** L-DOPA was compared with D1-agonist, SKF38393 (Sigma-Aldrich, Sweden). SKF38393 was given at the doses 1.5-2.5 mg/kg, dissolved in physiological saline and administered at a volume of 1 ml/kg body weight. Doses of SKF38393 were chosen so that they would produce approximately the same dyskinesia scores as L-DOPA (Monville et al., 2005). Fenobam (Synexis Inc. Durham NC) was administered in the same regimen as described above and given simultaneously to the L-DOPA/SKF38393 injection. The 5-HT1B agonist CP94253 (Tocris Bioscience, Bristol, UK), was given at 3 mg/kg and dissolved in physiological saline (Lindgren et al., 2009). It was administered s.c. 20 minutes prior to the L-DOPA/SKF38393 injection and the administration volume of 1 ml/kg body weight.

CP94253 has previously been shown to mildly attenuate dyskinesia (acutely) induced by both L-DOPA and D1 agonist, but only at relatively high doses (Carta et al., 2007; Jaunarajs et al., 2009). At subthreshold doses it will also reduce both the development of and the severity of dyskinesia, when administered in combination with subthreshold doses of the 5-HT1A-agonist, 8-OH-DPAT (Carta et al., 2007; Munoz et al., 2008). This is however only true for dyskinesia induced by L-DOPA and not for D1-agonist (Munoz et al., 2009).
Table 1. Experimental groups and drug treatments for paper II

Experimental Design 1 – Acute drug treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Targets</th>
<th>Doses (mg/kg)</th>
<th>L-DOPA/benserazide</th>
<th>Vehicle and i.p. injection volume</th>
<th>N per group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>-</td>
<td>No</td>
<td>physiological saline or 5% Tween 80/sterile water</td>
<td>6</td>
</tr>
<tr>
<td>L-DOPA-only</td>
<td>all DA rec.</td>
<td>10.0</td>
<td>Yes</td>
<td>1 ml/kg in saline</td>
<td>6-8x3*</td>
</tr>
<tr>
<td>MTEP</td>
<td>mGluR5</td>
<td>1.25 and 6.25</td>
<td>Yes</td>
<td>2 ml/kg in 5% Tween 80/sterile water</td>
<td>7 and 7</td>
</tr>
<tr>
<td>EMQMCM</td>
<td>mGluR1</td>
<td>1.25 and 5.0</td>
<td>Yes</td>
<td>2 ml/kg in 5% Tween 80/sterile water</td>
<td>7 and 7</td>
</tr>
<tr>
<td>LY379268</td>
<td>mGluR2/3</td>
<td>1.0 and 10.0</td>
<td>Yes</td>
<td>2.5 ml/kg in saline</td>
<td>4 and 6</td>
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<tr>
<td>Ro631908</td>
<td>NR2B</td>
<td>3.0 and 30.0</td>
<td>Yes</td>
<td>1 ml/kg in saline</td>
<td>3 and 7</td>
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<tr>
<td>isradipine</td>
<td>L-type calcium channel</td>
<td>0.5 and 5.0</td>
<td>Yes</td>
<td>2 ml/kg in sterile water, sonicated</td>
<td>9 and 4</td>
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Experimental Design 2 – Chronic drug treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Targets</th>
<th>Doses (mg/kg/day)</th>
<th>L-DOPA/benserazide</th>
<th>Vehicle and i.p. injection volume</th>
<th>N per group</th>
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</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>-</td>
<td>No</td>
<td>physiological saline or 5% Tween 80/sterile water</td>
<td>4x5 *</td>
</tr>
<tr>
<td>L-DOPA-only</td>
<td>all DA rec.</td>
<td>6.0</td>
<td>Yes</td>
<td>1 ml/kg in saline</td>
<td>8-9x5*</td>
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<tr>
<td>MTEP</td>
<td>mGluR5</td>
<td>5.0</td>
<td>Yes</td>
<td>2 ml/kg in 5% Tween 80/sterile water</td>
<td>10</td>
</tr>
<tr>
<td>EMQMCM</td>
<td>mGluR1</td>
<td>5.0</td>
<td>Yes</td>
<td>2 ml/kg in 5% Tween 80/sterile water</td>
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<td>LY379268</td>
<td>mGluR2/3</td>
<td>3.0</td>
<td>Yes</td>
<td>2.5 ml/kg in saline</td>
<td>10</td>
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<tr>
<td>Ro256981</td>
<td>NR2B</td>
<td>3.0</td>
<td>Yes</td>
<td>1 ml/kg in 37°C-sterile water</td>
<td>8</td>
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<td>isradipine</td>
<td>L-type calcium channel</td>
<td>0.2</td>
<td>Yes</td>
<td>Subcutaneous continuous-release pellets</td>
<td>9</td>
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</tbody>
</table>
**AUTORADIOGRAPHY**

Animals were killed by a sodium pentobarbital overdose one day after the last L-DOPA injection, unless otherwise stated. Brains were rapidly extracted and frozen in crushed dry ice (rat) or isopentane (NHP), stored at −80 °C, and cut on a cryostat into 16 µm or 20 µm thick coronal sections as in (Aubert et al., 2005; Johansson et al., 2001), respectively.

In both animal and human specimens, SERT autoradiography was assessed using [3H]citalopram (2 nM, Perkin Elmer, Sweden) as in (Chinaglia et al., 1993; D’Amato et al., 1987; Descarries et al., 1995; Lu et al., 1997). [3H]Citalopram binding have been shown to strictly and linearly correlate to the number of 5-HT varicosities labelled by [3H]5-HT uptake in adjacent tissue slices, not only under conditions of normal 5-HT innervation, but also across a wide range of conditions causing 5-HT hyperinnervation (Descarries et al., 1995) (see also Fig. 5). In the rat, autoradiographic analyses were carried out in the dorsal (sensorimotor) striatum and the overlying motor-premotor cortices. In NHP and human material, analyses were carried out in the post-comissural (motor) part of the basal ganglia, including posterior putamen, external and internal segments of the globus pallidus (GP). SERT autoradiography was performed using [3H]citalopram (2 nM, Perkin Elmer, Sweden) (D’Amato et al., 1987). Sections were incubated at room temperature for 15 min in 50 mM Tris-buffer (pH 7.5) containing 120 mM NaCl and 5 mM KCl. [3H]Citalopram (spec. act. 79 Ci/mmol) was added to new buffer for incubation 2 hours at room temperature. Non-specific binding was determined by pre-incubating some additional consecutive sections with 20µM fluoxetine. After [3H]citalopram incubation, sections were rinsed 4 x 6 minutes in ice cold Tris-buffer and dipped in distilled water. When dried, sections were put in lightproof x-ray cassettes. Thereafter slides were apposed to Biomax MS films and Biomax (Perkin Elmer, Sweden) for 7 days at -80°C. Films were developed in Kodak D19 (Kodak AB, Sweden), fixed in High speed fixer (Stena, Stockholm, Sweden) and dried. Images of autoradiographs were captured in a Nikon DM1200F camera and optical density was measured using the NIH Image J software (Fig. 6).

To determine the degree of nigrostriatal DA denervation, DA transporter (DAT) autoradiography was performed using 0.1 nM [125I]((E)-N-(3-iodoprop-2-etyl)-2-carboxymethyl-3-(4-methylphenyl)-nortropane (PE2I; Chelatec, France) in NHP and 2 nM [3H]GBR12935 (Perkin Elmer, Sweden) in rat and human tissue as described in (Aubert et al., 2005; Bowden et al., 1997; Cenci et al., 1998). In rat and human tissue sections were incubated at 4°C for 20 min in 50 mM Tris-buffer containing 450 nM NaCl, 0.02% bovine serum albumin and to block unspecific binding 1 µM flupentixol and 10 uM desipramine. To block non-specific binding some additional consecutive sections were pre-incubated with GBR12953. Thereafter sections were incubated in the same buffer containing 2 nM [3H]GBR12935 for 20 hours at 4°C. Sections were rinsed as described above and apposed to Biomax MS films and Biomax transcreens for 5 days at -80°C. Analysis was performed using Image J as described above.
IN SITU HYBRIDIZATION

Radioactive in situ hybridization was used to measure the expression levels of preproenkephalin-A (PPE-A), prodynorphin (PDyn) and BDNF mRNA in the striatum and cortex (Cenci et al., 1998; Kokaia et al., 1996). In addition, SERT mRNA was measured in midbrain (McQueen et al., 1997). In paper II animals were anesthetized and decapitated after 2 days of wash-out to evaluate the long-lasting effects of PDyn. In paper IV rats were killed four hours after the last L-DOPA injection, the time point at which L-DOPA-induced upregulation of BDNF had been reported by a previous study (Guillin et al., 2001). The tissue was prepared as described above for autoradiography. In situ hybridization was carried out according to our standard methods (Andersson et al., 1999) using previously characterised [35S]-labelled oligonucleotide probes specific for PDyn, BDNF mRNA or SERT mRNA. Briefly, the oligonucleotide probes were labelled at their 3’ end with α-35S dATP using 15 U Terminal deoxynucleotidyltransferase (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) (2 hours at 37°C). The labelled probes (specific activity >106 cpm/µg) were purified with chromaspin column (Chroma Spin Columns; Clontech Laboratories; Palo Alto, USA) and added to the hybridization cocktail at a final concentration of 107 cpm/ml. The slide-mounted sections were pretreated with 0.2 M HCl and 25% acetic anhydride in 0.1 M triethanolamine. They were then dehydrated and air-dried for approximately 10 min before adding the hybridization cocktail (40 µl per section). Slides were coverslipped with parafilm and incubated at 42°C in a humid chamber over night. After four washes (15 min each) in 1 X sodium citrate buffer (SSC) at 55°C, the slides were cooled down to RT, briefly rinsed in distilled water, dehydrated in 70/95% ethanol, and apposed to Biomax MR film for 3 weeks (at -20°C), together with

Fig. 6. Autoradiographs of [3H]citalopram binding in rat (A), monkey (B) and human (C) striatal sections. The radioligand binding densities were measure from striatum/putamen as outlined 1. In rat motor-premotor cortex was analysed (see outlined above the striatum). In monkey and human sections the globus pallidus (GP) was measured (outlined next to putamen in B and C).
radioactivity standards (14C-Microscale standards, Amersham International plc, Amersham, UK). Films were developed in Kodak D19 and fixed in High Speed Fixer (Stena, Sweden). After the film incubations, slides were dipped in photographic emulsion (LM-1 emulsion, Amersham, 1:1 dilution in distilled water), and exposed for 10 weeks at -20°C to confirm that the labelling detected with autoradiographic films corresponded to specific labelling of cell bodies. Emulsion-coated sections were developed in D-19, fixed, Nissl-counterstained, and coverslipped with D.P.X. mountant. The hybridisation signal was measured on digitised and calibrated film autoradiographs using the procedure described above.

The in situ hybridization of PPE-A of paper I was performed by the co-authors at Boehringer Ingelheim (S. Schuster) and was performed according to the procedures in (Aubert et al., 2007) but on thicker sections (60 µm – see above), with probe designed to recognize the rat PPE-A (Yoshikawa et al., 1984). [³⁵S]–labelled antisense and sense cRNA probes were prepared by in vitro transcription from 100 ng of linearised plasmid using [³⁵S]UTP (> 1000 Ci/mmol; NEN), and SP6, T3, or T7 polymerases. After alkaline hydrolysis to obtain 0.25 Kb cRNA fragments, the probes were purified on G50-Sephadex and precipitated in sodium acetate (0.1 vol)/absolute ethanol (2.5 vol). After hybridization sections were then exposed to Biomax film as described above and image analysis was performed by the system (Mercator, Explora Nova, La Rochelle, France).

**IMMUNOHISTOCHEMISTRY**

One day after the last drug injection unless otherwise stated, rats were anesthetized and perfused transcardially with buffered 4% paraformaldehyde (PFA). Brains were immersed for 2 h in the same fixative, cryoprotected in sucrose, and cut into 40 µm-thick coronal sections. Immunohistochemistry was carried out on using the antibodies described in Table 2.

Sections were rinsed in 0.02 M potassium phosphate buffer with 0.25% triton-X (KPBS-T). They were quenched with 3% hydrogen peroxide and 10% methanol hereafter they were blocked with 5% serum in the same buffer. Incubation with primary antibody was performed according to Table 2, hereafter the sections were rinsed and incubated with the appropriate biotinylated secondary antibody (1:200, 2 h at room temperature) in KPBS-T and 2.5% serum. After rinsing the sections were incubated by an avidin-biotin complex (ABC standard kit, Vector, 1 h). Visualization of the immunocomplexes was obtained using 3,3′-diaminobenzidine (DAB, Sigma Aldrich, Germany) as a chromogen. After another rinse the sections were mounted and dehydrated before coverslipped.

Immunoreactive cells for phospho-ERK1/2 and phospho-MSK-1 were counted by a blinded investigator using the Image J software (Westin et al., 2007). Six sample areas (0.82 x 0.66 mm) per side were visualised under a 10 X objective in a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan), and digitized through a Nikon DMX 1200F video camera.
Table 2: List of primary anti-bodies used.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Paper</th>
<th>Concentration</th>
<th>Incubation</th>
<th>Company</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pERK1/2, Thr202/Tyr204</td>
<td>II</td>
<td>1:250</td>
<td>36 h at 4 °C</td>
<td>Cell Signalling Technologies</td>
<td>(Westin et al., 2007)</td>
</tr>
<tr>
<td>Rabbit monoclonal anti-phospho MSK-1</td>
<td>II</td>
<td>1:200</td>
<td>48 h at 4 °C</td>
<td>Epitomics Inc.</td>
<td>(Westin et al., 2007)</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-Tyrosine Hydroxylase (TH)</td>
<td>I, II, V</td>
<td>1:1000</td>
<td>48 h at 4 °C</td>
<td>Pel-Freeze</td>
<td>(Andersson, et al, 1999)</td>
</tr>
<tr>
<td>Rabbit anti-5-HT</td>
<td>IV</td>
<td>1:15000</td>
<td>Overnight at 4 °C</td>
<td>Immunostar Inc</td>
<td>(Zhou et al, 1995)</td>
</tr>
<tr>
<td>Goat polyclonal anti-SERT</td>
<td>IV</td>
<td>1:200</td>
<td>Overnight at 4 °C</td>
<td>Santa Cruz</td>
<td></td>
</tr>
</tbody>
</table>

On human tissue section SERT-immunohistochemistry was carried out on fresh-frozen material. The slide-mounted sections were preincubated in ice-cold 4% PFA (1.5 h), rinsed in a Trizma-based buffer (TBS) and quenched in 0.3% hydrogen peroxide. Thereafter, the sections were blocked in 3% bovine serum albumin (BSA) before incubated with SERT antibody. This was followed by incubation with biotinylated horse anti-goat IgG (1:200, 1.5 h at room temperature) and by an avidin-biotin complex (ABC standard kit, Vector, 1 h). Visualization of the immunocomplexes was obtained using 0.05% 3,3′-diaminobenzidine (DAB, Sigma Aldrich, Germany) for 5 min and 0.001% hydrogen peroxide for 30 seconds.

Counts of 5-HT/SERT axon varicosities
Unbiased stereology (fractionator sampling design) (Gundersen et al., 1988; West et al., 1991) was used to estimate the total number of 5-HT positive axon varicosities in the striatum. Axon varicosities (defined as swollen parts of an axon, oval or circular in shape, Fig. 7B,C) were counted within random frames of 888 µm² (x, y steps of 800 µm) using a 100X oil immersion objective in a Nikon Eclipse 80i microscope connected to an Olympus DP72 digital video camera, merged with a stereology software (CAST v. 2.3.2.0, Visiopharm, Denmark) (Finkelstein et al., 2000). In the same animals, 5-HT immunoreactive cell bodies in the raphe B7 and B8 cell groups were counted manually as in (van Luijtenaar et al., 1992). Within the B7 and B8 regions, spherical, fusiform or multipolar 5-HT-immunoreactive cells with a diameter > 15 µm were counted (van Luijtenaar et al., 1992). The values obtained were corrected to adjust for split cell counts as in (Abercrombie, 1946). We used this method instead of a fractionator sampling design because stereological cell estimates would be imprecise due to the irregular shape of the sample volume (i.e. the B7 and B8 cell groups).

On human sections a random sampling procedure was used to count SERT immunopositive axonal varicosities under a 40 X objective (See Fig.7B,C). This analysis
Experimental methods and materials

was carried out in the GPe, a compact structure in which the tissue had remained well preserved in all cases by the end of the immunostaining. Briefly, random sample areas were selected using a stereology software (CAST v. 2.3.2.0, Visiopharm, Denmark, as described above, x and y steps of 800 µm) that in total covered 2.86% of the GPe area. The results were expressed as total number of varicosities per mm² tissue.

Fig. 7. SERT radioligand binding positively correlates with the density of SERT-immunopositive axon varicosities in the external part of the human GP (A). B and C are microphotographs from patients, B illustrating a high magnification and C varicosity density of 5-HT (SERT-immunopositive) innervation in GPe. Scale bar (in B and C): 20 µm.

Electron microscopy

Electron microscopy (EM) was performed in the large collaborative study of paper I in the laboratory of J. Surmeier and in paper IV by M. Parent at L. Descarries’ laboratory.

In paper I, D1-positive neurons were detected by the preembedding immunoperoxidase technique as previously described (Dumartin et al., 1998; Guigoni et al., 2007). Animals were deeply anesthetized with sodium chloral hydrate (150 mg/kg) 1 h after the last injection and animals were perfused transcardially with a mixture of 2% PFA and 0.2% glutaradehyde in phosphate buffer (Dumartin et al., 2000). Brains were cut into 60 µm frontal sections with vibratome. D1 was detected by immunohistochemistry using a monoclonal antibody raised in rat (Sigma; St Louis, MO, 1:5000, overnight) (Hersch et al., 1995; Levey et al., 1993). Peroxidase activity was revealed with 3,3'-diaminobenzidine. The analysis of the distribution of D1-postivie and D1-negative dendritic spines in striatal neurons was performed on digital images obtained with a computer linked directly to CCD camera on the electron microscope at a final magnification of 2500 to 6000 using the Metamorph software (version 4.6r5, Universal Imaging, Paris, France). The measures were performed on 2 animals per group and on a minimum of 100 randomly selected dendritic fields (Guigoni et al., 2007;
Scholz et al., 2008). A dendritic field is defined as an area that does not contain a cell body. Indeed, given the difference in size between cell bodies and dendrites, the presence of a cell body in the randomly selected counting areas would imbalance the counting procedure. The results were expressed as the proportion of D1-positive or D1-negative dendritic profiles present in the caudate-putamen of each individual.

In paper IV rats were perfused transcardially with 3% acrolein in PBS followed by 400 ml of buffered 4% paraformaldehyde one day after the last drug injection for morphological analysis of SERT varicosities. Brains were cut on a vibratome at 50 μm thickness. Coronal sections through the striatum were processed for pre-embedding EM immunocytochemistry with a SERT antibody (Santa Cruz Biotechnology, CA, USA; 1:500, 48 h incubation) and the immunoperoxidase-diaminobenzidine technique as in (Parent et al., 2010). SERT-positive profiles of axon varicosities were readily identified by their size (transverse diameter > 0.25 μm) and content of aggregated synaptic vesicles. At a working magnification of 11,500 X, a picture was taken of every such profile encountered, until approximately 40 profiles showing a full contour were available from each animal. Short (l) and long (L) axes, diameter (l + L / 2) and cross sectional area, were measured using Image J. Each profile was also categorized as showing or not a synaptic membrane specialisation. The junctions were classified as symmetrical or asymmetrical, and the synaptic target identified. The synaptic incidence observed in single thin section was extrapolated to the whole volume of varicosities using a stereological formula as in (Beaudet and Sotelo, 1981).

Studies of stimulated DA release in striatal slices

We used the method described by Goggi et al 2002 (Goggi et al., 2002) with minor modifications. Rats were rapidly anesthetized by CO2 inhalation and decapitated one day after the last drug injection. Vibratome-cut coronal slices (350 μm) were immediately prepared from the 6-OHDA-lesioned brain hemisphere. All extrastriatal tissue and the nucleus accumbens were removed with a sharp forceps. The striatal slices (n = 4, in separate vials) were pre-incubated at 37° in oxygenated Krebs-phosphate solution (KPS) followed by incubation with 0.1 μM [3H]DA for 15 min, and by 4 x 15 min washes in KBS. From each animal the four separate slices were then incubated for 10 min in each of the following conditions: (i) KPS only; 20 mM KCl; (iii) KCl together with 100 ng/ml BDNF (Sigma-Aldrich; BDNF was added 10 min before KCl); (iv) KCl, BDNF, and the protein kinase inhibitor, K252a, which acts as a potent inhibitor of tyrosine kinase receptors, among which the high-affinity BDNF receptor, TrkB belongs (K252a, 100 ng/ml, Sigma-Aldrich; added 20 min before BDNF i.e. 30 min before KCl). At the end of incubations, radioactivity counts were determined both in 50 μl- aliquots of the media and in the slice homogenate using liquid scintillation spectrometry. An index of DA release was given by the ratio between radioactivity in the media and the residual radioactivity in the tube (medium + slice homogenate).
STATISTICAL ANALYSIS

Unless otherwise stated, comparisons between treatments and/or groups were carried out using either one- or two-factor analysis of variance (ANOVA) followed by Student-Newman-Keuls’ or Tukey HSD test, respectively. For time-course experiments repeated measures ANOVA and Tukey’s honestly significant differences (HSD) test was used. Relevant differences were confirmed with non-parametric statistics (Mann-Whitney or Wilcoxon's signed-rank test, where appropriate). The number of spines in paper I was compared within groups with a two-tailed paired t-test and between groups with a two-tailed unpaired t-test. For monkey studies in paper III, comparisons were performed on using one-factor ANOVA with repeated measures followed by Bonferroni post hoc test. Area under curve (AUC) derived from time-course experiments were analysed using the unpaired t-test with Welch correction while outcome of group swap was analysed using paired t-test. The level of statistical significance was set at $p < 0.05$ and data are expressed as mean group value ± SEM, unless otherwise written.
GENERAL RESULTS AND COMMENTS

The first part of this thesis (Paper I-III) consists in a comparison of pharmacological modulators targeting the corticostriatal glutamate transmission. An aberrant glutamate transmission at the corticostriatal synapse is believed to contribute to LID and therefore pharmacological agents to these targets, of which some are already clinically tested, could bring about a preventive or alleviating therapy for LID. The following targets were evaluated and compared for their modulatory role on akinesia or dyskinesia as well as LID associated molecular and morphological alterations: 1) antagonist for L-type calcium channels 2) antagonist of the NR2B subtype selective NMDA receptors 3) agonist of the presynaptic mGluR2 4) antagonist of the postsynaptic mGluR1 and 5) antagonist of the mGluR5. Results suggest two non-DA therapeutic options: isradipine, an antagonist of L-type calcium channels of the Cav 1.2-1.3 subtype that could potentially act prophylactically in the early stage of the disease (when morphological alteration have not yet occur) and fenobam, an antagonist of the mGluR5 that could prevent the development or attenuate the severity of dyskinesia at a more severe stage of PD.

ANTAGONIZING L-TYPE CALCIUM CHANNELS WITH ISRADIPINE REDUCES THE DEVELOPMENT OF AIMS IN THE RAT MODEL OF LID (PAPER I).

In paper I, the specific target of the Cav1.2-1.3 L-type calcium channel was evaluated for a potential involvement in LID. This channel has been linked to the atrophy of spines and dendrites in the striatopallidal pathway after DA denervation (Day et al., 2006) as well as to glutamate signalling in MSNs (Rajadhyaksha et al., 1999). This paper addressed the implication of DA denervation-induced spine pruning in the development of LID using the Cav1.2-1.3 L-type calcium channels antagonist isradipine, a clinically used drug for hypertension that has shown good tolerance with minor side-effects (Fitton and Benfield, 1990). Isradipine was evaluated for its anti-dyskinetic effect along with its potency to prevent lesion-induced spine pruning in 6-OHDA lesioned rats. Animals were implanted with subcutaneous isradipine pellets at clinically relevant daily doses (0.5, 1 and 2mg/kg/day) at the same time as the 6-OHDA lesion (at the occurrence of spine pruning) or 7 weeks post-lesion (when spine pruning had already occurred) before treated chronically with L-DOPA. After a wash-out period animals were challenged with apomorphine to rule out an interfering with the anti-parkinsonian effect of isradipine. After the experiment the brains were processed for electron microscopy (EM) analysis to evaluate spines and dendrites of D1 positive (striatonigral) or D1 negative (striatopallidal) MSN using a preembedding immunoperoxidase technique (Dumartin et al., 1998; Guigoni et al., 2007). Alternatively brains were processed for PPE-A in situ hybridisation, a molecular marker for dyskinesia (Winkler et al., 2002).
**General results and comments**

**Isradipine can prevent the development of AIMs when given simultaneously with the 6-OHDA lesion.**

Animals implanted with isradipine pellets simultaneously with the 6-OHDA-lesion showed a dose-dependent reduction that reached 50% in both L-DOPA-induced rotational locomotion (Fig.1A) and AIMs (Fig.1B), compared to animals implanted with placebo-pellets or control animals (6-OHDA-only). The most effective dose (2 mg/kg/day), specifically dampened orolingual and axial subtype of AIMs whereas the more choreic subtype of limb was not affected (Fig.1.C,D,E). This suggests that isradipine primarily dampens the dyskinetic hyperactivity of the dystonic type, i.e. the subtype that is most dependent on striatopallidal pathway (Bezard et al., 2001a). On the contrary, when implanting the pellets 7 weeks post-lesion there were no alleviating effects on any AIM subtype or locomotion from the dose of 2 mg/kg/day (Fig. 2). When challenging the animals for apomorphine-induced rotations at the end of the experiment isradipine treatment did not reduced the numbers of rotations, suggesting a non-compromised therapeutic response to the DA-agent. Taken together, this data suggests that isradipine can dampen the development of dyskinetic movement induced by L-DOPA when given at the time of the DA-denervation.

**Isradipine can prevent dendritic spine pruning of striatopallidal neurons.**

To evaluate the grounding hypothesis behind the anti-dyskinetic effect of isradipine, EM analysis of D1-negative or positive spines was performed in animals implanted with placebo or isradipine pellets. A dendritic spine pruning of 20% was induced by the 6-OHDA-lesion induced in placebo treated animals. The decrease was specifically observed in the D1-negative spines, i.e. striatopallidal neurons. In line with our hypothesis, animals treated with isradipine showed no decrease in the number of striatopallidal spines, but instead expressed a similar number of dendrites as on the intact side. These results are in accordance with previous a study (Day et al., 2006) and establish a link between prevention of denervation-induced spine pruning and reduction of AIMs. *In situ* hybridization of PPE-A mRNA further showed increased levels in placebo treated rats with 6-OHDA lesion whereas the PPE-A mRNA levels were normalized in isradipine treated animals, suggesting a normalized activity of MSNs after isradipine treatment.

Taken together the results of this paper show that antagonizing the L-type calcium channel of the Cav 1.2-1.3 subtype concomitantly with the neurotoxic insult to the DA neurons, can prevent the dendritic spine loss in the striatum and dampen the severity of LID. On the other hand, when antagonizing the channel when spines and synapses are already lost, i.e. 7 weeks post-lesion the treatment is not effective. This is the first study suggesting that striatal dendritic alterations, secondary to DA denervation may play a role in the development of dyskinesia during L-DOPA treatment. Likewise, isradipine could represent a potential therapeutic option for preventing the development of LID in PD, especially since this drug is well tolerated in humans at comparable relevant doses (See Materials and methods, Drugs).
Fig. 1 (left): Isradipine (0.2 mg/kg/day) (6-OHDA+I) or placebo pellets (6-OHDA + P) implanted simultaneously with the 6-OHDA lesion. Effects upon turning behaviour (A-B) and AIMs (C-D). Left panel shows the time-courses of behaviours monitored every 20 min over 140 min. Right panel highlight the peak values 80 min after administration of L-dopa (6 mg/kg/day). Cumulative AIM scores (C-D) were subdivided into axial (E-F), limb (G-H) and oro-lingual (I-J) subscores. Data are mean ± SEM of day 1-14. *P < 0.05, **P < 0.01, ***P < 0.001. Fig. 2 (right): Effect of post-lesional implants of Isradipine (0.2 mg/kg/day) or placebo pellets upon turning behaviour (A-B) and AIMs (C-D). The time-courses of behaviours monitored every 20 min over 140 min and the peak values 80 min after administration of L-dopa (6mg/kg/day). Data are mean ± SEM of day 1-14. Cumulative AIM scores (C-D) were subdivided into axial (E-F), limb (G-H) and oro-lingual (I-J) subscores.
Pharmacological modulation of glutamate transmission in the rat model of LID: Effects on motor behaviour and striatal nuclear signalling.

Paper II of this thesis represents the first comparison of a range of compounds with postulated “anti-glutamatergic” effects acting on the corticostriatal glutamate transmission in the 6-OHDA rat model. Herein, the effects on dyskinesia as well as on the associated exuberant striatal activation of the ERK1/2 signalling pathway (Westin et al., 2007), and pDyn gene expression (Cenci et al., 1998), were examined. Five specific glutamate transmission targets were modulated: L-type calcium channels (Cav1.2-1.3), NR2B subunit specific NMDA receptors, mGluR2/3, mGluR1 and mGluR5. These targets were assumed to be able to inhibit the overactive glutamate transmission at the corticostriatal synapse based on previous reports. In paper I the L-type calcium channel antagonist isradipine had been shown to prevent structural abnormalities with a partial prophylactic efficiency against LID but so far its effect on striatal nuclear signalling or gene expression correlated to LID was unknown. The NR2B subunit of NMDAR had been linked to LID with altered phosphorylation or synaptic membrane trafficking (Dunah et al., 2004; Gardoni et al., 2006) but so far, the anti-dyskinetic potential of NR2B antagonist had shown diverging results (Nash et al., 2004; Nutt et al., 2008). Selective agonism of mGluR2/3 decreased excitatory transmission at the corticostriatal synapses via a presynaptic mechanism (Picconi et al., 2002) and so far agonists effects on dyskinesia were unknown. Antagonists to group I mGluRs had shown to exert modulatory effect on NMDAR and regulated striatal gene expression and to alleviate LID (Dekundy et al., 2006; Levandis et al., 2008; Mela et al., 2007). However, their potency to block pERK1/2 signalling pathways had not been studied. Moreover the relative effect compared to other ligands was unknown.

All compounds and doses were chosen based on previous behavioural data where available (See "drugs" in experimental methods and materials).

Targeting group I mGluR inhibits striatal nuclear signalling of LID in the rat.

6-OHDA lesioned rats were challenged acutely with L-DOPA (6-10 mg/kg/day) together with any of the substances at low and high doses (table 1) to determine a dose-response effect on striatal nuclear signalling pERK1/2 and the downstream pMSK-1 using immunohistochemistry. L-DOPA-treated rats showed an induction of pERK1/2 and pMSK-1 as previously demonstrated (Westin et al., 2007). The expression of pERK1/2 and pMSK-1 was compared among the groups on the side ipsilateral to the lesion after having ascertained that no differences occurred on the intact side. Neither of the substances: the mGluR2/3 agonist LY379268, the NR2B antagonists Ro631908, and the L-type calcium channel antagonist, isradipine were able to reduce the expression of pERK1/2 or pMSK-1 at any of the doses tested (Fig.3). Only antagonists of group I mGluR receptors MTEP and EMQMCM could reduce the altered signalling (Fig. 3B) and among these, MTEP was more potent than EMQMCM in normalising pERK1/2 signalling (Fig. 3A) at both a low and a high dose.
Antagonising mGluR5 is superior to other glutamate transmission targets in alleviating LID

Based on the results from the acute study above a suitable dose of the same substances was then chosen for chronic treatment and administered along with L-DOPA to detect any potential anti-dyskinetic or anti-akinetic effects in AIM and rotarod test. Brains were processed for PDyn in situ hybridization to measure a potential inhibition of the LID-associated gene induction.

Similar to their effects on nuclear signalling, the mGluR2/3 agonist LY379268, the NR2B antagonists Ro256981, and the L-type calcium channel antagonist, isradipine, showed no significant effect on alleviating the severity of AIMS (Fig. 4). On the other hand, the mGluR5 antagonist MTEP was able to both alleviate AIMS to 70%, and block the upregulation of striatal PDyn mRNA. This effect was larger than that of the mGluR1 antagonist, EMQMCM. More importantly, MTEP achieved these effects at a dose that enhanced the positive action of L-DOPA on the rats’ rotarod performance (Fig.4B).

These findings discourage a further pursuit of group II mGluR agonists, mGluR1 antagonist as well as NR2B antagonist as anti-parkinsonian therapies. In line with previous paper it further shows that isradipine, when given to animals with severe and stable DA-denervating lesions, cannot block the abnormal nuclear signalling responses induced by L-DOPA in striatal neurons, suggesting that treatment with isradipine would not have any anti-dyskinetic action in patients with advanced PD. Of the approaches
examined, specific antagonism of mGluR5 with MTEP instead was the most effective in inhibiting nuclear signalling pathway-activation and upregulation of late-response genes by L-DOPA, supporting the close link between these molecular changes and dyskinesia. We conclude that pharmacological approaches targeting mGluR5 appear promising for therapeutic development in PD.

Fig. 4. Chronic effects on behaviour. L-DOPA co-treatment with "anti-glutamatergic" substances in comparison with L-DOPA-only. (A) AIM scores as percentage of L-DOPA-only group. Only cotreatment with mGluR5 antagonist (L-DOPA + MTEP) attenuated the development of dyskinesia significantly (p < 0.001 for treatment effect). (B) Rotarod performance expressed as percentage of baseline performance. L-DOPA-only group performed significantly better on the rotarod compared to vehicle control. Co-treatment with mGluR1 antagonist EMQMCM and mGluR2/3 agonist LY379268 blocked the anti-akinetic effect of L-DOPA (p < 0.001 for treatment effect). MTEP, Ro256981 and isradipine had no such effects. (C) Striatal PDyn mRNA levels as percentage of L-DOPA-only. Chronic L-DOPA treatment up-regulates the PDyn mRNA levels compared to vehicle controls (p < 0.001 for treatment effect). MTEP cotreatment blocked L-DOPA-induced upregulation. For respective doses see Table 1. P < 0.05 vs. *, L-DOPA only; #, Vehicle; $, MTEP; +, EMQMCM; @, Ro631908. Values represent group mean ± SEM bars.

A mGLuR5 antagonist under clinical development is efficient in reducing LID in rats and monkeys

Since MTEP has no translational potential, another mGluR5 antagonist amenable to clinical use, would be needed in order to conduct a comprehensive preclinical validation of the mGluR5 as an anti-dyskinetic target. The third paper examined the effects of fenobam, a mGluR5 antagonist that has already been clinically tested, in 6-OHDA rat and MPTP NHP models of LID. Fenobam has been tested in humans for an anxiolytic effect and has shown good safety profile (Pecknold et al., 1982). It shares the same allosteric modulatory site as the prototypical mGluR5 antagonist, MPEP (Gasparini et al., 1999) that has shown anti-dyskinetic efficacy (Levandis et al., 2008; Morin et al., 2010). Paper III consisted in a collaboration with the group of E. Bezard, who performed the monkey experiments. All studies on rats were performed in Lund at our laboratory.
Fenobam reduces already established dyskinesia in rat and monkey models of PD. In this study 6-OHDA-lesioned rats or MPTP-treated monkeys that had been primed with L-DOPA to develop severe dyskinesia were acutely challenged with fenobam at different doses to determine the dose-response effects on dyskinesia and akinesia. The severity of dyskinesia and akinesia was assessed using the AIMS- and rotarod test in the rats, whereas the NHP dyskinesia disability- and PD motor scale was used for the monkeys. In both animal models, acute administration of fenobam was able to attenuate already established AIMS by 50-70% at doses of 30 mg/kg in rats and 10 mg/kg in monkeys. The effect consisted of a reduction in peak-dose dyskinesia, whereas the end-dose phase was not affected. Instead, fenobam prolonged the motor stimulant effect of L-DOPA as measured with L-DOPA-induced rotations (Fig.5) in the rat and with locomotor activity in the monkeys. This prolongation of the on-phase of L-DOPA could be of advantage in the clinic.

Fenobam reduces the development of LID without affecting the anti-akinetic effect of L-DOPA in rat and monkey models of PD.
In chronic experiments, animals were treated de novo with L-DOPA alone or in combination with fenobam (30 mg/kg/day in rat and 10 mg/kg/day in monkey) in a cross-over design. The first treatment arm (12 or 17 days in rats and monkeys respectively) was followed by a wash-out period before treatment arm 2, where the treatment groups were swapped to the other. Animals previously treated with fenobam were swapped to L-DOPA + vehicle and vice versa.

Chronic administration of fenobam to previously drug-naïve animals (de novo treatment) attenuated the development of peak-dose dyskinesia (Fig.6A,C in rats and E,G in monkeys) without compromising the anti-parkinsonian effect of L-DOPA as seen in the rotarod test (in rats) or the PD scale (in monkeys). In addition, fenobam was able to attenuate already established dyskinesia (Fig. 6B,D,F,H) since both rats and monkeys...
treated with L-DOPA + vehicle in arm 1, progressively reduced their dyskinesia severity when swapped to fenobam cotreatment in arm 2 (Fig.6). In rats, fenobam's attenuating effect seemed to persist even after withdrawal of the cotreatment in arm 2 (Fig.6D).

With a significant reduction of peak-dose dyskinesia by about 50% in the rat and 70% in the monkey, fenobam is as potent as MTEP in the rat LID model (Mela et al., 2007) and more potent than MPEP and MTEP in the monkey model of LID (Morin et al., 2010). Fenobam thereby represents the most effective “anti-glutamatergic” drug tested in experimental PD models so far and acts similarly in rat and primate models of LID.

Fig.6. Fenobam reduces the development of AIMs in de novo treated 6-OHDA-lesioned rats and MPTP-treated monkeys. After the first treatment arm (day 12), fenobam cotreatment significantly reduced the development of peak-dose AIM scores in rats (A). This effect disappeared when the treatment was switched to L-DOPA + vehicle in treatment arm 2 (D). In addition fenobam reduced already established AIMs at the first day of treatment arm 2 in animals previously treated with L-DOPA + vehicle (B,C). This effect was persistent throughout treatment arm 2 (D). P < 0.05 vs. * vehicle group, # same group in other treatment arm. Data are mean ± SEM between 40 and 80 min. In monkeys, fenobam cotreatment significantly reduced the development of peak-dose LID from day 9 onwards during the first treatment arm (E). This effect disappeared when animals were switched to L-DOPA + vehicle in treatment arm 2 (F). In addition fenobam reduced already established LID at the first day of treatment arm 2 in animals previously treated with L-DOPA + vehicle, (G, * = P < 0.05). This effect was persistent throughout treatment arm 2 (H, * = P < 0.05 between two black bars) while the animals receiving L-DOPA + vehicle in treatment arm 2 displayed dramatically increased LID in comparison with last day of treatment arm 1 (H, *: P < 0.05 between two grey bars). Data are the mean AUC ± SEM between 50 and 100 min post drug administration.
The second part of this thesis shifts the focus on to the 5-HT system, which has been implicated in LID. The alleviating effects of 5-HT1A or B receptor agonists, in both animal experiment and clinical trials (Bonifati et al., 1994; Carta et al., 2007; Dupre et al., 2008; Lundblad et al., 2005), confirms the importance of the 5-HT system in these motor complications and demonstrate 5-HT autoreceptors as potential therapeutic targets. Even though this non-DA system possesses an eminent potential for neuroplasticity (Mattson et al., 2004), its adaptive changes to chronic DA-agent treatment and the role in LID is still unknown. This thesis has revealed a new mechanism of maladaptive plasticity of the 5-HT projections, induced by chronic L-DOPA treatment, in the striatum and possibly other brain areas as well. Analysis of rat and monkey models of LID, as well as post-mortem tissue from PD patients, consistently showed a positive association between striatal 5-HT fibre density and severity of LID. The association seems to be selectively coupled to L-DOPA treatment and a presynaptic mechanism since dyskinesia induced with direct DA agonist (independent of the presynaptic conversion and release), does not correlate with a higher density.

Altogether, the 5-HT fibre innervation of the striatum should be seen as a susceptibility factor for developing LID.

**SERT radioligand binding is positively correlated with the severity of AIMs**

The fourth paper, (paper IV) started off by searching for possible correlations between the severity of L-DOPA-induced AIMs (in the rat) and the density of striatal DA or 5-HT axon fibres, which were labelled using tritiated ligands that selectively bind to DAT and SERT, respectively. The contribution of 5-HT in the development of LID is supported by the exacerbation of LID after intrastratial transplantation of raphe neurons (Carlsson et al., 2007). In contrast, a 5-HT specific lesion causes a dramatic reduction in dyskinesia (Carta et al., 2007). A decreased DAT/VMAT ratio has been linked to a higher risk for developing dyskinesia (Troiano et al., 2009) and DAT is thought to contribute to a large DA surge after L-DOPA administration (Lee et al., 2008; Sossi et al., 2009).

Saline treated 6-OHDA-lesioned animals showed a 60% reduction of SERT radioligand binding compared to intact control levels, in the striatum and motor-premotor cortex. In comparison, a 90% reduction in DAT radioligand binding was observed the same structures. For DAT, the denervation was independent on L-DOPA treatment and development of dyskinesia. However, SERT radioligand binding proved to be positively correlated with the L-DOPA-induced AIM scores (Fig. 7). Looking at the graph, some animals did, despite their 6-OHDA lesion, express SERT binding levels of the same or higher degree as intact controls (Fig.7B). This led us to speculate if L-DOPA treatment in itself could have any growth-promoting effects.
Chronic L-DOPA treatment increases serotonin innervation densities with ultrastructural and functional changes

Prompted by these first observations a possible growth-promoting effect of L-DOPA was hypothesized. L-DOPA is able to upregulate cortical BDNF levels (Guillin et al., 2001), and BDNF in turn has a growth-promoting effect on the 5-HT system specifically (Mamounas et al., 2000). To this end, rats were chronically treated with two different doses (one low clinically relevant dose and one high daily dose). Results showed a dose-dependent increase in SERT radioligand levels in the striatum that was positively correlated with the developed AIM scores, as in the previous experiment. This was paralleled by a dose-dependent upregulation of BDNF mRNA in the same structures. Also the motor-premotor cortex of dyskinetic L-DOPA treated animals, showed a dose-dependent upregulation of BDNF, consistent with previous data (Guillin et al., 2001).

To further confirm our hypothesis, extensive morphological analyses was performed by counting 5-HT immunopositive axonal varicosities in striatum. An index of 5-HT axon terminal sprouting was obtained by dividing the density of 5-HT-positive varicosities by the number of 5-HT-immunoreactive cell bodies in the raphe nuclei in each animal (Finkelstein et al., 2000). L-DOPA treated rats (all dyskinetic), revealed a growth-promoting effect on striatal 5-HT axonal fibres, with a greater number of axon varicosities per raphe cell body compared to saline treated controls or intact animals (Fig. 8). The effect was significant already at the lowest dose. This demonstrates a sprouting effect of 5-HT axon fibres induced by L-DOPA treatment.

Given the purported mechanism of 5-HT striatal terminals on DA release (Arai et al., 1995; Tanaka et al., 1999), together with a potentiation effect of BDNF on the transmitter release from 5-HT terminals (Goggi et al., 2003), we hypothesized that a sprouting effect would have malfunctional consequences of excessive DA release. To
this end, striatal slices from L-DOPA-treated (dyskinetic) or saline treated rats were prepared to measure the stimulus-evoked release of \(^{3}\text{H}\)DA. Dyskinetic animals separated from saline treated controls, by a larger stimulus-evoked \(^{3}\text{H}\)DA release that further was potentiated by the application of BDNF. When applying a TrkB antagonist, the BDNF-induced potentiation was blunted. This results link the maladaptive sprouting response to an aberrant DA release, which in turn is linked to LID.

In another experiment, brains were processed for EM analysis (performed by our collaborator Dr. Martin Parent at the laboratory of L. Descarries, Canada), to evaluate whether the axon plasticity coincided with abnormal ultrastructural features. Analysis at the EM level showed no difference between striatal SERT-positive terminals of L-DOPA-treated and saline-treated animals, in terms of general morphometric features, i.e. mitochondria and vesicle content. However, the proportion of SERT-immunoreactive varicosities making a synaptic contact with striatal neurons was 2.5-fold larger in the L-DOPA group compared to saline-treated controls. These results are in agreement to that reported in adult rats (Soghomonian et al., 1989), and illustrate with a growth-promoting effect (Descarries and Mechawar, 2008).

Fig. 8. L-DOPA dose-dependently induces 5-HT axon terminal sprouting in the striatum. Bright-field photomicrographs in A-E show 5-HT-immunoreactive axon terminals in the different treatment groups and in magnification (E; arrows illustrate two of the varicosities). Scale bar: 10 \(\mu\)m. Values in F give an index of 5-HT axon terminal sprouting in the lesioned striatum (total number of 5-HT-positive varicosities divided by the number of 5-HT-positive neurons in the B7 and B8 raphe cell group in each animal). Bars show group means + SEM. \(P < 0.05\) *.
**General results and comments**

*Increased SERT binding densities in parkinsonian monkeys and PD patients with LID*

The correlation of 5-HT axon density and severity of dyskinesia is not necessarily restricted to the rat model of PD but could also be applied to other animal models and PD patients. Both the monkey model of PD, as well as clinical studies have shown improvement of LID with 5-HT1A or 1B agonist (Fox et al., 2008; Munoz et al., 2008). As further support of the involvement of 5-HT, clinical observations have shown a larger L-DOPA-induced DA release related to LID (de la Fuente-Fernandez et al., 2004; Pavese et al., 2006) and late-onset patients are less susceptible for developing LID while entailing degenerative changes of 5-HT system (Mattson et al., 2004; van Luijttelaar et al., 1992).

This thesis shows a persistency over species with increased SERT radioligand binding densities measured in striatal tissue from dyskinetic monkeys (collaboration with E. Bezard) and patients compared to non-dyskinetic cases (Fig.9). In dyskinetic monkeys MPTP induced a 40-80 % reduction in striatal SERT radioligand binding in the striatum and globus pallidus (GP) respectively. Chronic L-DOPA treatment was able to increase the binding, blunting the lesion effect only in animals expressing dyskinesia (Fig. 9A,B). Likewise, post-mortem sections through the basal ganglia (a collaboration with Prof. A. Lees and S.S. Sullivan Queens square brain bank, London) revealed a 5-HT fibre denervation of approximately 40-60 % in both the striatum and the GP in PD cases compared to healthy controls (Fig.9C,D), which is consistent with previous studies (Kerenyi et al., 2003). However, patients with a clinical record of LID showed elevated SERT radioligand levels compared to non-dyskinetic patients, providing new evidence in this field.

Conclusively, this is the first paper to demonstrate that a non-DA system such as 5-HT system can undergo maladaptive plastic modifications during chronic L-DOPA treatment in the parkinsonian striatum. The combined effect of a larger numbers of 5-HT varicosities together with the increased synaptic incidence and higher stimulus-evoked DA release, (that is potentiated by endogenous BDNF levels), would lead to a prominent increase in unregulated DA release and thus provide a risk for dyskinesia complication.
Fig. 9. LID is associated with increased SERT radioligand binding densities both in parkinsonian monkeys (A and B) and in PD patients (C-G). Radioligand binding data are expressed as percentage of control values (intact monkeys or neurologically healthy human subjects). Autoradiographs of SERT radioligand binding on human tissue (E-G) show the regions used for densitometric analyses (1 = putamen and 2 = GP). $P < 0.05$; § vs. intact/healthy controls, # vs. saline, * vs. non-dys. Bars represent group means + SEM from A-B. Scale bar, 2 mm.
L-DOPA-induced dyskinesia is attributed to dysfunctional neuroplasticity affecting to different extent the presynaptic control of DA release and the postsynaptic response of dopaminoceptive neurons. Accordingly, dyskinesia can be improved by treatments that either stabilize extracellular DA levels or dampen the supersensitive response of striatal neurons. Depending on the specific profile of the plastic changes in the parkinsonian brain, a specific anti-parkinsonian treatment might be more or less appropriate reducing the risk for dyskinesia.

The relative contribution of pre- versus postsynaptic mechanisms to dyskinesia are however difficult to dissect when subjects are treated with L-DOPA, which causes both changes in DA release and pronounced activation of signalling pathways in dopaminoceptive cells. Paper V in this thesis is a manuscript comparing the expression of dyskinesias induced by L-DOPA or a direct D1-receptor receptor agonist (SKF38393), which bypasses any involvement of presynaptic factors. In addition two anti-dyskinetic compounds of the non-DA systems, the mGluR5 antagonist, fenobam (paper III), and the 5-HT1B receptor agonist CP94253, were evaluated for their pre- and postsynaptic mechanisms. In the light of paper IV, the pre- or postsynaptic component of 5-HT maladaptive plasticity was evaluated using SERT autoradiography for SERT radioligand binding.

Comparison of dyskinesia induced by L-DOPA or SKF38393
Rats with unilateral 6-OHDA lesions received either L-DOPA (6-10 mg/kg/day) or SKF38393 (1.5-2.5 mg/kg/day) treatment for 5 weeks, during which the development of dyskinesia was monitored. Both DA agents induced comparable severity of AIMs that reached a plateau during the third week of treatment. The dose of SKF38393 was adjusted to produce comparable AIMs as L-DOPA. While the two treatments produced similar limb and orolingual AIMs, L-DOPA induced more severe axial AIMs. Indeed L-DOPA affects targets other than just the D1 receptor such as the D2 receptor, which could explain this difference.

Even though the dyskinesia severity was similar between the two treatments, we hypothesised that the two DA agents would have induced partly different striatal plasticity. Animals that had become dyskinetic with L-DOPA treatment would likely have a prone presynaptic plasticity such as enhanced raphe-striatal innervations whereas those that became dyskinetic with SKF38393 treatment would be expected to show more postsynaptic plasticity in the MSN such as an upregulation of FosB (Cenci and Lundblad, 2006).

In line with paper IV, results confirmed that the degree of raphe-striatal projection density in L-DOPA treated animals was correlated to the severity of AIMs in such a way that dyskinetic animals expressed a higher fibre density compared to non-dyskinetic animals (Fig.10A,C). Such correlation was not seen in the SKF38393 treated animals (Fig.10B). In this treatment group dyskinetic animals did not differ from non-dyskinetic ones and were indistinguishable from saline treated controls (Fig.10C). These results confirmed the hypothesis of the raphe-striatal compartment being mostly
involved in the presynaptic mechanism of LID. Studies further evaluating the degree of postsynaptic striatal nuclear signalling induced by the two dyskinesiogenic treatments are in progress.

Fig. 10. SERT binding in the striatum after chronic treatment with DA agents. 6-OHDA surgery induced degeneration of 5-HT fibres in the striatum by 63% (E). After chronic L-DOPA treatment there was a positive correlation between severity of dyskinesia and SERT binding density (A). Chronic SKF38393 treatment did not affect the 5-HT fibre density in the striatum (B). P <0.05 * vs. intact, § vs. L-DOPA non-dys.

Post-synaptic mechanisms behind the anti-dyskinetic effect of mGluR5 antagonist and 5-HT receptor agonist

At the end of the chronic experiment rats were given a challenge injection of fenobam or CP94253 to reveal the location of mechanistic action of these non-DA modulators. We expected that fenobam would dampen the overactive corticostriatal glutamate signalling at a post-synaptic location, i.e. on the MSNs (Gubellini et al., 2004). CP94253 on the other hand would assume to exert its actions predominantly in a presynaptic location, reducing the activity of 5-HT neurons and blunt the peak of DA efflux after L-DOPA administration (Carta et al., 2007; Munoz et al., 2009). However, postsynaptic located 5-HT1B receptors have also been implicated in dyskinesia induced with D1-receptor agonist (Jaunarajs et al., 2009).

Results supported a predominant postsynaptic effect for both substances. While fenobam challenge reduced peak-dose AIMs (40-80 min) in the L-DOPA group (30%), it was more efficient in reducing peak-dose and total AIM scores (50%) in SKF38393 treated animals (Fig.11A,B). Thus fenobam seems to act predominantly at the level of MSN (Fig.1) in reducing abnormal molecular responses. At this location mGluR5 antagonists could normalize hyperactive GABAergic transmission in the basal ganglia output nuclei (Mela et al., 2007) and reduce nuclear signalling to oppose the expression of dyskinesia (Cenci, 2007).

Interestingly CP94253 was able to reduced both L-DOPA and SKF38393-induced AIMs to similar extent (approximately by 50%) (Fig.11C,D). This demonstrate a more efficient reduction of D1 agonist-induced dyskinesia than previously reported.
General results and comments

(Jaunarajs et al., 2009) and supports a predominant postsynaptic mechanism of action in addition to the general presynaptic effect (Carta et al., 2007; Lindgren et al., 2010).

Conclusively, a comparison between L-DOPA and D1-agonist induced dyskinesia seems to provide a good paradigm to separate presynaptic and postsynaptic alterations. L-DOPA-induced dyskinesia is associated with an elevation of striatal SERT-binding densities, (i.e. a presynaptic plasticity), in contrast to D1-agonist induced dyskinesia. Further molecular and biochemical analyses are in progress to evaluate postsynaptic plasticity markers.

**Fig 11.** Acute challenges with mGluR5 antagonist fenobam and 5-HT1B agonist CP94253 in 6-OHDA rats treated chronically with L-DOPA or SKF38393. Co-administration of the fenobam with L-DOPA or SKF38393 reduced the AIMS scores during peak (A and B). Dyskinesia induced with D1-agonist was more efficiently reduced during peak and was also reduced during the total AIMS session. Co-administration of CP94253 reduced peak dyskinesia induced by L-DOPA. There was a trend for a reduction of SKF38292-induced dyskinesia. However, this did not reach statistical significance. Instead the dyskineia of the entire testing session was significantly reduced (D’). P < 0.05 * vs. vehicle.


**DISCUSSION**

**PREVENTION OF DENDRITIC SPINE PRUNING CAN ALLEVIATE LID.**

A marked atrophy of dendrites and spines on MSNs occurs in the parkinsonian brain of both patients (McNeill et al., 1988; Stephens et al., 2005) and rodent models of the disease (Day et al., 2006; Neely et al., 2007). The mechanism could be seen as an adaptive response to protect the striatal neurons from sustained and excessive glutamatergic input, as that seen in LID (Neely et al., 2007), that could cause neurodegeneration. Therefore in a way spine pruning can be good. Nevertheless blocking this spine pruning with an L-type calcium channel blocker (isradipine) seems not to induce a frank cell loss in the striatum (unpublished observation of Surmeier) but instead has shown neuroprotective effects (Chan et al., 2007). Blocking spine pruning could possibly also prevent unfavourable effects of PD pharmacotherapy or restorative treatment (Soderstrom et al., 2010).

Paper I of this thesis highlights, for the first time, the effect of striatopallidal spine pruning in the pharmacotherapy of PD. Treatment with an antagonist for the L-type calcium channel isradipine, dampened the expression of dyskinesia and preserved striatal spines. However, isradipine was not able to attenuate LID when spine pruning had already occurred (i.e. 7 weeks post-lesion). These data propose that prevention of spines is crucial for the anti-dyskinetic effect of isradipine. However, it does not necessarily directly link the preserving of spines with alleviation of LID. Even if spines would be preserved they may not necessarily function physiologically and other dysfunctions of the MSNs could be preserved, such as the over-excitability of MSNs or altered morphological shape of spines. Such adaptations might not be fully reversed (even if spines are) and could explain the persistence of some dyskinetic symptoms in the rats.

Recent data has supported our results while extending the implications of spine pruning. Not only is the development of LID alleviated by isradipine, but so are graft-induced dyskinesias and the therapeutic efficacy of DA neuron transplantation (Soderstrom et al., 2010). In that study, the prevention of spine loss of the MSNs was not attributed to the striatonigral or the striatopallidal pathway. Spine preservation of the D1-expressing MSNs could therefore also occur. However, the profound loss of 50% that have been proven for D2-expressing MSNs (Day et al., 2006) was larger than that reported from randomly selected MSNs (Ingham et al., 1989; Neely et al., 2007), supporting a predominant loss for the D2-expressing neurons.

Interestingly, isradipine has shown a neuroprotective effect on nigral neurons. The substance can force DA neurons away from their unusual reliance on L-type Cav.1.3. calcium channels to drive their intrinsic basal activity and thereby protect them from neurodegenertation. However, this mechanism is probably not the reason behind...
isradipines' alleviating effect on LID in paper I, since all animals had a similar extent of DA denervation measured (Chan et al., 2007).

We conclude that isradipine can prevent the development of LID if given at an early stage of the disease, i.e. when spine pruning of MSNs has not yet occurred but is ineffective at a later stage of the disease. Isradipine is already used in the clinics (for treating hypertension) at a comparable dose and is therefore a suitable drug for further development in the pharmacotherapy of LID (Surmeier, 2007).

MGLUR5 AS A POTENTIAL THERAPEUTIC TARGET FOR ALLEVIATING LID.

Allosteric modulators of G-protein coupled receptors such as mGluRs have advantages over traditional receptor antagonists in that they rely on the action of endogenous transmitters, that are released in an activity-dependent fashion, and do not induce tonic activation of the receptors that could cause adverse effects (Gubellini et al., 2004). Paper II proposes mGluR5 as a superior target for treating LID compared to other glutamate drugs acting on the corticostriatal synapse i.e. NR2B subunit of the NMDA receptor, mGluR1, mGluR2/3 and L-type calcium channel. Group I mGluRs are expressed in several basal ganglia nuclei, such as the striatum, GP, SN and STN where they are believed to regulate neuronal activity (Rouse et al., 2000; Tallaksen-Greene et al., 1998). Still, only antagonists to mGluR5, and not mGluR1, attenuate dyskinesia observed in paper II and in a previous study (Dekundy et al., 2006). Such a discrepancy might be due to a positive reciprocal interaction of specifically mGluR5 to the NMDA receptor. Activation of the NMDA receptor potentiates mGluR5-mediated responses while mGluR5 stimulation enhances the currents of NMDA receptor (Alagarsamy et al., 1999; Pisani et al., 2001b). The NMDA receptor is in turn linked to LID with altered expression or phosphorylation of subunits (Dunah et al., 2000; Hallett et al., 2005; Oh et al., 1998). Therefore it seems plausible that MTEP (in paper II) or fenobam (in paper III) reduces L-DOPA-induced stimulation of NMDA receptors at the postsynaptic density of MSNs and thereby attenuates the expression of AIMs. In further support of this, a more potent anti-dyskinetic effect of fenobam was seen in dyskinesia induced by SKF38393 compared to dyskinesia induced with L-DOPA in paper V. Also previous reports have found a reversal of D1 agonist-induced locomotion by MPEP in reserpinsised rats (Ismayilova et al., 2006).

Notably, in vitro studies have reported that the neuroprotective effects of mGluR5 antagonists (Breysse et al., 2003; Miller et al., 1995) may reflect their ability to bind directly to the NMDA receptor and inhibit its signalling (O'Leary et al, 2000; Lea et al, 2005). However, these effects were seen with brain concentrations of MTEP, that were substantially higher than those produced by the doses in this thesis (Loscher et al., 2006). Moreover, in vivo and in vitro studies have indicated that MTEP is highly selective for mGluR5 and has much fewer off-target effects than any related mGluR5 antagonists (Cosford et al., 2003; Lea and Faden, 2006).
A postsynaptic antagonism of mGluR5 on MSNs would lead to a reversal of postsynaptic molecular alterations coupled to LID, such as striatal upregulation of ∆FosB (Jimenez et al., 2009; Levandis et al., 2008), pDyn mRNA (Mela et al., 2007) and glutamic acid decarboxylase (GAD65 and GAD67) (Yamamoto and Soghomonian, 2009). Furthermore, our group has previously shown that MTEP's attenuation of AIMs in rat is paralleled with a normalization of the hyperactive GABAergic transmission to SNpr (Mela et al., 2007). MTEP can also reverse metabolic changes in the output structures of basal ganglia seen in akinesia (Oueslati et al., 2005). These studies all support a central inhibition of LID's molecular and neurochemical mechanisms.

As further support for a role of mGluR5 in LID, a recent paper has measured mGluR5 radioligand binding in the brains of MPTP-treated monkeys or post-mortem tissue from patients. In this paper, cases with motor complications had higher radioligand levels in both putamen and GP compared to non-dyskinetic subjects (Ouattara et al., 2009; Samadi et al., 2008b).

**Fenobam as a potential therapy for LID**

During the experimental work of paper III, the two mGluR5 antagonists MPEP and MTEP were reported to alleviate LID also in monkey models of PD (Morin et al., 2010). This was consistent with the behavioural effect of fenobam in our monkey experiment, although we did observe a more potent reduction than that demonstrated in Morin et al (Morin et al., 2010). The reduction also appeared differently in our observations, with a removal of the peak-dose dyskinesia followed by a prolonged effect in the end-phase. This prolonged effect could be of advantage in the clinics where it could improve the on-phase and reduce the off-phases between the L-DOPA administrations. Discrepancies between fenobam and MTEP, MPEP might depend on differences in their chemical composition (Porter et al., 2005).

In support of the clinical development of fenobam, this substance has already been tested in the clinics as an anxiolytic back in the 70's, showing a good safety profile (Goldberg et al., 1983). However as an anxiolytic fenobam was withdrawn from further development due to lack of efficacy or incidence of psychostimulate side effects (Friedmann et al., 1980). Indications for further development of the molecule were made. The side effects could be due to the large variability in oral bioavailability of fenobam (Itil, 1978), demonstrating the importance of route of administration. Nevertheless, today more than 30 years later, fenobam has been characterized in a broad range of *in vitro* and *in vivo* models (Jacob et al., 2009; Montana et al., 2009; Porter et al., 2005) where it has shown improved therapeutic window over benzodiazepines (Porter et al., 2005). Taken together, fenobam represents a suitable drug for further clinical development towards a better anti-parkinsonian and anti-dyskinetic treatment. In addition, its anxiolytic use has potential for improved efficacy without side-effects.

Antagonists to mGluR5 have, in addition to an anti-dyskinetic effect, shown more therapeutic potencies in PD. For example, MPEP treatment to PD rats reverses visuospatial memory impairments (De Leonibus et al., 2008), which constitutes the most frequently reported cognitive deficits in PD (Berger et al., 2004; Giraudo et al., 1997).
Moreover mGluR5 antagonists could be effective in treating pain, depression, anxiety, and addiction, all of which are possible non-motor complications of PD (Berry-Kravis et al., 2009; Jacob et al., 2009; McGeehan et al., 2004; Montana et al., 2009). Notably, some adverse effects of MTEP have been detected for hippocampal-depended memory task in the physiological situation (Lu et al., 1997). However, in parkinsonian animals MTEP instead improved memory (De Leonibus et al., 2008). Thus, while blockade of mGluR5 in a normal brain may negatively affect cognition, the same treatment would ameliorate cognitive deficits in situations where this receptor is over-stimulated, which is the case in LID.

NEGATIVE EFFECTS OF MODULATIONS OF THE NR2B-SUBUNIT SELECTIVE NMDA RECEPTOR, MGLUR1 AND MGLUR2/3 IN THE TREATMENT OF LID.

An overactive glutamate transmission at the corticostriatal synapse is linked to development of LID and thus modulations of specific targets at this site could prevent the non-physiological signalling and attenuate LID (Chase et al., 2000). With an abundant expression in the striatum (Gubellini et al., 2004), where its expression is correlated with LID (Calon et al., 2003), we hypothesised that antagonising the NR2B subunit of the NMDA receptor would improve the symptoms of dyskinesia. Up to this point, monkey and clinical studies using NR2B subunit selective antagonists have demonstrated both worsening and improvement in dyskinesia (Hadj Tahar et al., 2004; Nash et al., 2004; Nutt et al., 2008). The discrepancies might reflect differences between monkey species and/or the degree of striatal DA denervation (Gerfen et al., 2002).

This thesis provides the first evidence of the effects on LID of a selective NR2B subunit antagonist in a rat model. Results were different from our initial hypothesis. Rats cotreated with the antagonist Ro256981 showed similar severity of AIMs and similar levels of phosphorylated proteins and genes as animals treated with L-DOPA only. The lack of effect on pERK1/2 is similar to that seen with NMDA antagonists (Gertler et al., 2008). Regardless of the dyskinesia, rats cotreated with Ro256981 showed a better performance on the rotarod demonstrating an anti-kinetic effect. This effect is coherent with previous report (Nash et al., 2004). Therefore, to rule out NMDA subunits as therapeutic targets for PD might not be necessary yet. Other studies have identified NR2A subunit as the most critical for the functional role of NMDA receptor in striatum with altered expression in LID (Hallett et al., 2005) (Schotanus and Chergui, 2008). Given that NR2A mutant mice display increased locomotion (Miyamoto 2001), antagonists for this subunit might be more beneficial in the treatment of PD or LID.

An alternative way of blocking the overactive glutamate transmission at the corticostriatal synapse could be by inhibiting presynaptic glutamate release with agonist to mGluR2/3 (Picconi et al., 2002). Contrary to our initial hypothesis, cotreatment with the mGluR2/3 agonist LY379268 showed no significant effect on L-DOPA-induced molecular changes, nor did it reduce AIMs in the rat. Instead, LY379268 tended to
reduce rotarod performance, this was despite the use of a lower dose to avoid such effect. Indeed, mGluR2/3 agonists seem to lack anti-parkinsonian efficacy (Ossowska et al., 2007) and instead induce cognitive and memory deficits (Higgins et al., 2004). Moreover, the expression of mGluR2/3 in the basal ganglia, remain unchanged with L-DOPA treatment and dyskinesia development (Samadi et al., 2008a). Taken together, this target does not have strong potential for PD therapy.

The results for the mGluR1 antagonist in paper III were partly negative as well. Despite the use of the highly potent and selective antagonist EMQMCM (Lavreysen et al., 2002), that has shown to inhibit haloperidol-induced catalepsy and attenuate freezing response in fear conditioning test (Dekundy et al., 2006; Pietraszek et al., 2005), we did only demonstrate a trend of this substance to reduce AIMs. In parallel, the related striatal nuclear signalling was only reduced to some extent, which is in line with Dekundy et al (Dekundy et al., 2006). Nevertheless, a less demanding statistical comparison provided significantly attenuation on AIMs by EMQMCM. However, this was achieved at a dose comprising the anti-akinetic effect of L-DOPA. The untoward effect of the substance in the rotarod test may be related to the high cerebellar expression of mGluR1 since cerebellar glutamate transmission is of importance in the control of motor coordination (Nakao et al., 2007). In addition, the receptors are also expressed presynaptically on the residual DA terminals where its stimulation could reduce the DA release (Conn et al., 2005). Therefore to target mGluR1 might attenuate the expression of dyskinesia to some degree but only at a dose that compromises the anti-akinetic efficacy of L-DOPA.

When comparing different glutamate target modulators, it is important to remember that pharmacological modulation of central glutamate receptors may yield different effects depending on the site of actions. Blocking the glutamate transmission at the corticostriatal synapse would hypothetically decrease LID severity (Calabresi et al., 2007) whereas blockade at other sites such as in the STN would not (Fig. 1). Therefore some agonists/antagonists tested here might provide a better therapeutic benefit if administered directly to the desired target. For example, if EMQMCM would be administered locally to the striatum, it might have provided a better effect.

MALADAPTIVE PLASTICITY OF THE SEROTONIN SYSTEM IN LID.

L-DOPA-induced dyskinesias can be described as a pathological neuroplasticity that adapt to a progressive DA denervation from the disease together with a non-physiological treatment (L-DOPA). The individual brain plasticity of PD patients is dependent on several factors, such as a patient's age and genetics (Linazasoro, 2005). In this thesis we have discovered a new mechanism of maladaptive plasticity in LID: a growth-promoting effect on raphe-striatal projections. While the parkinsonian DA denervation itself induced a degeneration of 5-HT axons, the consecutive dyskinesiogenic L-DOPA treatment, was persistently linked to a higher degree raphe-projection density in rat, monkey and human species. In rats for example, the 6-OHDA lesion itself caused a striatal 5-HT fibre denervation of 40-60%, which is comparable to
Discussion

that seen in patients (Scatton et al., 1983). Dyskinesiogenic L-DOPA treatment partly
reversed this denervation in a dose-dependent way. The effect was only seen in the 6-
OHDA lesioned side, whereas on the intact side, there were no group differences. This is
worth noticing when comparing different animal models of the disease as not all of them
cause a raphe-fibre denervation (Breese et al., 1984; Zhou et al., 1991).

The maladaptive plasticity occurred at terminal levels of the raphe neurons and
did not depend on any upregulation of the SERT protein at the level of the cell. In proof
for this, the levels of SERT mRNA in B7 and B8 were similar between L-DOPA and
saline treated rats. In addition, SERT radioligand binding positively correlated to the
number of 5-HT immunoreactive varicosities in both rats and human brain tissue (Fig.7
in experimental methods and materials). Increased levels of BDNF mRNA in both
striatum and cortex paralleled the dose-dependent increase in SERT radioligand binding.
Brain-derived neurotrophic factor in turn does not seem to alter protein levels SERT
protein (Daws et al., 2007) but has a potent growth-promoting effect on specifically 5-
HT fibres (Mamounas et al., 2000).

A sprouting response of 5-HT striatal terminals brings increased stimulation-evoked DA
release and enhanced synaptic incidence.
The growth-promoting effect possibly illustrates a compensatory mechanism of 5-HT
projections to either oppose the DA deficiency or to cope with the continuous L-DOPA
administrations. Indeed, striatal 5-HT terminals express the enzymes required to convert
L-DOPA to DA, and have ability to store it in synaptic vesicles (Arai et al., 1995;
Tanaka et al., 1999). However, DA-release from 5-HT terminals results in large
intermittent fluctuation in brain levels of DA, that constitute the prime causal role of
LID (Chase, 1998). Sprouting of the 5-HT system, as that seen in paper IV, would
assume to contribute largely to this aberrant DA release.

In paper IV, we propose that enhanced BDNF levels, induced by cortical
activity or L-DOPA treatment (Guillin et al., 2001), would have functional effects on the
5-HT terminals. In fact, BDNF does potentiate stimulus-evoked transmitter release from
5-HT terminals (Goggi et al., 2002). In a slice-experiment, we showed that dyskinetic
animals, as hypothesised, exerted a higher stimulus-evoked [3H]DA release compared to
saline controls. The [3H]DA release was further enhanced by the administration of
BDNF. This suggests a malfunction of the 5-HT axonal sprouting that would increase
the risk for LID.

Analysis of 5-HT axonal varicosities at the EM level, revealed normal
ultrastructure in terms of vesicular and mitochondrial content and relative distribution
onto dendritic spines and shafts of striatal neurons. However, L-DOPA treated
dyskinetic animals showed an increased synaptic incidence of 5-HT varicosities
compared to saline controls. Serotonin terminals normally form very few synaptic
contacts in the adult brain (Soghomonian et al., 1989) and an increase in synaptic
incidence more than two-fold would likely affect the striatal response to 5-HT
stimulation. In addition, these data support immature features of the newborn
varicosities (Descaries and Mechawar, 2008).
Taken together, in dyskinetic subjects the plasticity would hypothetically cause a five to seven-fold increase in striatal synaptic contacts (2.5 fold increase in synaptic incidence multiplied by two to three-fold increased in the number of 5-HT varicosities). Such increase would expect to have major consequences in the non-regulated DA release (Lindgren et al., 2010; Tanaka et al., 1999) and predispose for L-DOPA complications. Indeed, dyskinesia is linked a two-fold larger surge compared to non-dyskinetic subjects in DA extracellular levels along with a higher extracellular and striatal tissue levels of 5-HT and its main metabolite 5-HIAA (Lindgren et al., 2010). Our results thus support previous implications of the 5-HT role in LID (Bonifati et al., 1994; Munoz et al., 2008).

A recent study has in line with our results revealed higher 5-HT fibre innervation in dyskinetic rats using measurement of SERT-immunopositive binding (Lundblad et al., 2009). Moreover, two weeks of intrastratal infusion of L-DOPA in 6-OHDA-lesioned rats reverses the initial decrease of 5-HT uptake and tryptophan hydroxylase levels (Kaariainen et al., 2008).

The maladaptive plasticity seems not to be restricted to the striatum but was also seen in non-striatal areas, i.e. motor-premotor cortex in the rat and GP in monkeys and human. Therefore this effect possibly occurs in many 5-HT projection targets, affecting motor as well as non-motor symptoms of the disease. For example, a recent paper from our group showed reduced DA release in the SNpr after administered 5-HT1A and B agonist, highlighting a possible increased 5-HT density in this structure as well (Lindgren et al., 2010).

Clinical implications
A possible growth-promoting 5-HT response in PD patients supports the validity of 5-HT receptor agonists as a therapeutical strategy for reducing LID in PD. Moreover it could explain other implications found in dyskinetic cases such as the increased DA surge over PD disease duration that has been seen in patients (de la Fuente-Fernandez et al., 2004). It could also explain why young patients are more susceptible to develop dyskinesia. Young patients have a larger potential for plasticity in the brain (Linazasoro, 2005) with a higher 5-HT fibre innervation along with a larger BDNF expression compared to patients at late-onset (Mattson et al., 2004). Early-onset patients also show increased DA turnover, that is in turn influenced by the rate of DA release (Sossi et al., 2006).

A negative role of 5-HT in the development of dyskinesia has been revealed from transplantation studies in rats (with an extensive 5-HT fiber outgrowth in the striatum) (Carlsson et al., 2007), and in a recent clinical study (Politis, 2010). In accordance to this, we here provide the first evidence of enhanced striatal density of 5-HT projections in dyskinetic PD patients. Even though the 5-HT mechanisms have shown to play a role in the postsynaptic supersensitivity of LID (Dupre et al., 2008; Zhang et al., 2006) this growth promoting plasticity seems to be dependent on presynaptic mechanism since denser raphe-striatal innervation was not seen after D1-agonist-induced dyskinesia.
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Current data are in contrast to Kish et al (Kish et al., 2008) that did not see any association of reduced SERT or 5-HT levels in patients without dyskinesia. These negative findings may however reflect the low number of cases (only 3-4 patients in dyskinetic and non-dyskinetic group) and/or the limited amount of tissue processed for Western immunoblotting in this study.

In addition to defining a new mechanism of drug-induced maladaptive plasticity, our results will inspire further investigations on structural and functional changes of the brain 5-HT innervation induced by any neuropsychiatric interventions. Even though it will be difficult to prevent the plasticity to occur, given the multifunctional role of 5-HT and BDNF in several physiological functions (Mattson et al., 2004), our findings will help to devise biomarkers that can predict the risk of complications. If the degree of striatal 5-HT innervation could be measured at the time of diagnosis of PD, by utilizing PET scanning, the risk of developing LID could be predicted and support the choice of treatment. In such way it would be possible to initially treat patients with a greater risk for LID, i.e. those with high 5-HT innervation or young patients with greater plasticity potential (Linazasoro, 2005) with a less LID-causing treatment, e.g. DA agonists or MAO inhibitors.

Other effects of 5-HT and BDNF plasticity in PD.
Given the multifaceted role of both 5-HT and BDNF it seems plausible that the upregulation of these factors as that seen in paper IV would have several effects also beyond those seen in LID. Serotonin axons project ubiquitously to all brain regions including basal ganglia, limbic system and cortex, of which all could be involved in PD symptoms or drug complications when up to 50% of the projections may degenerate (Agid and Blin, 1987). A purported role of this 5-HT loss has been applied in some PD symptoms e.g. depression, sleep disturbances and autonomic symptoms but also postural instability or freezing phenomena (Melamed et al., 1996). A reduction in 5-HT has been suggested to cause depression in PD but the correlation is not at all clear (Boileau et al., 2008; Mayeux et al., 1984).

In paper IV, chronic L-DOPA treatment, when causing dyskinesia, was shown to instead increase 5-HT fibre density and possibly the levels of 5-HT (Lindgren et al., 2010) in both putamen and GP and possibly other non-motor areas. Such an effect could be involved in other non-motor symptoms in PD such as the drug related hallucinations, paranoid delusions, gambling behaviour (Nicholson and Brotchie, 2002; Voon et al., 2009).

Serotonergic neurons are indeed very responsive to BDNF that in turn has implications in LID. Brain-derived neurotrophic factor has well-established effects in the synaptic plasticity related to learning and memory (Martinowich and Lu, 2008), mechanisms that have been linked to LID. Dyskinetic subjects’ have an inability to depotentiate from LTP (Picconi et al., 2003). Moreover, a common single-nucleotide polymorphism in the pro-domain of human BDNF gene (Val66Met) has been implied in the time to onset of LID. Moreover this polymorphism has been detected in subjects with cognitive deficits (Guerini et al., 2009).
Our focus on BDNF's growth-promoting effect and neurotransmitter releasing effect on the 5-HT system, does not exclude other potential effects of BDNF. For example BDNF has a modulatory role in the angiogenesis in the striatum that has been linked to dyskinesia (Raab and Plate, 2007; Westin et al., 2006). In fact, plasticity of 5-HT system and microvascular plasticity most likely go hand in hand sharing some similar upstream regulators, i.e. vesicular endothelial growth factor (VEGF) and BDNF. For example, VEGF can also promote axonal outgrowth while BDNF can promote endothelial cell survival and angiogenesis (Raab and Plate, 2007).

PRE AND POSTSYNAPTIC PLASTICITY IN LID

Anti-dyskinetic drug trials in PD provide highly variable results in different PD patients. This could reflect an individual variability between the relative involvements of pre- versus postsynaptic malplasticity in the different parkinsonian brains. Thus, defining indexes of pre- versus postsynaptic mechanisms may allow for future anti-dyskinetic treatments to be individualized for maximal efficacy.

One important marker of maladaptive presynaptic plasticity is the L-DOPA-induced DA efflux released from 5-HT axon terminals (Lindgren et al., 2010; Tanaka et al., 1999). In addition, as showed in paper IV, a sprouting effect on 5-HT axon terminals along with a potentiated stimulus-evoked DA release is associated with dyskinesia induced with L-DOPA. However, D1 agonists can induce similar dyskinesia as L-DOPA and induce the striatal signalling markers of dyskinesia such as PDyn and FosB (Andersson et al., 1999; Hope, 1998). This confirms the well-established postsynaptic contribution to dyskinesia.

Paper V provides a comparison between D1 agonist and L-DOPA-induced dyskinesia in order to separate pre- or postsynaptic mechanisms. Several parameters were included in the comparison, namely, the topographic distribution of the dyskinetic movements, their response to anti-dyskinetic drugs targeting glutamatergic or 5-HT mechanisms and their correlations with indexes 5-HT innervation.

Both DA agents produced a similar overall severity of dyskinesia, but found significant differences in the expression of axial AIMs, which were significantly more severe following L-DOPA treatment. These data may suggest that a maximally severe expression of axial AIMs requires costimulation of D1 and D2 receptors, although further experiments are will be needed to prove this assumption.

When comparing dyskinetic behaviour with the levels of SERT radioligand binding, a higher striatal 5-HT innervation was positively correlated to dyskinesia induced L-DOPA as opposed to D1 agonist-induced dyskinesia. Thus, individual variations in dyskinesia severity can partly be explained by differences in 5-HT innervation densities only when L-DOPA is used as the inducing drug, whereas D1 agonist-induced dyskinesia does not require a dense 5-HT innervation to occur. Further investigations are required to elucidate the correlates of postsynaptic markers associated with dyskinesia such as the levels of FosB and levels of PDyn mRNA in the MSNs of the striatum.
The challenges with the anti-dyskinetic drugs, showed that the 5-HT1B receptor agonist CP94253, exerted a similar reduction for dyskinesia induced by SKF38393 compared to L-DOPA, suggesting a postsynaptic effect and an extra-raphe implication of this receptor in LID. In accordance with this, Zhang and co-authors demonstrated upregulated levels of 5-HT1B receptor in striatonigral neurons after dyskinesiogenic L-DOPA treatment, a mechanism that was dependent on D1 signalling pathway (Zhang et al., 2008). Vice versa, the expression of 5-HT1B receptor is abolished after anti-dyskinetic treatment with citalopram (Kuan et al., 2008). Thus, it seems like 5-HT1B, similar to the 5-HT1A receptor (Antonelli et al., 2005; Dupre et al., 2008; Mignon and Wolf, 2002) is able to act through non-raphe signalling to reduce LID in addition to its presynaptic effect on DA release. For example, agonists of the 5-HT1B receptor have been shown to reduce the activity in corticostriatal pathways or by inhibiting GABA release on GPi (Chadha et al., 2000).

Metabotropic glutamate receptor 5 is expressed on MSNs modulating NMDA and DA signalling (Pisani et al., 1997; Testa et al., 1994; Voulalas et al., 2005). Antagonizing this receptor was shown to attenuate dyskinesia and its molecular adaptations in animal models (Dekundy et al., 2006; Mela et al., 2007; Rylander, 2010; Samadi et al., 2008b). Yet, it is not known whether the anti-dyskinetic effect exerted by mGluR5 antagonist act through pre or postsynaptic effects of L-DOPA. Our data herein show a superior effect of fenobam in reducing AIMs induced with SKF compared to AIMs induced with L-DOPA. This supports the hypothesis of a postsynaptic effect of this receptor antagonist in its alleviation effect on LID.

**CONCLUDING REMARKS**

The present work has identified some novel glutamatergic and 5-HT mechanisms that contribute to the development of LID in experimental models of PD. We show that mGluR5 is implicated in the abnormal signaling responses that are induced by L-DOPA in striatal MSN. These responses include a hyperactivation of ERK1/2 signaling and an upregulation of PDyn mRNA. Previous work had demonstrated that these molecular changes are dependent on D1-type DA receptors. Accordingly, our work shows that pharmacological antagonism of mGluR5 significantly attenuates dyskinesia when this is induced by either a D1 receptor agonist or L-DOPA. These results prompt further investigations on the molecular mechanisms through which the D1 receptor and mGluR5 interact to modulate striatal signaling and synaptic plasticity in PD and LID.

The novel 5-HT mechanism identified by our work consists in a maladaptive structural plasticity of 5-HT axon terminals, which exhibit a sprouting response, an increased synaptic incidence, and altered release properties in L-DOPA-treated, dyskinetic rats. This abnormal plasticity of degenerated 5-HT terminals seems to be a consistent feature of LID across species. Our results support the pursuit of therapeutic avenues targeting the 5-HT system to reduce LID. Moreover, our findings encourage further mechanistic investigations on the role of the 5-HT innervation in the development of both LID and graft-induced dyskinesia in PD.
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