Levodopa- and Neuroleptic-Induced Dyskinesias

Studies on Pharmacological Modification and Processing of Opioid Neuropeptides

BY

REBECKA KLINTENBERG
ABSTRACT


Dyskinesias or abnormal involuntary movements are a debilitating complication of long-term levodopa treatment of Parkinson’s disease (PD) that is widely experienced and may compromise the efficacy of the drug therapy. Tardive dyskinesia is another important adverse effect seen with antipsychotic drug treatment. The neural mechanisms underlying levodopa- and neuroleptic-induced dyskinesia are not clear and involvement of the endogenous opioid neuropeptide system has been implicated. In this thesis, the role of the opioid system is investigated in models of dyskinesia and PD using behavioral, neurochemical and advanced analytical chemistry techniques. In addition, the motor effects of a new partial dopamine agonist with normalizing properties on both reduced and elevated dopamine transmission are studied and a new model for tardive dyskinesia is presented.

Using microdialysis in combination with micro-electrospray mass spectrometry, the in vivo processing of the opioid neuropeptide dynorphin A(1-17) was studied and 32 metabolites were detected in the striatum. Altered in vivo metabolism of the peptide was found in a model of PD with more metabolites formed in the dopamine-depleted striatum. Moreover, dynorphin A(1-17) was differently processed in levodopa-, bromocriptine and saline-treated animals.

Levodopa treatment caused an increase in the mRNA expression of the precursor of dynorphin, preproenkephalin-B as well as the precursor of enkephalin, preproenkephalin-A, in all sub-regions of the dopamine-depleted striatum. A non-selective opioid receptor antagonist, naloxone, was found to reduce levodopa-induced dyskinesia with maintained antiparkinsonian response and a normalization of hyperkinesia. Moreover, the new drug GMC1111 showed dopamine stabilizing properties in models of levodopa-induced dyskinesia and PD. This might prove useful in the treatment of PD.

Altogether, these results suggest that the endogenous opioid system is involved in the pathophysiology of levodopa-induced dyskinesia.

Key words: Opioid peptides, dyskinesia, Parkinson’s disease, mass spectrometry, metabolism

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PAPERS DISCUSSED

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


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ABBREVIATIONS

aCSF  artificial cerebrospinal fluid
AD   acute dystonia
ANOVA analysis of variance
CNS  central nervous system
C-terminal carboxyl-terminal
Dyn  dynorphin
ESI  electrospray ionization
GABA γ-aminobutyric acid
GPe  globus pallidus externa
GPI  globus pallidus interna
i.m. intramuscular
i.p. intraperitoneal
LC  liquid chromatography
L-DOPA L-dihydroxyphenylalanine
Leu  leucine
NMDA N-methyl-D-aspartate
Met  methionine
MFB  medial forebrain bundle
MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA  messenger ribonucleic acid
MS  mass spectrometry
N-terminal amino-terminal
6-OHDA 6-hydroxydopamine
PD  Parkinson’s disease
POMC proopiomelanocortin
PPE-A preproenkephalin-A
PPE-B preproenkephalin-B
s.c. subcutaneous
S.D.  standard deviation
S.E.M. standard error of the mean
SNC substantia nigra pars compacta
SNr  substantia nigra pars reticulata
STN  subthalamic nucleus
TD  tardive dyskinesia
TH  tyrosine hydroxylase
TOF  time-of-flight
VCM vacuous chewing movements
VTA ventral tegmental area
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1 INTRODUCTION

The presence of a specific dopamine-containing neuronal system was first demonstrated by Carlsson, Falck and Hillarp (1962) (Carlsson et al., 1962). Since then we have learned that dopamine is involved in many functions of the central nervous system (CNS) such as motor control, learning, memory, cognition, emotions and reward (Missale et al., 1998). The importance of the dopaminergic system in motor control is clearly seen in Parkinson’s disease (PD). This disease was first described by James Parkinson in 1817 (Parkinson, 1817) and is caused by degeneration of dopaminergic neurons projecting from the substantia nigra pars compacta (SNC) to the striatum (Hornykiewicz, 1973). The loss in dopamine leads to the cardinal symptoms of the disease (Kish et al., 1988), rigidity, akinesia, tremor and bradykinesia. In the 1960s the precursor of dopamine, L-3,4-dihydroxyphenylalanine (levodopa) was introduced as an effective drug in the treatment of PD (Cotzias et al., 1967) but soon after its introduction motor complications such as levodopa-induced dyskinesia were reported (Marsden, 1994).

The dopamine system has also been the target of antipsychotics, which were shown to be receptor antagonists (Carlsson and Lindqvist, 1963). Like the levodopa-treatment of PD, drug treatment with antipsychotics has been associated with the development of motor side effects that compromise the use of the drugs. Tardive dyskinesia (TD) is seen after chronic treatment with classical neuroleptics and is potentially irreversible (Casey, 1985).

The pathophysiology of levodopa-induced dyskinesia and TD is not fully elucidated but opioid neuropeptides have been suggested to be involved (Liminga et al., 1989; Henry and Brotchie, 1996; McCormick and Stoessl, 2002). In this thesis, the role of opioid neuropeptides has been investigated in levodopa-induced dyskinesia by using behavioral and neurochemical studies as well as advanced analytical methods to study the processing of an opioid neuropeptide in the brain. However, levodopa-induced dyskinesia is a complex syndrome and may be pharmacologically modified using different strategies. Therefore, the effects on motor behavior of a novel partial dopamine agonist have been investigated in animal models of PD. Moreover, a new animal model of TD is presented.

1.1 Levodopa-induced dyskinesia in Parkinson’s disease

Initially, levodopa treatment provides stable symptomatic relief of the symptoms of PD (Barbeau, 1969; Cotzias et al., 1969; Yahr et al., 1969; Jankovic, 2002). However, as the disease progresses, levodopa therapy is often associated with the development of adverse fluctuations in motor response such as “on-off phenomena” and early “wearing off” that coexist with levodopa-induced dyskinesia (Nutt, 1990). Eventually, dyskinetic movements affect more than 50 % of patients on long-term levodopa treatment (Fahn, 2000) and they may sometimes even become
more disabling than the disease itself (Nutt, 1990). The development of motor complications will lead to a decrease in health-related quality of life (Scheife et al., 2000). Thus, the motor complications seen with chronic levodopa-treatment gradually compromise its efficacy.

There are three main types of levodopa-induced dyskinesia: Peak-dose dyskinesia, diphasic dyskinesia and off-period dystonia. The peak-dose dyskinesias are the most common ones, occurring while the patient experiences the peak-dose effect of the levodopa administration. These involuntary movements usually affect the neck, face and limbs and are mainly of the choreic or choreoathetoid type, but may also be dystonic or ballistic. Diphasic dyskinesia is observed at the onset and wearing off of a therapeutic dose of levodopa and may be choreic, dystonic or ballistic. Diphasic dyskinesia may be very severe. Off-period dystonia is often seen early in the morning or during the day between the levodopa doses. This type of dyskinesia is characterized as a static dystonic posture most often displayed in legs or feet and sometimes in the arms and trunk. It is almost always painful in contrast to the other types of levodopa-induced dyskinesia (for reviews see Nutt, 1990; Nutt, 2001; Adler, 2002 and Fahn, 2000).

Early onset of PD (patients younger than 50 years) and initial treatment with more than 600 mg/day of levodopa have been associated with higher risk to develop dyskinesia (Grandas et al., 1999). Moreover, the severity of the disease seems to be a contributing factor with dyskinesias most prominent on the side most affected by the disease (Horstink et al., 1990).

The pattern of levodopa administration may also affect the development of dyskinesias. In the normal brain, dopamine receptor stimulation is essentially tonic in nature interrupted by burst firing. This pattern seems to be important in reward and in the learning system of the brain (Schultz, 1998) and is disturbed when using standard dopamine replacement strategies. More continuous dopamine receptor stimulation by long-term infusion of levodopa or dopamine agonists or administration of control-release formulations has been proposed to induce less dyskinesia (Chase, 1998; Nutt et al., 2000),

Other strategies to combat levodopa-induced dyskinesia include the substitution of levodopa by certain dopamine agonists. In a five-year study, initial treatment with a dopamine agonist (ropinirole) was associated with a reduced risk of dyskinesia compared to traditional levodopa-treatment (Rascol et al., 2000).

Non-dopaminergic treatment strategies of levodopa-induced dyskinesia include N-methyl-D-aspartate (NMDA) receptor antagonists, opioid receptor antagonists, α2 adrenergic receptor antagonists, cannabinoid receptor agonists or antagonists and serotonin enhancing agents (Brotchie, 1998). Moreover, surgical intervention
such as pallidotomy or deep brain stimulation of globus pallidus interna (GPI) has been used to decrease levodopa-induced dyskinesia (Limousin-Dowsey et al., 1999; Parkin et al., 2002). Stimulation of the subthalamic nucleus (STN) might indirectly reduce levodopa-induced dyskinesia by lowering the effective dose of levodopa (Limousin-Dowsey et al., 1999).

Compounds with stabilizing properties on dopamine neurotransmission or partial dopamine agonists have been suggested to be useful in the treatment of PD (Jenner, 2002) and might induce less dyskinetic movements.

1.1.1 Animal models of parkinsonism

1.1.1.1 MPTP-induced parkinsonism

In the late 1970s and early 1980s several cases of severe parkinsonism in younger patients were reported in California (Davis et al., 1979; Langston et al., 1983). The patients had injected a meperidine analog with heroin-like effects that was contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin that destroys the dopaminergic neurons of the SNc. MPTP was soon shown to be toxic both to primates (Burns et al., 1983; Langston et al., 1984a; Jenner et al., 1987) and mice (Hallman et al., 1984), observations that led to the development of commonly used primate and mouse models of PD.

The neurotoxic effects of MPTP seem to be generated by the 1-methyl-4-phenylpyridinium ion (MPP+) an active metabolite of MPTP (Langston et al., 1984b). MPTP readily passes the blood-brain barrier both after systemic injection and inhalation (Langston and Ballard, 1983) and is metabolized by monoaminooxidase type B (MAO-B) (Salach et al., 1984) to 1-methyl-4-phenyl-2,3-dihydropyridine (MPDP) that is readily auto oxidized to MPP+ (Castagnoli et al., 1985). Accumulation of MPP+ in dopaminergic neurons is achieved by the catecholamine uptake system (Javitch et al., 1985). In the dopamine neuron, MPP+ has been shown to interfere with complex I of the mitochondrial electron transport chain, leading to oxidative stress (Nicklas et al., 1985; Tipton and Singer, 1993; Betarbet et al., 2002).

The MPTP-induced parkinsonism in primates resembles the symptoms seen in idiopathic PD with rigidity, akinesia, bradykinesia and postural tremor in some cases, that could be reversed by levodopa (Jenner et al., 1984; Jenner et al., 1987). The animals also show motor complications seen in humans with PD such as levodopa-induced dyskinesias and dystonia (Clarke et al., 1987; Crossman, 1987; Boyce et al., 1990; Pearce et al., 1995).

Most often MPTP is given as intramuscular or intraperitoneal injections. However, infusion into the carotid artery on either side might be used to generate a hemiparkinsonian PD model (Bankiewicz et al., 1986). Moreover, several groups
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have treated primates with low doses of MPTP during longer time periods to better replicate the slow neurodegenerative process of PD (Albanese et al., 1993; Bezard et al., 1997).

Interestingly, the classical neuroleptic drug haloperidol has a similar structure to MPTP and has been shown to inhibit complex I of rat brain mitochondria (Burkhardt et al., 1993). Moreover, chronic systemic administration of another inhibitor of complex I of the electron transport chain, the naturally occurring pesticide rotenone, has been used as a new model for PD in rats (Betarbet et al., 2000). This emphasizes the involvement of normal mitochondrial function as a defense against toxins and suggests the involvement of environmental factors in the development of PD.

1.1.1.2 6-OHDA-induced parkinsonism

The neurotoxin 6-hydroxydopamine (6-OHDA) induces degeneration of nigrostriatal dopamine neurons after intracerebral injection into the substantia nigra (Ungerstedt, 1968). 6-OHDA has been widely used to induce lesions of the nigrostriatal dopamine system as an animal model for PD both in rats and primates.

The molecule is transported into catecholamine neurons by a specific catecholamine transporter and induces degeneration of both dopamine and noradrenaline neurons. The neurotoxicity of the compound is caused by its potent inhibitory properties on complex I and IV of the electron transport chain in the mitochondria and by formation of free radicals (Glinka et al., 1997). In order to prevent any 6-OHDA uptake into noradrenergic neurons and to maximize the dopamine depletion by 6-OHDA, animals can be pre-treated with the noradrenaline uptake inhibitor desipramine. As systemically administered 6-OHDA cannot pass the blood-brain barrier the toxin must be stereotactically applied by injection into the brain. Further selectivity for the nigrostriatal dopaminergic system might be accomplished depending on the site of injection of the toxin in the nigrostriatal pathway (Perese et al., 1989; Deumens et al., 2002). 6-OHDA-induced lesions of the medial forebrain bundle (MFB) cause a more extensive dopamine depletion than injections into the SNC or sub-regions of the caudate-putamen complex (Deumens et al., 2002). Moreover, the extent of the lesion is dose-dependent (Przedborski et al., 1995).

6-OHDA-induced lesions might be done unilaterally or bilaterally. Unilateral 6-OHDA lesions of the nigrostriatal pathway result in spontaneous ipsilateral rotation in primates as well as changes in the performance of behavioral tasks and reduced spontaneous motor activity (Annett et al., 1992). A tendency towards rotational behavior to the lesioned side is also seen in unilaterally 6-OHDA-lesioned rats indicating an imbalance in dopamine transmission in the striatum of the two hemispheres (Ungerstedt and Arbuthnott, 1970).
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Furthermore, administration of a dopamine receptor agonist to unilaterally dopamine-depleted animals results in contralateral rotations both in rats (Ungerstedt, 1971b) and primates (Annett et al., 1992) interpreted as a postsynaptic supersensitivity on the lesioned side caused by the denervation (Ungerstedt, 1971b). The rotational response seen after for instance apomorphine administration might be used as a measurement of the degree of the lesion as a strong correlation between the numbers of apomorphine-induced rotations in the 6-OHDA model of parkinsonism and the number of remaining tyrosine hydroxylase immunoreactive cells in the SNc has been reported (Carman et al., 1991). On the other hand, drugs leading to an increased dopamine release cause ipsilateral rotations in unilaterally 6-OHDA-lesioned animals (Ungerstedt and Arbuthnott, 1970; Annett et al., 1992).

Moreover, repeated treatment with dopamine receptor agonists to unilaterally 6-OHDA-lesioned animals results in increased contralateral turning behavior. This sensitization of rotational response is regarded as the equivalent of levodopa-induced dyskinesias in PD by some investigators (Henry et al., 1998). Recently, a new rat model of levodopa-induced dyskinesia after unilateral 6-OHDA lesions has been characterized where also abnormal involuntary movements and spontaneous forelimb use are rated (Lundblad et al., 2002; Winkler et al., 2002).

In contrast to the unilateral 6-OHDA model, bilateral injections of the toxin into the MFB might be poorly tolerated in rats as manifested by the development of aphagia (deficit in swallowing) and adipsia (deficit in drinking) (Ungerstedt, 1971a). However, the lesions might be better tolerated if injected into other parts of the nigrostriatal dopaminergic pathway. On the other hand, bilateral 6-OHDA lesions of the nigrostriatal dopamine pathway do not cause the same problems in primates where it has proven to be a useful model of PD (Mitchell et al., 1995). The animals show a stable parkinsonian syndrome with akinesia, bradykinesia and rigidity that is reversed by levodopa treatment (Mitchell et al., 1995). The advantage of the bilateral 6-OHDA primate model over the unilateral model is that more complex motor behavior such as levodopa-induced dyskinesias can be studied.

1.2 Tardive dyskinesia

The introduction of neuroleptics in the early 1950’s led to improvements in the treatment of schizophrenia but shortly after their introduction a variety of extrapyramidal side effects was reported. One of these was named tardive dyskinesia (TD) (Faurbye et al., 1964) and is seen after long-term treatment with antipsychotics. The most serious aspect of TD is that it may persist for months or years after drug withdrawal and may even become irreversible (Casey, 1985).

TD is characterized by abnormal, involuntary movements that might be choreiform, athetoid, ballistic or myoclonic in nature. These movements most commonly involve the face, mouth and tongue (orofacial type) and are manifested for instance...
as tongue protrusions, chewing and grimacing. The dyskinesias may also be localized to the upper and lower limbs, the trunk and the neck (Kulkarni and Naidu, 2001).

The prevalence of TD varies between 0.5% and 62% in different studies where the large deviation might be attributed to differences in age, length of neuroleptic medication and methods of recording these adverse events (Llorca et al., 2002). Increased risk to develop TD is seen in elderly patients (Woerner et al., 1998) and this is the strongest known risk factor for the development of TD. Other risk factors include female gender, diabetes, mood disorders, prolonged exposure to neuroleptics, a history of acute extrapyramidal side effects, structural brain pathology and severe mental illness but the importance of these are not clear (see Cavallaro and Smeraldi, 1995 and Llorca et al., 2002 for reviews). Genetic factors have also been suggested to be involved in the susceptibility to TD development (Waddington and Youssef, 1988; Tamminga et al., 1990; Rosengarten et al., 1994) and recently TD was proposed to be associated with dopamine D3-receptor polymorphism (Lerer et al., 2002).

Some of the atypical antipsychotics seem to induce less TD compared to the classical antipsychotics even among susceptible patients (Kane, 2001; Friedman, 2003) but are associated with other disadvantages such as agranulocytosis with clozapine treatment, weight gain and other manifestations of a metabolic syndrome (Tandon and Jibson, 2003). Despite the improvements with the new generation of drugs, TD remains an important clinical problem (Llorca et al., 2002). Extrapyramidal side effects are associated with poor compliance in schizophrenia (Buchanan, 1992), which may lead to relapse. Moreover, the nature of these extrapyramidal side effects might increase the social stigma of the disease and also mimic characteristics of the underlying illness and complicate the differential diagnosis.

1.2.1 Animal models of tardive dyskinesia

One problem in the drug development process is the lack of good and economical animal models for screening new antipsychotics for their propensity to induce TD. Moreover, a good animal model of TD can give important insights into the neural mechanisms behind this syndrome. Ultimately, a homologous model of the syndrome is desirable. Such a model should show features such as late onset, symptoms without agonist provocation, interindividual vulnerability and a potentially persistent and irreversible time course (Casey, 2000).

Long-term treatment with neuroleptics induces a gradual increase in spontaneous vacuous chewing movements (VCMs) in rats, which may persist for months after drug discontinuation. This VCM model for TD (Waddington et al., 1983; Gunne et al., 1986) is considered an analogous model since it mimics some but not all of the clinical features seen in human TD (Casey, 2000). It has been criticized and sug-
gusted to be a model of acute dystonia (AD) rather than TD (Rupniak et al., 1983),
or even parkinsonian oral tremor (Salamone et al., 1986), i.e. reflecting only acute
neuroleptic-induced syndromes. However, the antipsychotic-induced VCMs are in
the same frequency range as the orofacial TD symptoms in humans (See and Ell-
son, 1990). Moreover, as in TD a subgroup of rats is more susceptible (Hashimoto et
al., 1998) and similar responses in VCMs and TD symptoms are seen after chronic
treatment with atypical neuroleptics (Gunne et al., 1986; Turrone et al., 2002).

The development of a homologous model of TD in *Cebus apella* primates (Gunne
and Barany, 1976) offered further insight into the mechanisms underlying the dis-
order. Moreover, a TD syndrome has been reported in other primate species includ-
ing *Macaca mulatta* (Bedard et al., 1982) and *Macaca speciosa* (Domino, 1985).
However, the preceding chronic neuroleptic treatment, before symptoms became
evident, was generally long with those species with a slowly increasing dividend of
TD responders. For instance, in *Cebus apella* 56% developed the syndrome after 4
years of chronic treatment with haloperidol (Gunne et al., 1988). The mean time
lag from the start of treatment to the appearance of persistent dyskinesia, in the frac-
tion of Cebus primates that developed the syndrome, was 2.1 years (range between
5 months and 4 years) (Gunne et al., 1988; Johansson et al., 1990). This makes the
*Cebus apella* model valuable for studies of TD-related brain mechanisms, but too
time-consuming and expensive for the screening of new antipsychotics.

1.2.2 Hypotheses for tardive dyskinesia

Several hypotheses on the pathophysiology of TD have been suggested and the
major outlines of some of these are briefly summarized in this thesis.

The dopamine supersensitivity hypothesis of TD (Klawans, 1973) is based on the
ability of classical neuroleptics to block dopamine D2-receptors with resulting post-
treatment supersensitivity. However, supersensitivity of receptors occurs rapidly
after the start of treatment and is perceptible even after a single dose (Asper et al.,
1973) whereas TD has a delayed onset and may persist for months after drug with-
drawal and even become irreversible.

Another hypothesis on the pathophysiology of TD is the γ-aminobutyric acid
(GABA) hypothesis, which is based on the findings of reduced glutamic acid decar-
boxylase (GAD) activity and GABA levels in the substantia nigra, STN and globus
pallidus of animals with neuroleptic-induced dyskinesia (Gunne et al., 1984).
These results might indicate a neuroleptic-induced degeneration of the striatonigral
and/or striatopallidal GABAergic pathways.

In the excitotoxicity hypothesis for TD, the glutamate neurotransmitter is pos-
tulated to cause cell damage and degeneration through excessive stimulation of
postsynaptic NMDA receptors (Gunne and Andren, 1993). It has also been found
that chronic neuroleptic treatment increases glutamate release in the striatum (See and Lynch, 1995). Moreover, drugs that block excitotoxicity have been shown to inhibit the development of persistent VCMs (Andreassen et al., 1996; Andreassen and Jorgensen, 2000).

Finally, oxidative stress has been proposed to be involved in the underlying neural mechanism behind dyskinesia (Lohr et al., 2003), leading to a free radical hypothesis of TD.

### 1.3 Comparing neuroleptic-induced and levodopa-induced dyskinesia

Although neuroleptics and levodopa show opposing effects on dopamine neurotransmission there are certain similarities between the involuntary movements that they might induce. First, the abnormal movements seen in both syndromes are of the same type, although the most common form of levodopa-induced dyskinesia is choreic and the most frequent form of neuroleptic-induced dyskinesia is stereotypic. Secondly, abnormal movements may coexist in both conditions at the same time or at different time points. Moreover, both syndromes are heterogeneous and cannot be explained by a single pathophysiological mechanism and they worsen with stress and improve with relaxation (Rascol and Fabre, 2001).

Further support for a link between neuroleptic- and levodopa-induced dyskinesia is found in recent publications on the role of dopamine D2-receptor occupancy in the development of extrapyramidal side effects. A blockade of dopamine D2-receptors exceeding 80 % in the striatum and a low dissociation rate from the D2-receptor seems to be linked to the development of extrapyramidal side effects such as TD (for reviews see Strange, 2001; Turrone et al., 2002; Westerink, 2002). This indicates that the dopamine depletion in the striatum, either caused by sustained receptor blockade of neuroleptics or loss of dopaminergic innervation as in PD, is an important feature in the development of neuroleptic-induced dyskinesia as well as levodopa-induced dyskinesia in PD.

It has been hypothesized that the dopamine depletion induces dysfunctions in GABA and glutamate neurotransmission (Gunne and Andre, 1994). Changes within the glutamate system have been suggested to induce a faulty learning phenomenon in the striatum ultimately leading to dyskinesia (Calon et al., 2000). Moreover, chronic alteration of dopaminergic neurotransmission might change the expression of other genes. In fact, both long-term levodopa treatment and chronic treatment with typical neuroleptics have been shown to induce dyskinesia in animal models and increase the striatal immunoreactivity levels of the transcription factors FosB/ΔFosB (Atkins et al., 1999; Rodriguez et al., 2001; Westin et al., 2001). These transcription factors have been demonstrated to induce high levels of preproenkephalin-B (PPE-B) gene expression (Andersson et al., 1999) and ΔFosB seems to play an important role in mediating long-term adaptations in the brain (Nestler et al.,
1999). In conclusion, certain changes in neurotransmission and in gene expression are seen in both TD models and models of levodopa-induced dyskinesia.

1.4 The basal ganglia

The basal ganglia include the putamen, the caudate nucleus, globus pallidus, subthalamic nucleus and substantia nigra and make up a major center in the complex extrapyramidal motor system. Apart from their central role in motor behavior, the basal ganglia have a cognitive, emotional, associative and motivational function (Herrero et al., 2002).

Segregated circuits that originate in the cerebral cortex, pass through the basal ganglia and the thalamus and project back to the cortex. In these circuits, the striatum is the input structure and the GPi and the substantia nigra pars reticulata (SNr) function as output stations. The motor circuit (Figure 1) has great importance in movement disorders such as levodopa- and neuroleptic-induced dyskinesia. The striatum receives massive excitatory input from most areas of the cortex and dopaminergic impulses from the substantia nigra via the nigrostriatal pathway. The two output projections from the striatum are organized into one direct and one indirect pathway. The direct pathway projects from the striatum to the SNr and GPi whereas the indirect pathway projects from the striatum to the SNr and GPi via the external part of globus pallidus (GPe) and the STN (for review see Wichmann and DeLong, 1996; Obeso et al., 2000).

![Figure 1](image_url)

**Figure 1.** Simplified schematic drawing of the neuronal pathways interconnected the different subnuclei of the basal ganglia. Black arrows indicate excitatory pathways and gray arrows indicate inhibitory pathways. Dopamine activates the direct pathway by D1-receptors and inhibits the indirect pathway by D2-receptors.
The two efferent projection pathways show some different properties. First, the dopamine D1-receptor is expressed in the striatonigral pathway (direct pathway) whereas the dopamine D2-receptor is found in the striatopallidal projection (indirect pathway) (Gerfen et al., 1990; Le Moine and Bloch, 1995). Secondly, substance P and dynorphin is coexpressed with GABA in the direct pathway whereas the indirect pathway contains enkephalin (Gerfen and Young, 1988; Le Moine et al., 1990; Le Moine and Bloch, 1995). Finally, the two pathways show opposing effects on the output function of the basal ganglia. Activation of the GABAergic neurons of the direct striatonigral pathway provides an inhibitory effect on GPi/SNr neurons that leads to a disinhibition of the thalamus and thereby increased excitatory influence on the cortex. GABA is a transmitter in the striatopallidal pathway in the projections from putamen to GPe and from GPe to STN but glutamate is the neurotransmitter of neurons projecting from the STN to the GPi/SNr. Therefore, stimulation of neurons in the indirect pathway will cause an inhibition of the GPe, disinhibition of the STN, excitation of GPi/SNr and an increased inhibition of the thalamocortical neurons (for review see Wichmann and DeLong, 1996; Obeso et al., 2000).

The degeneration of the nigrostriatal dopamine system in PD seems to cause overactivity of the striatopallidal pathway that leads to inhibition of neurons in the GPe. Hence, the STN will be disinhibited leading to subthalamic overactivity and subsequently to overactivity of GPe neurons. In contrast, dyskinetic movements such as ballism, levodopa-induced dyskinesia and chorea in Huntington’s disease, are all associated with underactivity of both the GPi and the STN (Crossman, 2000).

Although the classical model of basal ganglia organization has been criticized (Parent et al., 2001), the major outlines might still be valuable in trying to understand the underlying neural mechanisms in dyskinesia.

1.5 Dopamine

The main dopamine systems in the CNS originate in the ventral tegmental area (VTA) (A10) and the substantia nigra (A9). These areas show discrete projections to mesolimbic, mesocortical and striatal regions of the brain. Moreover, a separate tuberoinfundibular pathway projects from the hypothalamic neurons to the pituitary gland (Cooper et al., 1996).

1.5.1 Dopamine receptors

Dopamine exerts its effects by activation of dopamine receptors, which are G-protein coupled receptors. The dopamine receptors are classified according to their effect on adenylyl cyclase, their sequence homology and pharmacology into two major classes, the D1-like (D1 and D5 subtypes) and D2-like (D2, D3 and D4 subtypes) (Jaber et al., 1996). The D1-family stimulates adenylyl cyclase whereas the D2-family inhibits the formation of adenylyl cyclase (Kebabian and Calne,
1979; Stoof and Kebabian, 1981) leading to enhanced and decreased formation of the second messenger cyclic adenosine 3', 5'-monophosphate (cAMP) via different G-proteins respectively (Jaber et al., 1996).

Both classes of receptors are present postsynaptically and the D2-subtype has also been found presynaptically (Stoof et al., 1982) which is probably true for the D3-subtype as well (Sokoloff et al., 1990; Meller et al., 1993; Levant, 1997). The presynaptic dopamine receptors are part of a negative feedback mechanism and might also be named autoreceptors. Stimulation of autoreceptors located on soma or dendrites causes reduced firing of dopamine neurons whereas stimulation of autoreceptors in the terminal region will inhibit dopamine synthesis and release. An interesting difference between postsynaptic and presynaptic dopamine receptors is that the autoreceptors are more sensitive to the effects of dopamine. This means that a dopamine agonist might provide mainly autoreceptor-mediated effects in low doses manifested as diminished dopamine function. On the other hand, high doses will stimulate postsynaptic dopamine receptors, leading to enhanced dopaminergic neurotransmission (Cooper et al., 1996). Substances with autoreceptor-selective properties include 3-(3-hydroxyphenyl)-N-n-propylpiperidine (3-PPP), a partial dopamine agonist that has shown some antipsychotic properties in its minus enantiomer form (Lahti et al., 1998).

The D1-receptor is the most widespread dopamine receptor and is highly expressed in the striatum as well as in limbic areas. The D5-receptor is expressed at a much lower level than the D1-receptor and shows a more restricted distribution to the hippocampus and the lateral mamillary nucleus and the parafascicular nucleus of the thalamus. The D2-receptor is mainly found in the striatum. The D3-receptor is predominately expressed in the limbic areas such as the nucleus accumbens whereas the D4-receptor is primarily found in the prefrontal cortex, medulla, hypothalamus and the amygdala. The D3- and D4-receptors have been proposed as potential targets for novel antipsychotics because of their localization to cortical and limbic areas and low abundance in the striatum. The basis of this is the proposal that the antipsychotic effects of neuroleptics are generated by blocking dopamine receptors in the mesolimbic system and the extrapyramidal side effects are due to the dopamine D2-antagonist action of the drugs in the basal ganglia (Jaber et al., 1996). However, as discussed in this thesis, the dopamine hypothesis of TD shows many drawbacks and the atypical antipsychotics might exert some of their effects through serotonergic receptors.

1.6 Opioid neuropeptides

The opium poppy (Papaver somniferum) has been cultivated for several thousand years and used for medical purposes and because of its euphoric effects. Opium is extracted from the milky juice of the plant and contains more than 20 alkaloids. In 1806 Sertürner isolated a substance from opium that he named morphine after
Morpheus, the Greek god of dreams. Other alkaloids such as codeine, papaverine and noscapine were later found in opium and led to the use of the isolated alkaloids rather than opium preparations. Morphine, the main alkaloid of opium, is still widely used for its analgesic properties.

Opiate is a term used for drugs derived from opium such as morphine, codeine and semisynthetic morphine or thebaine analogues. The term opioid is more general and refers to receptor agonists and antagonists with morphine-like activity and to endogenous or exogenous opioid peptides. The three major families of endogenous opioid peptides (endomorphins) are the enkephalins, β-endorphins and dynorphins (Reisine and Pasternak, 1996).

1.6.1 Opioid receptors

In the 1970s three research groups simultaneously reported on stereospecific opioid binding sites in the mammalian brain (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). The existence of several endogenous opioid receptors was soon proposed (Martin et al., 1976) and the three major classes of opioid receptors, mu (μ), kappa (κ) and delta (δ) were molecularly cloned in the early 1990s (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Minami et al., 1993). Human chromosomal assignment of these receptors has been achieved (Befort et al., 1994; Wang et al., 1994; Yasuda et al., 1994) and the three opioid receptor genes show high sequence homology (Wei and Loh, 2002).

All opioid receptors belong to the family of G-coupled receptors with an extracellular N-terminal region, seven transmembrane domains and an intracellular C-terminal part. These receptors might be pre- or postsynaptically located and they show differences both in their specific localization and relative abundances across brain regions (Mansour et al., 1988). Mapping of the localization of the opioid receptor subtypes using autoradiography in the rat revealed a wide distribution of μ-opioid receptors. They show a high density in caudate-putamen, neocortex, nucleus accumbens, thalamus, hippocampus, amygdala and superior and inferior colliculi and are thought to be involved in pain regulation and sensimotor integration. The δ-opioid receptors have a more restricted distribution with dense appearance in neocortex, caudate-putamen, nucleus accumbens and amygdala. The κ-opioid receptor subtype shows an intermediate distribution and is predominately found in the caudate-putamen, nucleus accumbens, amygdala and hypothalamus (Mansour et al., 1988). Hence, limbic structures and all sub-regions of the striatum have a rich distribution of opioid receptors.

1.6.2 Biosynthesis of endogenous opioid peptides

Soon after the discovery of the opioid binding sites, three classes of endogenous opioid peptides, enkephalins, β-endorphins and dynorphins, were identified as ligands of the opioid receptors (Hughes et al., 1975; Terenius and Wahlström, 1975;
Lord et al., 1977; Goldstein et al., 1979). The endogenous opioid peptides show different affinity to the different classes of opioid receptors (Table 1). Methionine-enkephalin (met-enkephalin) and leucine-enkephalin (leu-enkephalin) bind and activate δ-opioid receptors with high potency whereas β-endorphin has high affinity for both μ- and δ-opioid receptors. Dynorphin shows the highest affinity for the κ-opioid receptor but may also interact with other members of the opioid receptor family (Zhang et al., 1998). However, the receptor affinities may also depend on the concentration of the opioid peptide. The amino acid sequence Tyr-Gly-Gly-Phe appears to be essential for interaction with the opioid receptors and the C-terminal extension might determine the receptor selectivity (Schwyzer, 1986). However, this core sequence is not shared by the potent endogenous μ-opioid receptor ligandsendorphin 1 and 2 (Zadina et al., 1997).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Preferred receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met-enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Met</td>
<td>δ</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Leu</td>
<td>δ</td>
</tr>
<tr>
<td>β-endorphin</td>
<td>Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Gly-Glu</td>
<td>μ/δ</td>
</tr>
<tr>
<td>Dynorphin A</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Pro-Lys-Leu-Trp-Pro-Trp-Phe</td>
<td>κ</td>
</tr>
<tr>
<td>Dynorphin B</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr</td>
<td>κ</td>
</tr>
<tr>
<td>Endomorphin-1</td>
<td>Tyr-PRO-Trp-Phe</td>
<td>μ</td>
</tr>
<tr>
<td>Endomorphin-2</td>
<td>Tyr-Pro-Trp-Phe</td>
<td>μ</td>
</tr>
</tbody>
</table>

Table 1. Amino acid sequence of some endogenous opioid peptides

The biosynthesis of neuropeptides differs from that of the classical neurotransmitters. Generally, neuropeptides are formed from large, biologically inert protein precursors that are synthesized in the ribosomes on the endoplasmatic reticulum in the cell body of the peptidergic neuron and subsequently cleaved by various proteases to generate shorter, bioactive peptides. Promelanocortin (POMC) is the precursor molecule for β-endorphin and β-lipotrophin. Moreover, it encodes the non-opioid peptides adrenocorticotropic (ACTH) and various forms of melanocyte stimulating hormones (MSH) (Nakanishi et al., 1979). Cleavage of PPE-B will generate dynorphin A, dynorphin B and α-neoeendorphin that all begin with the leu-enkephalin sequence, as well as other opioid peptides (Figure 2) (Kakidani et al., 1982). Pre-proenkephalin-A (PPE-A) encodes multiple copies of met-enkephalin, one copy of leu-enkephalin as well as extended forms of these peptides (Noda et al., 1982).
An interesting aspect of neuropeptide processing is that the peptide might undergo enzymatic conversion to a fragment with retained or modified biological activity. For instance, dynorphins with affinity for κ-opioid receptors can be converted to the hexapeptide leu-enkephalin-arg, selective for δ-opioid receptors (Nyberg, 2002).

1.7 Involvement of opioid neuropeptides in dyskinesia

Postsynaptic modifications downstream of the nigrostriatal dopaminergic neurons have been proposed to be involved in the development of levodopa-induced dyskinesia (Calon et al., 2000). Along this line it has been suggested that increased opioid peptide transmission might underlie dyskinesia after chronic levodopa-treatment and that opioid antagonists might be useful as adjuncts to levodopa (Henry and Brotchie, 1996). As mentioned above, the opioid peptides dynorphin and enkephalin are co-transmitters in the GABAergic striatal output pathways, where dynorphin is expressed by neurons in the direct pathway and enkephalin is expressed by neurons in the indirect pathway (Gerfen and Young, 1988; Le Moine and Bloch, 1995).

There is a great body of evidence of changes in the expressions of opioid peptide precursors following dopamine depletion of the nigrostriatal dopaminergic system. Up-regulation of PPE-A mRNA, the precursor of enkephalin, but no effect or down-regulation of the mRNA expression of the dynorphin precursor, PPE-B have been detected in the striatum of MPTP or 6-OHDA-lesioned animals (Young et al., 1986; Engber et al., 1991; Gerfen et al., 1991; Herrero et al., 1995; Jolkkonen et al., 1995; Tel et al., 2002).

Furthermore, the development of levodopa-induced dyskinesia and behavioral sensitization to dopaminergic agents has been associated with a normalization or some further elevation of PPE-A mRNA expression and increase of PPE-B mRNA
expression in the dopamine depleted striatum in animal models of PD (Bordet et al., 1997; Duty and Brotchie, 1997; Cenci et al., 1998; Morissette et al., 1999). Using autoradiography, rats displaying levodopa-induced dyskinesia showed lower levels of binding to κ-opioid receptors in the striatum on the dopamine-depleted side, indicating an increased striatal synthesis of endogenous ligands (Johansson et al., 2001).

Moreover, high expression of PPE-A has been seen in the striatum of levodopa-treated patients postmortem (Nisbet et al., 1995; Calon et al., 2002) and a significant correlation between increased PPE-A mRNA expression and levodopa-induced dyskinesia was established (Calon et al., 2002). Increased transmission of endogenous opioid neuropeptides was demonstrated in striatum and thalamus of patients with levodopa-induced dyskinesia using positron emission tomography (PET) (Brooks et al., 2000).

Administration of opioid receptors antagonists has been shown to reduce dyskinesia in animal models (Newman et al., 1997; Henry et al., 2001). Moreover, repeated treatment with the long-acting dopaminergic receptor agonists lisuride or bromocriptine, known to produce less dyskinesia when administered de novo, do not cause increased PPE-A or PPE-B mRNA expression in the striatum of 6-OHDA-lesioned animals (Henry et al., 1999).

A correlation between opioid neuropeptides and other types of dyskinetic movements has been seen. Enkephalinergic neurons in the striatum were shown to be involved in the pathophysiology of VCMs (Andreassen et al., 1999). Moreover, administration of selective μ- and δ-opioid receptor antagonists into globus pallidus suppresses VCMs in rats (McCormick and Stoessl, 2002). Structural changes in dynorphinergic connections have been shown in the nucleus accumbens shell of rats showing VCMs (Meredith et al., 2000). In rats with persistent VCMs increased PPE-A and PPE-B mRNA expression was detected in the striatum (Egan et al., 1994). Also, intranigral infusion of enkephalin analogues will induce oral dyskinesia in rats (Liminga et al., 1989) and administration of the endogenous μ-opioid receptor agonist endomorphin in globus pallidus of rats has been shown to elicit orofacial dyskinesia (Mehta et al., 2001).
2 AIMS OF THE THESIS

The general aims of this thesis were to investigate the pathophysiology of levodopa-induced dyskinesia and neuroleptic-induced dyskinesia, with focus on levodopa-induced dyskinesia. In order to achieve insight into the neural mechanisms behind these disorders, behavioral and pharmacological assessments were combined with biochemical techniques and most importantly the combination of the microdialysis technique and mass spectrometry.

The specific aims of this thesis were to:
1) Find a good and economical model for tardive dyskinesia.

2) Investigate the involvement of opioid neuropeptides in levodopa-induced dyskinesia in animal models of PD.

3) Investigate the motor effects of a novel dopamine stabilizer in animal models of PD.

4) Examine the in vivo processing of dynorphin A(1-17) in an animal model of PD utilizing microdialysis and liquid chromatography micro-electrospray mass spectrometry.

5) Study the effect of different treatment regimens on the metabolism of dynorphin A(1-17) using the same technique as in 4).
3 MATERIALS AND METHODS

A summary of the methods used in the present work is listed below (Table 2). An introduction to animals (paper I-V), animal models of parkinsonism (paper II, III, IV and V), behavioral assessments (paper I-V), microdialysis (paper IV and V), electrospray mass spectrometry (ESI MS) (paper IV and V) and liquid chromatography (LC) (paper IV and V) follows thereafter. All other experimental procedures are explained in detail in the individual papers.

<table>
<thead>
<tr>
<th>Method</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass spectrometry</td>
<td>IV, V</td>
</tr>
<tr>
<td>Liquid chromatography</td>
<td>IV, V</td>
</tr>
<tr>
<td>Microdialysis</td>
<td>IV, V</td>
</tr>
<tr>
<td>6-OHDA lesioning</td>
<td>II, III, IV, V</td>
</tr>
<tr>
<td>MPTP-treatment</td>
<td>II</td>
</tr>
<tr>
<td>In situ hybridization</td>
<td>II, V</td>
</tr>
<tr>
<td>Behavioral assessments</td>
<td>I, II, III, IV, V</td>
</tr>
</tbody>
</table>

Table 2. Methods used in the present thesis

3.1 Animals

Either male Sprague-Dawley rats (paper IV and V) (B&K, Sollentuna, Sweden) weighing between 245 and 317 g at the beginning of the studies or adult in-house bred common marmosets (*Callithrix jacchus*) (paper I, II, III) of both gender were used in the experiments. The rats were maintained in a temperature (20±2°C) and humidity (50-70%)-controlled environment with a 12-hr light/dark cycle. Food (Lactamin R3) and water were available *ad libitum*. The rats were allowed to acclimate for a week before the start of experiments. The marmosets weighed between 290 to 595 g each and were housed in pairs in a temperature (27±1°C) and humidity (relative 50%)-controlled environment with a 12-hour light-dark cycle (the light was on between 6 a.m. and 6 p.m.). They received fortified milk solution, bread, monkey pellets and fresh fruit every day and had free access to water. All experiments were approved by the Ethical Committee on Animal Research at Uppsala University and followed the N.I.H. guidelines detailed in the *Guide for the Care and Use of Laboratory Animals* and the Federal Animal Welfare Act. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2 Animal models of parkinsonism

MPTP-treated or unilaterally or bilaterally 6-OHDA-lesioned animals were used in this thesis.
3.2.1 MPTP-treatment (paper II)

MPTP hydrochloride was dissolved in 0.9 % saline and injected subcutaneously (s.c.) once daily at a dose of 2.0 mg/kg/day. The neurotoxin was given for three consecutive days followed by a two-day break. Thereafter, the MPTP-treatment was repeated for two additional days.

3.2.2 6-OHDA lesion (paper II, III, IV, V)

Lesioning was performed by placing the anesthetized animals in a stereotaxic instrument. All surgery was performed under aseptic conditions. The animals were maintained at 37°C and monitored for any distress during the postoperative period. Only animals with a positive response to amphetamine (Apopetol, Sweden) (0.2 mg/kg s.c. to marmosets and 0.5 mg/kg s.c. to rats) were used in subsequent tests.

In marmosets, 6-OHDA (6-OHDA HBr with ascorbic acid, RBI, MA, USA) was dissolved in saline and infused into five sites within the right nigrostriatal bundle (unilateral lesions, paper II, III) or into both right and left hemispheres (bilateral lesions, paper III) under ketamine (80 mg/kg, Ketalar, Parke-Davis) and xylazine (4.5 mg/kg, Rompun vet, Bayer) anesthesia according to the method of Annett et al. (1992). A time period of eight weeks was allowed between the first and the second lesion in the bilaterally treated animals.

The rats (paper IV and V) were anesthetized by intraperitoneal (i.p.) injections of ketamine/xylazine (100 mg/kg, Ketalar, Parke-Davis/ xylazine 5 mg/kg, Rompun vet, Bayer) and pretreated with the noradrenaline uptake inhibitor desipramine (25 mg/kg, Sigma-Aldrich Sweden AB) and monoamine oxidase-B inhibitor pargyline (5 mg/kg, RBI, MA, USA) i.p. 30 min prior to surgery to maximize the selective dopamine depletion by 6-OHDA. A few drops of lidocaine (Xylocain, AstraZeneca, 40 mg/ml) were applied for local anesthesia when the incision was made to expose the skull. 6-OHDA (6-OHDA HBr with ascorbic acid, RBI, MA, USA) was dissolved in saline to a concentration of 5 mg/ml and intracerebrally infused into the MFB. Stereotaxic coordinates from bregma for the MFB were anterior-posterior –2.8 mm, medial-lateral + 2.0 mm and depth from dura 8.3 mm (Paxinos and Watson, 1998). 2.5 µl of the 6-OHDA solution was infused during 5 min and the probe was left in the brain for another 5 min before it was removed.

3.3 Behavioral assessments

In 6-OHDA-lesioned animals rotational behavior was assessed by visual inspection for 60 (paper II, III, IV and V) or 120 (paper V) min after injection of a given drug. Only full 360° turns were counted. The animals were always placed in a cage separate from the home cage where they were allowed to habituate before the experiment started. The animals were either videorecorded (paper III and V) or
rated by an observer present in the room (paper II, III) to whom the animals were acclimatized.

When dyskinetic movements, acute dystonia, parkinsonism, motor activity and other behaviors were scored, the animals were present in their home cage except in paper II where they were placed in a separate cage. Throughout the behavioral assessments trained observers conducted the ratings and animals were allowed to habituate to the observer before the experiment started. As different species and behaviors were studied in each paper, different behavioral scoring systems were used.

3.4 Microdialysis

Since the introduction of the microdialysis technique (Ungerstedt and Pycock, 1974; Ungerstedt, 1984) it has been widely used in CNS research. The microdialysis probe consists of a stainless steel tube, connected to a semipermeable membrane tube (Figure 3). The probe mimics the function of a blood capillary and a fluid is slowly perfused through the probe by a microsyringe pump. This perfusion solution should be as close as possible in composition to the extracellular medium of the tissue in which the probe is placed. When the microdialysis probe is placed in the brain, artificial cerebrospinal fluid (aCSF) is used as the perfusion solution. The aCSF diffuses out of the probe, equilibrates with the surrounding cerebrospinal fluid and analytes outside of the probe might diffuse over the membrane into the probe reservoir and be sampled. The driving force for the diffusion is the formation of a concentration gradient over the membrane. The perfusion fluid is sampled and used for the determination of the extracellular concentration of different transmitters (Benveniste et al., 1989). Moreover, the microdialysis probe can be used to deliver a substance into a tissue of interest.

![Figure 3](image-url)

**Figure 3.** Compounds may diffuse in both directions over the semipermeable membrane of the microdialysis probe (adapted from CMA Microdialysis AB).
The term relative recovery (R) refers to the ratio of the concentration of an analyte in the perfusion solution and the concentration of the same analyte in the medium where the probe is placed and might be calculated by the following formula:

\[ R = \frac{C_{p,f}}{C_{e,f}} \]

Where \( C_{p,f} \) is the concentration in the perfused sample and \( C_{e,f} \) is the concentration in the external solution. The recovery of an analyte through the microdialysis probe is dependent of several factors such as membrane length of the probe and metabolism rate. Low flow rate of the perfusion fluid increases the recovery (Chaurasia, 1999).

One advantage of the *in vivo* microdialysis technique is that it provides the possibility of continuous monitoring of biochemical events in specific brain structures in anesthetized or awake and freely moving animals. Moreover, minimal perturbation to the animal occurs as sampling is achieved without adding or withdrawing fluid from the tissue (Chaurasia, 1999).

One common problem in studying the processing of peptides *in vivo* is the susceptibility of peptides to degradation by proteolysis. As the cut-off of the microdialysis membrane typically is too low (in this thesis probes with a cut-off of 20 kDa were used) to allow enzymes to pass over the membrane, no further enzymatic processing will occur once the analyte has been sampled. The coupling of microdialysis to ESI MS has been shown to be a valuable tool for the study of extracellular brain metabolism of peptides and provides mass specific detection of the peptides as well as high sensitivity (Emmett et al., 1995; Andren and Caprioli, 1999).

### 3.5 ESI mass spectrometry

In mass spectrometry (MS) ions are separated according to their mass-to-charge ratio (m/z). The analyte must hence be transformed into ions and thereafter separated in the gas phase of the mass spectrometer. Since electrospray ionization (ESI) was introduced as an ionization technique in MS in 1984 (Yamashita and Fenn) it has been highly useful in the MS analysis of proteins and peptides. The advantages of ESI are that it allows the transfer of ions from the liquid phase to the gas phase, it produces multiply charged ions and it is a gentle ionization technique (for more on ESI MS see Cole, 1997).

In the present thesis, MS analysis was performed on a Mariner Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA, USA) ESI time-of-flight (ESI-TOF) instrument fitted with the microelectrospray module. The use of micro-electrospray will provide higher sensitivity than conventional ESI (Emmett and Caprioli, 1994).
3.6 Liquid chromatography

One disadvantage with the microdialysis technique is that the microdialysate often contains high levels of salts that are incompatible with some of the analytical techniques. Another disadvantage is that the technique shows low analyte recovery for some substances and low sample volumes. These two features might lead to low sensitivity in the analysis. In order to circumvent these problems, narrow nanoliter flow per min reversed-phase liquid chromatography (nanoLC) columns are used to desalt and concentrate the biological samples. In this thesis, these nanoLC columns were also used as spray needles when analyzing microdialysis samples with ESI-TOF MS to minimize the dead volumes of the system (Figure 4).

![Diagram](Image)

**Figure 4.** Nano-flow LC-electrospray set up.

The columns used in these experiments were made from 75 μm i.d. fused silica capillaries with a 30 μm i.d. tip (PicoTip, New Objective, Woburn, MA, USA) that were packed in-house with 3 cm of C18 material (10 μm particle size) (ODS-AQ 120A S-10, YMC Corp., Morris Plains, NJ, USA). A previously described packing method (Moseley et al., 1991) was modified and basically a slurry of the C18 particles and isopropanol was loaded into the capillary via a stainless-steel reservoir using the SMART® system as a pumping device. The packing procedure was monitored using a stereomicroscope.

In our experiments, an LC pump (JASCO, Tokyo, Japan) equipped with a splitter (Accurate, LC Packings, Amsterdam, The Netherlands) was used to give a final flow rate of the mobile phase through the column of 800 nL/min. The mobile phase used was 5% methanol/0.25% acetic acid. Ten microliters of the microdialysate samples were injected via a 10 μl sample loop of an injector (Rheodyne 7725i, Rohnert Park, CA, USA) onto the column for desalting and concentration. After 25 min of washing, a solution of 75% methanol/0.25% acetic acid was loaded onto the 10 μl sample loop of the injector and injected onto the column to elute the peptides isocratically. Blank control samples (aCSF) were injected between each microdialysate sample to check that carry over effects did not occur.
4 RESULTS AND DISCUSSION

4.1 The development of a novel animal model for tardive dyskinesia (paper I)

Paper I is the first demonstration of persistent TD symptoms after chronic administration of haloperidol decanoate in common marmosets. The first signs of TD appeared after 2.5-14 months of neuroleptic treatment and were generally localized in the buccolingual region and shown as tongue protrusions, masticatory movements and perioral twitchings (Table 3). Over time these symptoms aggravated and the dyskinesias were increasingly more evident as choreic movements of the extremities.

<table>
<thead>
<tr>
<th>Monkey</th>
<th>First dyskinesia symptoms (months)</th>
<th>Type of dyskinesia</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>4</td>
<td>TP, PT</td>
</tr>
<tr>
<td>33</td>
<td>12</td>
<td>TP</td>
</tr>
<tr>
<td>47</td>
<td>8</td>
<td>PT</td>
</tr>
<tr>
<td>48</td>
<td>2.5</td>
<td>TP, PT, DMA, DML</td>
</tr>
<tr>
<td>52</td>
<td>6</td>
<td>MM</td>
</tr>
<tr>
<td>53</td>
<td>14</td>
<td>MM, TP, DMA, DML</td>
</tr>
<tr>
<td>84</td>
<td>6</td>
<td>MM, PT, OT, TP, DMA, head shakings</td>
</tr>
<tr>
<td>89</td>
<td>6</td>
<td>MM, PT, OT, head shakings</td>
</tr>
<tr>
<td>107</td>
<td>5</td>
<td>MM, PT, OT, TP, DMA, DML, head shakings</td>
</tr>
<tr>
<td>109</td>
<td>dead after 5 months</td>
<td>------</td>
</tr>
<tr>
<td>115</td>
<td>6</td>
<td>OT, MM, DMA, PT, TP, head shakings</td>
</tr>
<tr>
<td>117</td>
<td>12</td>
<td>MM, DMA, DML, PT, TP, head shakings, DMA, OT</td>
</tr>
<tr>
<td>118</td>
<td>12</td>
<td>MM</td>
</tr>
</tbody>
</table>

Note. Abbreviations: TP= tongue protrusion, PT= periocular twitching, DMA= dyskinetic movements in arms, DML= dyskinetic movements in legs, MM= masticatory movements, OT= perioral twitching.

Table 3. The table shows the time point of occurrence of the initial symptoms of tardive dyskinesia and the localization of the dyskinesias in each animal during the time course of the study.

When TD symptoms had been recorded, the periodic treatment was interrupted and symptoms persisted for at least 5 months after drug discontinuation, while injection of the anticholinergic drug biperiden produced a non-significant tendency towards a few hours’ aggravation of TD signs. An injection of non-depot haloperidol produced a reduction in TD symptoms that was statistically significant with the dose of 0.12 mg/kg for the first 30 min after injection. This is in coherence with results in humans (Burnett et al., 1980; Cavallaro and Smeraldi, 1995) and Cebus apella primates (Gunne and Barany, 1976), where anticholinergics intensify and neuroleptics ameliorate the TD symptoms.
Moreover, injections of non-depot haloperidol caused a syndrome interpreted as acute dystonia (AD) in all animals. The animals showed sustained retrocollis, clonic and tonic contractions, hypermotility, repetitive turnings, intense backward movements, biting of the perch and upside down climbing. The severity of the syndrome varied with the haloperidol dose and differed in duration and intensity between the individual animals. A statistically significant change in AD score from baseline measurements was seen for 6 hours. As in humans (Lieberman et al., 1988) and *Cebus apella* primates (Gunne and Barany, 1976), injections of an anticholinergic drug reduced the AD symptoms. Thus, it was possible to separate TD and AD both by the different responses to pharmacological treatment as well as by the behavior displayed after antipsychotic administration.

This animal model of TD has several advantages compared to other animal models of the syndrome. First, the animals showed symptoms reminiscent of TD in humans with dyskinesias in the oro-facial region as well as choreic movements of the extremities in contrast to the various rodent models of the disorder, where mainly chewing movements are displayed (Schelkunov, 1967; Glassman and Glassman, 1980; Gunne et al., 1986). Secondly, it shows several features associated with the clinical aspects of TD such as late onset, interindividual vulnerability, a persistent and potentially irreversible time course and spontaneous occurrence, without provocation from another drug (Casey, 2000). However, all animals in our study did develop TD, which is not the case in humans treated with neuroleptics. Compared to the homologous *Cebus apella* model of TD, a shorter time period was required before the occurrence of persistent TD in marmosets. 92% of the animals in our study developed TD within a year of haloperidol treatment and all animals showed TD after 14 months. This makes the marmoset model less time consuming and expensive. Finally, the common marmoset is conveniently handled, housed and easily bred and it displays a high level of motor activity.

In conclusion, this animal model of TD might prove useful, both for the evaluation of the propensity of new antipsychotics to induce TD and for the study of the pathophysiology behind this potentially irreversible syndrome.

### 4.2 Pharmacological modification of levodopa- and apomorphine-induced dyskinesia (paper II and III)

Two different strategies to modify levodopa- or apomorphine-induced dyskinesia have been employed in this thesis. One strategy is based on the proposal of increased opioid neuropeptide transmission in the neural mechanism behind levodopa-induced dyskinesia and the subsequent use of opioid receptor antagonists as adjuncts to levodopa (Henry and Brotchie, 1996) (paper II). The other pharmacological modification of dyskinesia and parkinsonism involves the use of a novel partial dopamine agonist with stabilizing properties on dopamine transmission (paper III).
4.2.1 Effect of an opioid receptor antagonist on levodopa-induced dyskinesia

The aim of paper II was to examine whether the non-selective opioid antagonist naloxone could modify levodopa- or apomorphine-induced dyskinesia in common marmosets, dopamine-depleted on one or both sides. MPTP-treatment induced marked bilateral parkinsonian symptoms that were stable during the time course of the study. These symptoms were relieved by oral administration of levodopa/benserazide that also caused an increased motor activity. However, the antiparkinsonian treatment induced hyperkinesia and severe and reproducible peak-dose dyskinesia that was mainly choreic in nature (Figure 5).

![Figure 5](image-url)

*Figure 5.* Mean (± S.E.M.) motor activity (A), parkinsonism (B) and dyskinesia (C) score in MPTP-treated marmosets at baseline, after levodopa/benserazide administration or with concomitant naloxone injection. Non-parametric Mann-Whitney tests were used for statistical evaluation. *P<0.05 versus untreated, #P<0.05 versus levodopa/benserazide.

Naloxone was administered subcutaneously 30 min after the levodopa dose when peak-dose dyskinesia was evident. The injection of the opioid receptor antagonist caused a reduction of dyskinetic movements within 5-15 min that lasted for 90 min and reached statistical significance for the highest dose of naloxone (0.5 mg/kg) (Figure 5). A normalization of motor activity and maintained antiparkinsonian response were also seen after naloxone treatment. This indicates that different mechanisms underlie the antiparkinsonian action of levodopa and the development of levodopa-induced dyskinesia.
In marmosets with a unilateral 6-OHDA lesion of the nigrostriatal pathway, administration of apomorphine induced a marked contralateral rotational behavior that was significantly reduced by coadministration of naloxone. A maximal reduction in contralateral rotations by 35% was seen with the highest dose of naloxone compared to apomorphine alone.

Our results are in agreement with another animal study where administration of the unselective antagonist naltrexone as well as selective δ- and μ-opioid receptor antagonists reduced levodopa-induced dyskinesias in MPTP-treated marmosets without attenuating the antiparkinsonian effect of levodopa (Henry et al., 2001). Moreover, limited clinical data indicate an ameliorating effect on levodopa-induced dyskinesia after naloxone administration without any changes in the antiparkinsonian response of levodopa (Trabucchi et al., 1982; Sandyk and Snider, 1986). Moreover, naloxone has been shown to reduce hyperlocomotion in the 6-OHDA rodent model (Carey, 1991) as well as oral movements in animal models of TD (Pollock and Kornetsky, 1991; Stoessl et al., 1993). However, varying results with naloxone have been obtained in patients with TD (Blum et al., 1984; Blum et al., 1987; Lindenmayer et al., 1988).

It is concluded that naloxone can reduce levodopa-induced dyskinesia, while maintaining the antiparkinsonian effect of levodopa. Hence, the endogenous opioid system might be involved in the development of levodopa-induced dyskinesia.

4.2.2 Motor effects of GMCI111 in two animal models of Parkinson’s disease

The new drug 2-amino-6-(N,N-di-n-propylamino)thiazol[4,5-f]indan (GMCI111) has shown free radical scavenging properties in vitro (van Vliet et al., 2000b). It has an interesting pharmacological profile with high affinity to dopamine D3-receptors and it displays partial agonism at dopamine D2-sites and antagonism at D3-receptors (van Vliet et al., 2000a; van Vliet et al., 2000b). As oxidative stress leading to formation of free radicals has been proposed to be involved in the pathophysiology of PD (Jenner and Olanow, 1996) a molecule that combines dopaminergic agonistic and free radical scavenging properties might reduce the symptoms of the disease and also prevent further neurodegeneration. Partial dopamine D2-agonists such as aripiprazole have been suggested to be useful antipsychotic drugs with a potency to ameliorate schizophrenic symptoms and to reduce extrapyramidal side effects (Inoue and Nakata, 2001; Burris et al., 2002). Depending on their intrinsic activity and affinity for the receptors, partial agonists might also be useful in the treatment of PD where they might function both as dopamine agonists and antagonists and hence normalize both elevated and reduced dopaminergic neurotransmission. Drugs with these properties might have a beneficial effect on levodopa-induced dyskinesia but still show antiparkinsonian effects.
Rebecka Klintenberg

In order to evaluate its effects on motor behavior GMC1111 was given to common marmosets with either unilateral or bilateral 6-OHDA lesions. In unilaterally dopamine-depleted animals GMC1111 did not induce any rotational behavior after oral or subcutaneous administration. However, given with apomorphine, oral administration of GMC1111 abolished apomorphine-induced rotations in the two highest doses, indicating a good oral bioavailability (Table 4).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Contralateral rotations</th>
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<tr>
<td>Apomorphine 0.2 mg/kg</td>
<td>3.12 ± 3.56</td>
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<tr>
<td>GMC1111 0.3 mg/kg + saline</td>
<td>0.01 ± 0.03*</td>
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<tr>
<td>GMC1111 1.0 mg/kg + saline</td>
<td>0.06 ± 0.16*</td>
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<tr>
<td>GMC1111 3.0 mg/kg + saline</td>
<td>0.00 ± 0.00*</td>
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<tr>
<td>GMC1111 0.3 mg/kg + apomorphine 0.2 mg/kg</td>
<td>2.16 ± 2.72</td>
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<tr>
<td>GMC1111 1.0 mg/kg + apomorphine 0.2 mg/kg</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>GMC1111 3.0 mg/kg + apomorphine 0.2 mg/kg</td>
<td>0.01 ± 0.03*</td>
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</table>

Table 4. Mean number ± S.D. (n=6) of contralateral rotations per min during 60 min after peroral administration of GMC1111 and concomitant s.c. injection of apomorphine or saline. One-way analysis of variance (ANOVA) followed by paired t-test was used for statistical evaluation. * means P<0.01 versus apomorphine.

Subcutaneous administration of GMC1111 to unilateral 6-OHDA-lesioned marmosets nearly abolished the contralateral rotational behavior in animals with a low rotational response to apomorphine, but the drug had no effect in animals with a high level of rotations after apomorphine treatment (Figure 6).

The response to apomorphine did not change over the time period studied, indicating that the changes seen after GMC1111 treatment were not due to behavioral sensitization to apomorphine after the start of the experiment. The results with GMC1111 in unilaterally lesioned animals show that the drug may act as a dopamine receptor antagonist under these conditions probably attributed to the dopamine D2-receptor partial agonist features of the drug.

In bilaterally 6-OHDA-lesioned animals, peak-dose dyskinesias were seen after levodopa administration. A low dose of GMC1111 by itself reduced the parkinsonian symptoms to approximately the same degree as levodopa/benserazide (Figure 7), indicating a dopamine agonist effect of the drug. A trend towards a dose-
response related decrease in levodopa-induced dyskinesia with increasing dose of GMC1111 given with levodopa was seen, but this failed to reach statistical significance (Figure 8).

**Figure 6.** Graph representing data on rotational behavior induced by apomorphine at baseline or after concomitant subcutaneous administration of GMC1111 at concentrations of 0.1, 0.3, 1.0 and 3.0 mg/kg in unilaterally 6-OHDA-lesioned marmosets. The low rotating group of animals (- - -) was also given GMC1111 at a concentration of 0.03 mg/kg whereas the high rotating group of animals (--- ---) was not. Data shown as individual data points (n=6) during the 60 min observation period immediately after drug administration. One-way analysis of variance (ANOVA) followed by paired t-test was used for statistical evaluation. ***P<0.001 versus apomorphine. # P<0.05. ### P<0.001 versus saline.

**Figure 7.** Mean parkinsonism score (± S.E.M.) in bilaterally 6-OHDA-lesioned marmosets at baseline and 30 min after concomitant administration of GMC1111 and saline. Kruskal-Wallis test followed by Mann-Whitney test was used for statistical evaluation. * P<0.05. **P<0.01 versus untreated. # P<0.05 versus levodopa-treated.
The results obtained in this study show that GMC1111 has a good oral bioavailability and may show both dopamine receptor agonist and antagonist actions depending on the condition studied. This is in agreement with what is expected from a partial agonist at dopamine receptors where it acts as agonist or antagonist depending on the level of endogenous receptor activation (Hoyer and Boddeke, 1993). However, these studies were performed using the racemate of GMC1111 and the enantiomers have showed different intrinsic activities and affinities for dopamine receptors. In fact, the two enantiomers might function as an atypical antipsychotic and antiparkinsonian agent respectively and need further evaluation.

GMC1111 seems to have some features in common with another compound, (S)-(−)-3-methylsulfonylphenyl-1-propylpiperidine ((−)-OSU6162) that has shown presynaptic stabilizing properties on dopaminergic function. Like GMC1111, (−)-OSU6162 was also found to attenuate levodopa-induced dyskinesias in MPTP-treated marmosets (Ekesbo et al., 1997) and cynomolgus monkeys (Hadj Tahar et al., 2001) without a return of parkinsonism. Moreover, (−)-OSU6162 increased the duration of the on state of levodopa in the latter study.

In conclusion, the enantiomers of GMC1111 need further investigation but the drug seems to be capable of normalizing both elevated and reduced dopaminergic neurotransmission that might be useful both in the treatment of Parkinson’s disease and in antipsychotic medication.
4.3 *In vivo* processing of dynorphin A(1-17) in animal models of Parkinson’s disease and levodopa-induced dyskinesia (paper IV and V)

Based on the results in paper II and the great body of publications indicating a role of opioid neuropeptides in the pathophysiology of levodopa-induced dyskinesia, we wanted to further investigate how opioid neuropeptides are metabolized *in vivo* in animal models of PD (paper IV) and in levodopa-induced dyskinesia (paper V). As the processing of opioid peptides might generate products with selectivity towards different opioid receptors than the parent peptide, it is important to study the proteolytic biotransformation of the peptide. In fact, the conversion of neuropeptides to bioactive fragments represents an important regulatory mechanism in neuromodulation (Hallberg and Nyberg, 2003). In this thesis, behavioral effects of bromocriptine, levodopa/benserazide or saline were compared to the *in vivo* processing pattern of the opioid neuropeptide and to the mRNA expression of the peptide precursors in the same animals. Paper IV is the first publication of the *in vivo* biotransformation of dynorphin A(1-17) (Dyn A) in PD and paper V is the first report of changes in opioid peptide metabolism in levodopa-induced dyskinesia.

Exogenous Dyn A was dissolved in aCSF and infused through microdialysis probes placed bilaterally in the striatum of unilaterally 6-OHDA-lesioned rats in both studies (Figure 9). One side of the striatum was thus dopamine-depleted and the other hemisphere untreated. To achieve high sensitivity and molecular specificity in the analysis of the microdialysis samples nanoLC micro-ESI MS was used. This resulted in sensitivity in the range of low femtomol to low picomol for standard solutions of Dyn A, Dyn A(1-13), Dyn A(2-13) and Dyn A(1-5). When analyzing the infusion solution, Dyn A was found as 3+, 4+, 5+ and 6+ charged ions.

![Figure 9. Anatomical localization of the microdialysis probes bilaterally within the caudate-putamen in paper IV and V (adapted from Paxinos and Watson, 1998).](image)
Altogether, 32 metabolites were formed *in vivo* from Dyn A both as N-terminal, C-terminal and internal fragments (Table 5). The mass accuracy of the *in vivo* processed fragments in paper IV varied between +39 and −37 mDa using an external calibration.

<table>
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<tr>
<th>Metabolite</th>
<th>Saline (untreated)</th>
<th>Saline (6-OHDA)</th>
<th>Bromocriptine (untreated)</th>
<th>Bromocriptine (6-OHDA)</th>
<th>Levodopa (untreated)</th>
<th>Levodopa (6-OHDA)</th>
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Table 5. Metabolites of dynorphin A(1-17) found in microdialysates from the 6-OHDA-lesioned and untreated striatum in paper IV and V

The main finding of paper IV is that different metabolites are formed in the 6-OHDA-lesioned striatum compared to the untreated hemisphere (Table 5). The fragments Dyn A(1-8), 1-16-5-17, 10-17, 7-10 and 8-10 were detected in the 6-OHDA-lesioned striatum but not on the untreated side. Thus, more metabolites were formed from Dyn A in the dopamine-depleted hemisphere than in the untreated one, which might indicate altered *in vivo* processing on the PD-like side. Some of the major metabolites of Dyn A found in paper IV are shown in Figure 10.
Figure 10. Mass spectra of the major in vivo processed metabolites of Dyn A in the striatum detected with micro-ESI TOF MS. The spectra were obtained as an average taken at the apex of the peak for the extracted ions. The major fragments were (A) Dyn A(8-17) doubly-charged, (B) Dyn A(8-17) triply-charged, (C) Dyn A(8-17) 4+ charged, (D) Dyn A(1-6) doubly-charged, (E) Dyn A(2-17) triply-charged, (F) Dyn A(2-17) 4+ charged, (G) Dyn A(2-17) 5+ charged, (H) Dyn A(8-15) doubly-charged, (I) Dyn A(8-15) triply-charged, (J) Dyn A(1-11) triply-charged, (K) Dyn A(1-11) 4+ charged and (L) Dyn A(1-5) singly-charged.
At the first time point analyzed, the main metabolites formed were Dyn A(1-4), 1-5, 1-6, 2-17, 7-17, 8-17, 8-15 and 2-5. These metabolites were further processed over time into shorter fragments both from the C-terminal and N-terminal end. These results seem in agreement with the in vitro processing pattern of Dyn A where cleavage of the peptide bond between Arg⁶ and Arg⁷ to form Dyn A(1-6) and Dyn A(7-17) and cleavage of the N-terminal tyrosine to yield Dyn A(2-17) were the major pathways of biotransformation, followed by slow transformation of Dyn A(2-17) into shorter C-terminal fragments (Chou et al., 1994; Chou et al., 1996). Dynorphin converting enzyme (DCE) has earlier been shown to be specific for the conversion of Dyn A into leu-enk, leu-enk-Arg⁶ and leu-enk-Arg⁶-Arg⁷ (Silberting et al., 1992) but the enzymes involved in the biotransformation of Dyn A were not investigated in this thesis.

In paper V, levodopa administration twice daily for 10 days in unilaterally 6-OHDA-lesioned rats induced contralateral rotations that increased over time (Figure 11). Different metabolites were found in the microdialysate after saline, levodopa or bromocriptine treatment (Table 5). The levodopa-treated group of animals showed the greatest sensitization in behavioral response and the lowest levels of processed fragments expressed as percentage of Dyn A whereas higher relative concentrations were detected in animals administered saline or bromocriptine both in the dopamine-depleted and untreated striatum.

![Figure 11](image)

**Figure 11.** Graph showing mean number of contralateral rotations (± S.E.M.) during 120 min after levodopa/benserazide (filled bars), bromocriptine (hatched bars) or saline (white bars) administration after 1, 5 or 9 days of treatment.

In order to further evaluate the proteolytic processing of Dyn A extensive studies using specific enzyme inhibitors are needed both in normal animals as well as in animal models of PD and levodopa-induced dyskinesia. In conclusion, N-terminal, C-terminal and internal fragments seemed to be formed from Dyn A in the
4.4 Effect of levodopa-treatment on PPE-A and PPE-B mRNA expression (paper II and V)

In papers II and V, the effects of repeated levodopa administration on the dynorphinergic or enkephalinergic systems as measured by PPE-A and PPE-B mRNA expression were examined in sub-regions of the striatum of unilaterally 6-OHDA-lesioned rats and common marmosets. Our studies show that depletion of the nigrostriatal pathway in marmosets is associated with increased expression of PPE-A mRNA in the putamen and nucleus caudatus in the 6-OHDA-lesioned hemisphere whereas lower levels of PPE-B mRNA were found in all examined sub-regions of the dopamine-depleted hemisphere. The levodopa-treatment further increased PPE-A mRNA expression and restored the PPE-B mRNA to normal levels in the 6-OHDA-lesioned hemisphere of common marmosets (Figure 12).

**Figure 12.** Histograms showing the quantification of the expression of PPE-A mRNA (upper) and PPE-B mRNA (lower) in the intact and 6-OHDA-lesioned hemispheres of marmosets. The results are given as the mean (± S.E.M.). # P<0.05 versus intact saline-treated hemisphere, * P<0.05 versus dopamine-depleted saline-treated hemisphere. One-way analysis of variance (ANOVA) was followed by Newman-Keuls test for pairwise comparisons. Acc-nucleus accumbens; Put-putamen; Caud-nucleus caudatus.
In rats, the expression of PPE-A mRNA was elevated in caudate-putamen and nucleus accumbens in the dopamine-depleted hemisphere after the 6-OHDA lesion. No changes were seen in the expression of PPE-B mRNA after dopamine depletion in these animals. The levodopa treatment increased the mRNA levels of both PPE-A and PPE-B in caudate putamen and nucleus accumbens in the dopamine-depleted hemisphere.

Our results from rats and common marmosets on the expression of PPE-A and PPE-B mRNA are in agreement with other studies (Young et al., 1986; Engber et al., 1991; Gerfen et al., 1991; Herrero et al., 1995; Jolkkonen et al., 1995; Bordet et al., 1997; Duty and Brotchie, 1997; Cenci et al., 1998; Morissette et al., 1999; Tel et al., 2002) and indicate an increased expression of opioid neuropeptides in the development of levodopa-induced dyskinesia.

4.5 General discussion

The results from paper II, IV and V presented in this thesis suggest that the opioid neuropeptide system is being affected in parkinsonism and in levodopa-induced dyskinesia, which is in agreement with several other studies. How might increased expression of opioid peptides influence levodopa-induced dyskinesias? There seems to be an association between these dyskinesias and a reduced firing rate of neurons in the output structures of the basal ganglia (GPi and SNr) as well as in the STN (Crossman, 2000). Increased transmission of enkephalin could contribute to the development of dyskinesia by reducing the release of GABA in the GPi (Manef et al., 1994). This could disinhibit the GPi, leading to an inhibition of the STN and hence to reduced activity in GPi/SNr. Increased dynorphin transmission could contribute to the underactivity of GPi/SNr by decreasing the release of glutamate from STN terminals in the output regions of the basal ganglia (Manef et al., 1995).

However, it is not clear how different endogenous opioid ligands and receptors are involved. Conflicting data have been obtained concerning the effects of selective opioid receptor antagonists on dyskinesia. Using 6-OHDA-lesioned rats, intranigral (Newman et al., 1997) or systemic administration (Henry and Brotchie, 1996) of a κ-opioid receptor antagonist has been shown to reduce levodopa- or apomorphine-induced behavioral sensitization. However, Henry and colleagues did not establish any antidyskinetic effects with a κ-opioid receptor antagonist in marmosets (Henry et al., 2001). Using autoradiography in a rat model of levodopa-induced dyskinesia, the most clear-cut differences between dyskinetic and non-dyskinetic rats were seen at κ-opioid receptor sites in the striatum and in the substantia nigra on the dopamine-depleted side (Johansson et al., 2001). Moreover, a selective μ-opioid receptor antagonist did not show any antidyskinetic effects in 6-OHDA-lesioned rats (Henry and Brotchie, 1996) whereas both μ- and δ-opioid receptor antagonists reduced levodopa-induced dyskinesias in MPTP-treated marmosets (Henry et al.,
2001). The different results might reflect species differences between primates and rodents for instance in the *in vivo* processing of endogenous peptides.

In study IV and V of this thesis, differences in the striatal processing pattern were seen between dyskinetic and non-dyskinetic animals. However, the importance of these findings needs to be further evaluated. It is also important to reflect on whether the alterations seen in opioid peptide transmission and metabolism in dyskinetic animals might be a result of compensatory mechanisms in the brain rather than the cause of dyskinesia. Based on the results from paper II, IV and V we hypothesize that biochemical damage to the opioid neuropeptide system might be a part of the etiology of levodopa-induced dyskinesia.

One possible way of pharmacological modification of levodopa-induced dyskinesia might be the use of partial agonists such as GMC1111, which seemed capable of normalizing both elevated and reduced dopamine neurotransmission in this thesis. This might prove useful in the treatment of both PD and psychosis. TD seen after long-term antipsychotic medication might share the same neural mechanism as levodopa-induced dyskinesia. The novel TD model presented in this thesis may be used to study the common features of the two disorders.
5 CONCLUSIONS

The main conclusions from the results presented in this thesis are:

1) Long-term treatment with haloperidol decanoate was shown to induce a persistent TD syndrome in common marmosets that could be ameliorated by nondepot haloperidol. The AD-like symptoms seen after acute haloperidol administration were reduced by an anticholinergic drug and thus responded differently to pharmacological modification. The TD-model presented here shows several similarities to the human condition and advantages over existing models of the disorder and might be a practically useful and economical model for screening new antipsychotics for their propensity to induce TD.

2) Levodopa-treatment increased PPE-A and PPE-B mRNA in the dopamine-depleted striatum of rats and common marmosets. Moreover, the unselective opioid antagonist naloxone reduced levodopa-induced dyskinesia in MPTP-treated primates and normalized the motor activity without modifying the antiparkinson effect. Moreover, naloxone reduced apomorphine-induced rotations in an animal model of PD. This suggests a possible role for the endogenous opioid system in the pathogenesis of levodopa-induced dyskinesia.

3) The new drug GMC1111 showed a good oral bioavailability and dopamine agonist and antagonist properties on motor behavior in an animal model of PD. The drug seemed to modify the dopaminergic activity in a normalizing direction where Parkinson symptoms and levodopa-induced dyskinesia were reduced. Henceforth, GMC1111 might prove valuable in the treatment of PD and may have a potential as a new antipsychotic.

4) Exogenous Dyn A was metabolized in vivo in an animal model of PD and 32 N-terminal, C-terminal and internal metabolites were found using microdialysis in combination with nanoLC ESI-TOF MS. More fragments were seen in the dopamine-depleted striatum indicating that the opioid neuropeptide system may be affected in parkinsonism. Moreover, differences in the striatal processing pattern were seen after subchronic treatment with levodopa, bromocriptine or saline. Taken together, this implies the existence of biochemical changes within the opioid neuropeptide system in levodopa-induced dyskinesias.
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“Road trippin’ with my two favorite allies
Fully loaded we got snacks and supplies
It’s time to leave this town
It’s time to steal away
Let’s go get lost
Anywhere in the U.S.A.”

-RHCP
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