The rat dyskinesia model: neurochemical and behavioural characterisation

Das Rattendyskinesie-Modell: Neurochemische und verhaltenspharmakologische Charakterisierung

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CHAPTER I

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting about 4 million people worldwide, which is expected to double by 2030 (Dorsey *et al.* 2007). The neuropathological hallmark of PD involves the loss of dopaminergic neurons projecting from the substantia nigra pars compacta (SNc) to the striatum resulting in a depletion of striatal dopamine (DA) (Ehringer and Hornykiewicz 1960). The emerging cardinal motor features in patients suffering from PD are characterised by resting tremor, bradykinesia, rigidity, postural instability, "freezing" as well as loss of postural reflexes (Fahn 2003).

The primary therapeutic treatment strategy involves the restoration of dopaminergic neurotransmission in the basal ganglia motor loops. Many dopaminergic drugs are currently available but L-3,4-dihydroxyphenylalanine (L-DOPA) continues to be the most effective pharmacological treatment strategy, which counteracts all major symptoms of PD. However, L-DOPA therapy often has to be curtailed due to the development of debilitating treatment limiting side effects such as wearing off phenomena and other motor complications such as dyskinesia. L-DOPA-induced dyskinesia is characterised by abnormal involuntary movements mainly comprising an idiosyncratic mixture of choreic and dystonic movements. Dyskinesia is developed progressively over time and 40 % of PD patients are adversely affected by dyskinesia within 4 – 6 years following initiation of L-DOPA treatment (Ahlskog and Muenter 2001). Dyskinesia was found to be associated with a substantial increase of health care costs in PD patients, rising from €11 412/year in patients with no dyskinesia to €24 990/year in those with severe dyskinesia (Pechevis *et al.* 2005). The costs were more pronounced for non-medical costs (e. g. social and carer services) rather than for medical costs. Induction of L-DOPA-induced dyskinesia is mainly associated with risk factors such as the degree of dopaminergic neurodegeneration within the basal ganglia motor loops, which is equivalent to the severity of the disease (Obeso *et al.* 2000b), the dose and beginning of L-DOPA therapy (Rascol 2000) as well as the young onset of PD (Kumar *et al.* 2005).

L-DOPA-induced dyskinesias can be classified into three different patterns (Obeso *et al.* 2000a): 1) The most common form called peak-dose or "on"-period dyskinesia

most severely occurs during the two hours following exogenously applied L-DOPA reflecting the maximum plasma and brain levels of L-DOPA, which in parallel results in a drug-induced relief in motor response (Nutt 1990). 2) Diphasic dyskinesia mainly comprises stereotypic movements of the lower extremities and tends to appear both prior to the maximum L-DOPA response as well as when the therapeutic effect of L-DOPA wears off. 3) “Off”-period dystonia consists of painful dystonic muscle cramps affecting different segments of the body such as feet, arms and face and can result in a sustained abnormal posture.

Alternative pharmacological approaches to the use of L-DOPA for the treatment of PD patients include administration of DA agonists and compounds affecting the enzymatic metabolism of DA such as monoaminoxidase (MAO)-B or catechol-O-methyltransferase (COMT) inhibitors. However, none of these drugs has surpassed the therapeutic benefit derived from the gold standard L-DOPA. Moreover, monotherapy with DA agonists can also induce dyskinesia in PD patients, albeit with a reduced incidence of motor complications (Rascol *et al.* 2000).

At present, the glutamatergic non-competitive NMDA antagonist amantadine is the only compound which is used to alleviate L-DOPA-induced dyskinesia in patients suffering from PD. However, amantadine is not registered for the treatment of L-DOPA-induced dyskinesia, this is rather an off-label use. Pharmacotherapy with amantadine is limited due to the development of central side effects including dizziness, confusion and hallucinations. There is a controversy in the literature concerning the duration of amantadine’s antidyskinetic effect. Stocchi *et al.* (2008) reported that the effect of amantadine is often transient and lost within 1 year, whereas Verhagen Metman *et al.* (1999) showed that amantadine’s effect on motor response complications is maintained for at least 1 year after treatment initiation.

Non-pharmacological approaches for the treatment of L-DOPA-induced dyskinesia involve surgical interventions such as deep brain stimulation (DBS) targeting the globus pallidus internus (GPi) and the subthalamic nucleus (STN). The STN seems to be the preferred target in PD since a relief of tremor, bradykinesia, rigidity, “off” time as well as dyskinesia is provided (Fahn 2008). A meta analysis of the outcome of STN-DBS comprising 921 PD patients revealed a decrease in Unified Parkinson’s Disease Rating Scale (UPDRS) motor scores of 28 % (Kleiner-Fisman *et al.* 2006). Average reduction in L-DOPA equivalents and dyskinesia scores were observed to be 56 % and 69 %, respectively. However, alleviation of dyskinesia due to stimulation

of the STN is thought to simply result from the reduction of daily L-DOPA dosage (Follett 2004), but it is also possible that DBS of the STN provides antidyskinetic efficacy on its own (Fahn 2008). This is supported by a case report of Figueiras-Mendez *et al.* (1999), who demonstrated a substantial improvement of dyskinesia following bilateral STN stimulation without a reduction of L-DOPA dosage. Surgical procedures are generally subjected to patients suffering from intermediate or advanced PD, who respond well to L-DOPA but develop severe debilitating motor fluctuations and dyskinesia. Only a small percentage of PD patients undergo DBS since neurosurgeries are associated with a substantial risk of adverse events, are expensive and require regular follow-up adjustments of the stimulator to maintain the best outcome.

Animal models of L-DOPA-induced dyskinesia

Different animal species such as monkeys, rats and mice are used to investigate abnormal involuntary movements or dyskinesia, which to a greater or lesser extent mimic dyskinetic movements demonstrated in PD patients. Peak-dose dyskinesia is the one subtype which is closely modelled in animals, whereas the two other forms are rarer or not at all represented.

Non-human primate models show dyskinetic behaviour closely resembling that observed in humans. The most commonly used monkey model for L-DOPA-induced dyskinesia is generated by systemic administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to achieve dopaminergic neurodegeneration and subsequent treatment with L-DOPA to induce dyskinetic movements (Bedard *et al.* 1986; Clarke *et al.* 1987; Pearce *et al.* 1995). However, ethical, logistical and financial considerations limit the abundant employment of primate models in preclinical studies. Thus, it is reasonable to perform monkey studies as one of the crucial steps, i. e. target validation, as well as for candidate selection to start pre-development. Between target validation and candidate selection, it is not necessary to use the monkey model.

More accessible models to investigate L-DOPA-induced dyskinesia in early stages of the drug discovery process involve rodents, the use of which saw a significant upturn during the last decade. The unilateral 6-hydroxydopamine (6-OHDA)-lesioned rat dyskinesia model is popularly used to investigate either mechanisms underlying the induction and expression of dyskinetic behaviour (Buck and Ferger 2008; Buck and

Ferger 2009a; Cenci *et al.* 1998; Lundblad *et al.* 2002) or to assess potential drug targets (Buck and Ferger 2009b; Dekundy *et al.* 2007). The 6-OHDA rat dyskinesia model was first described by Dr. Angela Cenci in 1998 (Cenci *et al.* 1998), in which 6-OHDA was unilaterally injected in the medial forebrain bundle (MFB) to destroy dopaminergic neurons projecting from the SNc to the striatum. In the meantime, some groups modified stereotaxic surgery by infusing 6-OHDA into the striatum (Winkler *et al.* 2002) or SNc in order to achieve a varying degrees of lesion. Dopaminergic neurodegeneration, which is achieved following 6-OHDA administration in the MFB, is almost complete in the SNc (Winkler *et al.* 2002) ipsilateral to the injection site resulting in an almost complete DA depletion of > 99 % (Buck and Ferger 2008). According to this protocol, the lesion is highly reproducible with a low postoperative mortality rate. Two to three weeks following the lesion surgery, rats are chronically treated with L-DOPA (6 mg/kg) in combination with benserazide (12-15 mg/kg).

A previously published rat model for L-DOPA-induced dyskinesia is not based on dopaminergic degeneration but rather on the depletion of DA levels by reserpine and subsequent treatment with high doses of L-DOPA (Johnston *et al.* 2005). This treatment schedule led to an induction of vertical and horizontal movements. Antidyskinetic drugs were shown to selectively reduce vertical movements, whereas a reduction in horizontal activity was associated with worsening of the therapeutic L-DOPA effect. This model is suggested for use as a simple rodent assay to screen potential antidyskinetic compounds.

Dyskinetic mice can be generated by intracerebral injection of 6-OHDA followed by L-DOPA treatment (Lundblad *et al.* 2004; Lundblad *et al.* 2005), which gives the option to investigate transgenic mouse lines in relation to dyskinesia. However, the 6-OHDA mouse model is characterised by a high postoperative mortality ranging from 30 – 82 % depending on the injection site (Lundblad *et al.* 2004). In addition, there are some genetic mouse models of PD which were recently investigated for their ability to induce dyskinesia by chronic administration of L-DOPA. For example, regulator of G-protein signaling 9-2 (RGS9-2) knock-out mice develop abnormal involuntary movements after treatment with reserpine or haloperidol to produce supersensitivity of striatal DA D_2 receptors and subsequent administration of the DA D_2 receptor agonist quinpirole and the non selective DA D_1/D_2 receptor agonist apomorphine (Kovoor *et al.* 2005). The more recently reported model of dyskinesia in aphakia mice

is due to the selective loss of dopaminergic neurons in the midbrain as a consequence of deletions within the Pitx3 gene and chronic L-DOPA treatment (Ding *et al.* 2007).

The drug discovery process for treatment of L-DOPA-induced dyskinesia

The in vivo drug discovery process first involves proof-of-mechanism studies to investigate the target of interest. The ability of tool compounds to modulate the target in a non-disease related model is assessed. Alterations of biochemical markers including changes of neurotransmitters measured by in vivo microdialysis and/or post mortem neurochemistry support the proof-of-mechanism study. At this early stage of in vivo drug testing it is advisable to screen obvious deleterious side effects of a compound class, e.g. by a personal observation test. Further proof-of-concept (PoC) experiments reveal the efficacy of the compounds in a disease-related model. The unilateral 6-OHDA dyskinesia model is recommended to test compounds and is appropriate to uncover PoC mechanisms, since the model produces a stable and reproducible behavioural outcome. As mentioned earlier, the 6-OHDA model can be applied to rats or mice. Mice bear the advantage of requiring a lower absolute amount of compound to be synthesised by the project chemist. However, the behavioural outcome seen in mice is not as clear and easy to assess as observed in rats. In PoC studies, tool compounds can also be administered into the ventricles via intracerebral cannulae if they cannot cross the blood-brain-barrier. This issue may be resolved by local administration of the compound into the ventricles via intracerebral cannulae. Finally, most promising drug candidates are screened in an in vivo testing procedure including a vehicle group as well as a group treated with the positive control amantadine. Drug testing in the dyskinesia model can be carried out using a cross-over design in which a sufficient wash-out phase according to the pharmacokinetic parameters of the compound is included and in which the animals are matched prior to the experiment according to their dyskinesia score to exclude compound effects from the last testing day. At this phase of the drug discovery process, it is advisable to know the pharmacokinetic parameters of the compound including brain or preferably CSF levels at different time points to assess the maximum level of the compound resulting in optimisation of the experimental design to get the best results. CSF levels seem to be more accurate to predict unbound drug concentration in the brain. Thus, CSF exposure is a relevant surrogate marker for the

in vivo assessment of CNS druggability (Lin 2008), which is of relevance for the assumption that the free drug level is sufficient for interaction with its receptor target.
During the PoC study, not only behavioural effects of the compound should be monitored, but also effects on molecular markers of dyskinesia. L-DOPA-induced dyskinesia was found to be associated with molecular changes such as upregulation of striatal prodynorphin mRNA, elevated levels of phospho-extracellular signal-regulated kinase 1/2 as well as induction of FosB-like immunoreactive proteins (Cenci *et al.* 1998; Westin *et al.* 2001; Westin *et al.* 2007; Winkler *et al.* 2002). Antidyskinetic compounds have been shown to attenuate these L-DOPA-induced molecular correlates (Mela *et al.* 2007; Rylander *et al.* 2009; Valastro *et al.* 2009).
Furthermore, it is of fundamental importance to control confounding factors when drugs are tested on their ability to reduce L-DOPA-induced dyskinesia to enhance the predictive validity of the animal model. Recently, Jenner (2008b) emphasized that alterations in motor functions due to drugs causing sedation or alterations in cardiovascular parameters can restrain dyskinetic movements and lead to false positive results in the MPTP-lesioned primate model. Indeed, the same scenario can also be applied to the 6-OHDA dyskinesia model. Thus, a prerequisite for a reliable outcome of experiments investigating antidyskinetic drugs is the assessment of alterations in motor activity due to the drug treatment itself. In addition, an important issue to consider is the potential dampening effect of a compound on the beneficial effect of L-DOPA. Interaction with L-DOPA should be measured in behavioural tests such as the cylinder test, stepping test or rotational test. Results from the latter test are regarded to be treated carefully since an automatic rotational system cannot distinguish between rotational behaviour, in which the animals turn while having their limbs on the ground, and axial dyskinesia, in which the animals show twisting of the upper body. A potential interaction with the therapeutic effect of L-DOPA can be also detected using in vivo microdialysis measurements, in which alterations of extracellular L-DOPA as well as DA levels should be monitored. It is suggested that changes of extracellular DA levels in the striatum are analysed under conditions which resemble the disease state as close as possible. We have previously shown that extracellular DA levels in the 6-OHDA-lesioned striatum significantly increased following administration of L-DOPA, whereas DA levels in the intact striatum did not change (Buck and Ferger 2008). Thus, interaction with DA levels derived from exogenously applied L-DOPA can only be observed in the DA degenerated striatum.

Moreover, as one of the last steps before continuing with the monkey study, the selected drug candidate should be investigated in a chronic model to exclude effects of tachyphylaxy. Eventually, the successful drug candidate is tested on L-DOPA-induced dyskinesia in the MPTP-lesioned primate model.

Treatment of L-DOPA-induced dyskinesia

There is an unmet medical need to identify novel targets for the treatment of dyskinesia in PD. New treatment strategies aim at non-dopaminergic pathways to alleviate motor complications associated with L-DOPA therapy, whilst maintaining the anti-parkinsonian effect of L-DOPA. Currently, several approaches have been evaluated in clinical trials such as adenosine A_{2a} antagonists, 5-HT_{1A} agonists, mGluR5 antagonists, NMDA antagonists, MAO inhibitors and α_2 adrenoceptor antagonists which aim to treat symptoms of PD and L-DOPA-induced dyskinesia or at least try to avoid dyskinesia (please see figure 1 and table 1).

Currently, the NMDA antagonist amantadine is the only clinically used compound for the treatment of L-DOPA-induced dyskinesia. Amantadine has shown antidyskinetic efficacy in the MPTP-lesioned primate model (Blanchet *et al.* 1998; Hill *et al.* 2004; Visanji *et al.* 2006) as well as in the 6-OHDA-lesioned rat (Buck and Ferger 2009b; Dekundy *et al.* 2007; Lundblad *et al.* 2002) and mouse (Lundblad *et al.* 2004) dyskinesia model suggesting a high predictive validity of these models in the case of amantadine. However, treatment with amantadine is limited due to the development of psychiatric side effects. Accordingly, amantadine exacerbated psychosis-like behaviour induced by L-DOPA in the MPTP-lesioned primate model (Visanji *et al.* 2006). The NMDA antagonist Neu120 (Neurim Pharmaceuticals) is currently in a phase II clinical trial, the results of which are not yet disclosed.

Another approach targeting glutamatergic neurotransmission involves antagonism at mGluR5 receptors. At present, the mGluR5 antagonists ADX48621 (Addex) and AFQ056 (Novartis AG) are undergoing phase I and phase II clinical trials, respectively. Effects of these compounds in preclinical models have not yet been published, but tool compounds have demonstrated antidyskinetic efficacy. Indeed, the mGluR5 antagonists 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) (Mela *et al.* 2007; Rylander *et al.* 2009) as well 2-methyl-6-(phenylethynyl)-pyridine (MPEP) (Levandis *et al.* 2008) attenuated L-DOPA-induced dyskinesia in the 6-OHDA rat dyskinesia model. The antidyskinetic effect of MTEP and MPEP was paralleled by a

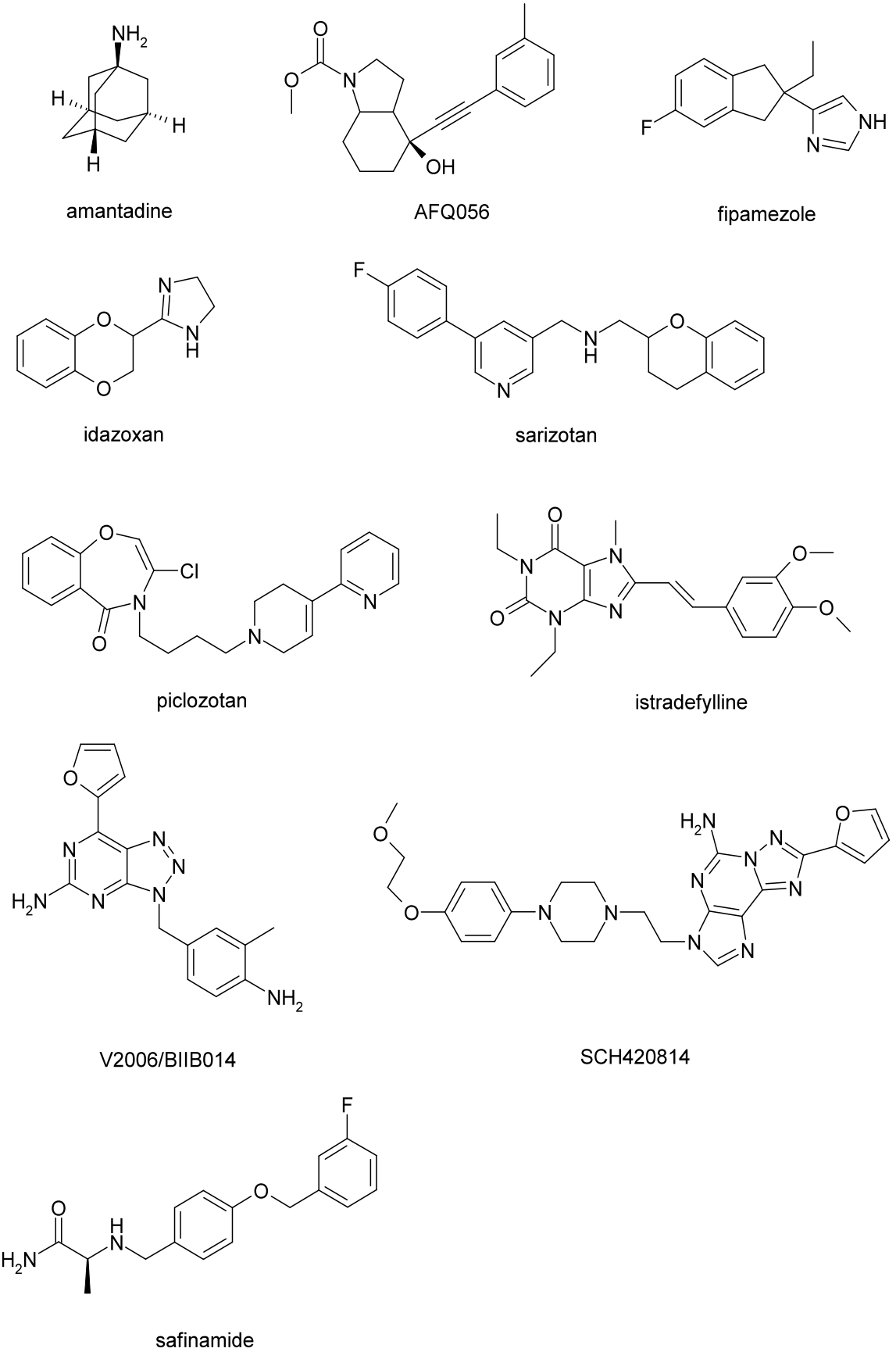


Fig. 1. Chemical structures of antidyskinetic compounds, which are/have been investigated in clinical trials. Some of the chemical structures are taken from conference reports and commercial databases but not verified e. g. by patent literature.

		Reduction of L-DOPA-induced dyskinesia			
Drug	**Mechanism**	**6-OHDA rats**	**MPTP primates**	**Clinical trial**	**Company**
Glutamatergic					
Amantadine	NMDA antagonist, Sigma antagonist	Y [37;66;155]	Y [27;112;272]	Y [68;154;208;220; 250;267;269]	
Memantine	NMDA antagonist	N/D	N/D	N [173]	
Dextromethorphan	NMDA antagonist, Sigma antagonist	Y [210]	N/D	Y [268-270]	
Riluzole	Glu release inhibitor	Y [66]	N/D	Y [174]/N [14;32]	
Talampanel	AMPA antagonist	N/D	Y [139]	N/D	
Remacemide	NMDA antagonist	N/D	N/D	N [212]	
Neu120	NMDA antagonist	N/D	N/D	Not disclosed (II)	Neurim Pharmaceuticals
AFQ056	mGluR5 antagonist	N/D	N/D	Not disclosed (II)	Novartis AG
ADX48621	mGluR5 antagonist	N/D	N/D	Not disclosed (I)	Addex
Noradrenergic					
Idazoxan	α_2 antagonist	Y [37]	Y [21;91;102;109]	Y [222]/N [164]	Pierre Fabre SA
Fipamezole	α_2 antagonist	N/D	Y [242]	Y (II)	Juvantia Pharma Ltd/ Santhera Pharmaceuticals AG
Clonidine	α_2 agonist	Y [66]	Y [97]	Y [194]	
Propranolol	β_1/β_2 antagonist	Y [37;66]	Y [97]	Y [42]	
Serotonergic					
Sarizotan	$5HT_{1A}$ agonist, D_3/D_4 antagonist	Y [166]	Y [26;98]	Y [13;95;204]/N [96] (III)	Merck KGaA
Piclozotan	$5HT_{1A}$ agonist	N/D	N/D	Y (II)	Asubio Pharmaceuticals

Table 1. Compounds for the alleviation of L-DOPA-induced dyskinesia. Comparison between the 6-OHDA rat dyskinesia model, the MPTP-lesioned primate model and clinical trials.

		Reduction of L-DOPA-induced dyskinesia			
Drug	**Mechanism**	**6-OHDA rats**	**MPTP primates**	**Clinical trial**	**Company**
Buspirone	$5HT_{1A}$ agonist, partial D_2 agonist	Y [66]	N/D	Y [29;134]	
Fluoxetine	SSRI	N [66]	N/D	Y [75]	
Paroxetine	SSRI	N/D	N/D	N [57]	
Fluvoxamine	SSRI	N/D	N [118]	N/D	
Adenosine					
Istradefylline	A_{2A} antagonist	N [158]	N [100;128]	N [106;148;254] (III)	Kyowa Hakko
V2006/BIIB014	A_{2A} antagonist	N/D	N/D	Not disclosed (II)	Biogen Idec
Preladenant	A_{2A} antagonist	N/D	N/D	Not disclosed (II)	Schering-Plough Corp
SYN115	A_{2A} antagonist	N/D	N/D	Not disclosed (II)	Synosia/Roche Holding AG
Cannabinoids					
Nabilone	CB agonist	N/D	Y [90]	Y [249]	
Rimonabant	CB_1 antagonist	N/D	Y [265]	N [175]	
Opioids					
Naloxone	Opioid antagonist	Y [155]	Y [137]/N [97;236]	N [89]	
Naltrexone	Opioid antagonist	N/D	Y [108]/N [237]	N [165;225]	
Miscellaneous					
Clozapine	Atypical neuroleptic	Y [155]	Y [101]	Y [19;20;73;74]	
Quetiapine	Atypical neuroleptic	N/D	Y [202]	Y [16]/N [130]	
Safinamide	MAO-B inhibitor, DA reuptake inhibitor, glu release inhibitor	N/D	N/D	Y (III)	Merck Serono/Newron Pharmaceuticals

blockade of upregulation of striatal prodynorphin mRNA levels (Mela *et al.* 2007) and a reduction of striatal levels of FosB/Delta FosB immunoreactivity (Levandis *et al.* 2008), respectively, both of which are strongly associated with the dyskinesiogenic action of L-DOPA.

The 5-HT_{1A} agonist sarizotan (Merck KGaA) reduced L-DOPA-induced dyskinesia in 6-OHDA-lesioned rats after local administration into the STN (Marin *et al.* 2009) as well as in MPTP-lesioned primates (Bibbiani *et al.* 2001; Gregoire *et al.* 2009). Two phase II clinical trials showed an improvement of L-DOPA-induced dyskinesia by administration of sarizotan (Bara-Jimenez *et al.* 2005; Goetz *et al.* 2007), but two large phase III trials (PADDY-1 and PADDY-2) involving more than 1000 PD patients failed to meet the primary endpoints. The reason for this discrepancy may be due to the low dose applied in the phase III study (1 mg) compared to the effective dose in the phase II trials (2 and 5 mg, respectively). Indeed, Iravani *et al.* (2006) demonstrated that the 5-HT_{1A} agonist (R)-(+)-8-OH-DPAT alleviated L-DOPA-induced chorea but only at the cost of reversal of the motor improvement of L-DOPA. Thus, there appears to be a major challenge to keep the balance between an excessive DA overflow, which prones dyskinesia, and a pronounced reduction in DA levels, which abolishes the beneficial effect of L-DOPA. Additionally, sarizotan also exhibits antagonist properties for DA receptors with the highest affinity for the DA D_4 subtype suggesting that sarizotan's dose has to be well titrated to exclude a worsening of L-DOPA's therapeutic effect via blockade of dopaminergic receptors. A 5-HT_{1A} agonist without antagonism at dopaminergic receptors is piclozotan (Asubio Pharmaceuticals), which offers agonistic properties at DA D_3 receptors in addition to being a 5-HT_{1A} agonist. Piclozotan is an injectable formulation and is currently under investigation in a phase II clinical trial. A short duration phase IIa study successfully revealed the PoC by improving both "on" time without dyskinesia as well as "off" time (press release).

The α_2 adrenoceptor antagonist idazoxan (Pierre Fabre SA) demonstrated antidyskinetic efficacy in the 6-OHDA rat dyskinesia model (Buck and Ferger 2009b) as well as in the MPTP-treated monkey (Bezard *et al.* 1999; Fox *et al.* 2001; Grondin *et al.* 2000; Henry *et al.* 1999). Additionally, the "on" time of L-DOPA was extended in the latter model. However, there is a controversy concerning the efficacy of idazoxan in clinical trials, one phase II clinical trial reported an alleviation of L-DOPA-induced dyskinesia in PD patients (Rascol *et al.* 2001), whereas another did not (Manson *et*

al. 2000). Additionally, a phase III clinical trial was initiated and terminated early due to unknown reasons. Idazoxan is currently in development for the treatment of schizophrenia. Another α_2 adrenoceptor antagonist, fipamezole (Juvantia Pharma Ltd/Santhera Pharmaceuticals AG), reduced L-DOPA-induced dyskinesia in the monkey (Savola *et al.* 2003) as well as in PD patients in a phase IIa clinical trial. Very recently, Santhera announced the positive outcome of its phase IIb trial FJORD in a press release. This study demonstrated efficacy of fipamezole in alleviation of L-DOPA-induced dyskinesia compared to placebo without clinically relevant worsening of motor disability. However, these results were achieved by analysing the US subgroup due to a strong evidence of inhomogeneity between US and Indian study populations. The original primary outcome comprising the US and Indian study population show a tendency towards reduction in dyskinesia but did not reach statistical significance.

The adenosine A_{2A} antagonist istradefylline (Kyowa Hakko) was shown to be effective in symptomatic animal models of PD as well as in clinical studies (reviewed in Jenner *et al.* (2009)). Istradefylline enhanced locomotor activity in MPTP-treated and reserpinised mice (Shiozaki *et al.* 1999), antagonised haloperidol-induced catalepsy in rats (Hauber *et al.* 2001) as well as potentiated L-DOPA- and apomorphine-induced rotational behaviour in 6-OHDA-lesioned rats (Koga *et al.* 2000) indicating an antiparkinsonian effect of istradefylline. Moreover, administration of istradefylline with L-DOPA provoked an additive relief in motor disability in 6-OHDA-lesioned rats, but dyskinetic behaviour in these rats remained unchanged by treatment with istradefylline (Lundblad *et al.* 2003). Similar results were obtained in the MPTP-lesioned primate such as a reversal of motor disability by administration of istradefylline (Grondin *et al.* 1999a; Kanda *et al.* 1998) and a potentiation of the antiparkinsonian effect of L-DOPA following cotreatment of istradefylline and L-DOPA (Grondin *et al.* 1999a; Kanda *et al.* 2000). However, L-DOPA-induced dyskinesia was not affected by administration of istradefylline indicating that this compound is effective in reduction of parkinsonian symptoms and potentiation of the therapeutic effect of L-DOPA, but has no antidyskinetic effect by itself, even though it does not induce dyskinesia by itself or worsen dyskinesia induced by L-DOPA. Some clinical trials are available investigating the antiparkinsonian properties of istradefylline. A first PoC study including 15 patients showed that istradefylline provided no antiparkinsonian benefit as a monotherapy and in combination with optimal-dose

infusion of L-DOPA (Bara-Jimenez *et al.* 2003). However, istradefylline coadministered with low-dose infusion of L-DOPA improved cardinal parkinsonian symptoms up to the antiparkinsonian response achieved by optimal-dose L-DOPA. Notably, 45% less dyskinetic movements were observed at this treatment regime. These results are in contrast to the findings from the monkey studies, in which istradefylline provided an antiparkinsonian benefit as monotherapy (Grondin *et al.* 1999a; Kanda *et al.* 1998). In agreement with the primate studies is the potentiation of L-DOPA efficacy by istradefylline resulting in an additive antiparkinsonian effect. Taken together the results from various phase II (Hauser *et al.* 2008; Lewitt *et al.* 2008; Stacy *et al.* 2008) and a large phase III clinical trial, istradefylline was shown to reduce “off” time, whereas no effect on UPDRS scores was observed. Additionally, the “on” time with dyskinesia was increased by istradefylline, but most patients showed non-troublesome dyskinesia. However, istradefylline has not been investigated in conditions that have be proven to be beneficial in primate studies such as adjunct therapy to L-DOPA (Jenner *et al.* 2009). Taken together, istradefylline has demonstrated validity in preclinical studies to predict clinical efficacy in terms of potentiation of the L-DOPA effect as well as no worsening of dyskinesia. The adenosine A_{2A} antagonists V2006/BIIB014 (Biogen Idec), preladenant (SCH420814) (Schering-Plough Corp) and SYN115 (Synosia/Roche Holding AG) are currently in phase II clinical trials, from which data are not yet disclosed.

Safinamide (Merck Serono/Newron Pharmaceuticals) is a reversible MAO-B inhibitor with additional sodium and calcium channel blocker activities being developed as an add-on treatment for patients suffering from PD. Safinamide is currently in a phase III clinical trial (SETTLE) for adjunctive use with L-DOPA. Study 016, the first phase III trial of safinamide as adjunctive therapy to L-DOPA revealed that safinamide significantly improved motor function. Safinamide met its primary endpoint by increasing daily “on” time in patients with motor fluctuations, who are in mid- to late-stage PD, without any increase in “on” time with troublesome dyskinesia. Secondary endpoints were also met including decrease in daily “off” time as well as decrease in “off” time following first morning L-DOPA dose.

Mode of action of antidyskinetic drugs

The exact mechanism of action of the gold standard amantadine is unclear but it is suggested to decrease enhanced glutamatergic transmission within the direct striatopallidal pathway of the basal ganglia motor loops resulting in reduction of dyskinesia.

Sarizotan is a representative of the compound class 5-HT_{1A} agonists primarily developed for the treatment of L-DOPA-induced dyskinesia. The mechanism of action of 5-HT_{1A} agonists seems to be due to the reduction of L-DOPA-derived DA overflow in the rat striatum (Kannari *et al.* 2001), where DA is released as 'false neurotransmitter' from serotonin (5-HT) terminals. The role of DA released from serotonergic neurons as an important presynaptic factor of L-DOPA-induced dyskinesia in the rat PD model has been discovered (Carta *et al.* 2008). In fact, the removal of 5-HT afferents by the serotonergic neurotoxin 5,7-dihydroxytryptamine resulted in an almost complete reversal of L-DOPA-induced dyskinesia (Carta *et al.* 2007). Moreover, serotonergic neurons release less of the original neurotransmitter 5-HT (5-HT) when releasing DA which causes an upregulation of the activity of 5-HT neurons favouring in turn the excessive swings of DA release. In fact, this vicious circle can be interrupted by administration of 5-HT_{1A} agonists.

Several mechanisms of action have been discussed for the antiparkinsonian effects of adenosine A_{2A} antagonists (Jenner *et al.* 2009). Adenosine acting at A_{2A} receptors stimulates neurons projecting from the striatum to the globus pallidus (GP) opposing the inhibitory effects of DA. In PD, the dopaminergic input is lost and striatopallidal neurons become overactive due to the excitatory influence of adenosine. A_{2A} antagonists are suggested to dampen the excessive activity of striatopallidal neurons by blocking stimulatory A_{2A} receptors and thus reconstitute the imbalance between indirect and direct pathway of the basal ganglia network. Another approach involves presynaptic A_{2A} receptors in the striatum and GP modulating GABAergic neurotransmission resulting in restoration of GP and STN activity. There is increasing evidence that A_{2A} receptors form heterodimer complexes with the DA D_2 receptor as well as mGluR5 receptors. Accordingly, a combination of A_{2A} antagonists and mGluR5 antagonists may have an additive antiparkinsonian benefit. The precise mechanism of dyskinesia alleviation by mGluR5 antagonists remains unknown. However, mGluR5 gene expression was found to be upregulated in the striatum of dyskinetic rats (Konradi *et al.* 2004) and an increase of mGluR5 specific binding was

shown in monkeys rendered dyskinetic by MPTP plus L-DOPA treatment (Samadi *et al.* 2008). Additionally, Mela *et al.* (2007) reported that MTEP attenuated the L-DOPA-induced increase of extracellular GABA levels in the substantia nigra pars reticulata (SNr) of dyskinetic rats measured by in vivo microdialysis. Conversely, the GABA overflow in the GP as part of the indirect pathway of the basal ganglia motor loops was altered neither by L-DOPA treatment nor by administration of MTEP. These findings indicate that the antidyskinetic action of mGluR5 antagonists may be due to a decrease of the GABA overflow in the direct pathway of the basal ganglia network.

The reversible MAO-B inhibitor safinamide is reported to have a novel dual mechanism of action based on the increase of dopaminergic neurotransmission by inhibition of MAO-B and DA uptake and reduction of glutamatergic activity by blocking glutamate release.

Conclusion

There is an unmet medical need for drugs alleviating L-DOPA-induced dyskinesia in terms of quality of life for the PD patient as well as socio-economic burden. At present, no compounds against L-DOPA-induced dyskinesia are registered. Amantadine is the only compound for the treatment of L-DOPA-induced dyskinesia, which was originally developed as virustatic agent and is now used off-label for L-DOPA-induced dyskinesia. In order to predict clinical efficacy of antidyskinetic drugs, several preclinical animal models are available, which have proven predictive validity. However, one has to critically evaluate findings from preclinical models and consider several confounding factors to exclude false positive results. Additionally, the mechanisms underlying L-DOPA-induced dyskinesia are complex and still remain unknown. Thus, drugs aiming at various targets may be conceivable to be effective against L-DOPA-induced dyskinesia.

Objective of the present thesis

The objective of my thesis is to gain insight into neurochemical and behavioural mechanisms underlying the expression of dyskinetic movements and thus characterise the rat L-DOPA dyskinesia model in depth. Various pathogenic factors, which contribute to the manifestation of dyskinetic movements, will be investigated.

Furthermore, I will explore and evaluate novel drug targets for the treatment of L-DOPA-induced dyskinesia in the rat dyskinesia model.

CHAPTER II

General methodology

Animals

The present studies were conducted in male Wistar rats (HsdCpb:WU, Harlan Winkelmann, Borchen, Germany). The animals were housed in groups of 4 per cage under a 12 h light/dark cycle (lights on 06:00 – 18:00) in temperature (23 ± 2°C) and humidity (55 ± 5%) controlled rooms with free access to food (GLP Vitamin fortified, Provimi Kliba AG, Kaiseraugst, Switzerland) and water throughout the experiment.
All in vivo studies were approved by the appropriate institutional governmental agency (Regierungspräsidium Tuebingen, Germany) and performed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International)–accredited facility in accordance with the European Convention for Animal Care and Use of Laboratory Animals.

6-OHDA lesion surgery

Rats were anaesthetised with a mixture of ketamine (70 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.) and mounted in a stereotaxic frame (David Kopf, Tujunga, CA, USA) on a flat-skull position. Anaesthesia was maintained by using 0.4 -2 % isoflurane in N_2O/O_2 (70:30). Animals were treated with a mixture of pargyline hydrochloride (25 mg/kg; i.p.) and desipramine hydrochloride (6.25 mg/kg; i.p.) 30 minutes prior to the infusion of 6-OHDA. 6-OHDA hydrobromide (3 µg/µl of free base) was dissolved in 0.02% ascorbate solution. Injections were performed at the rate of 0.3 µl/min into the left median forebrain bundle at the following coordinates relative to the bregma according to the rat brain atlas of Paxinos and Watson (1998): (1) AP: - 4.4 mm, ML: + 1.2 mm, DV: - 7.8 mm (from skull) (2.5 µl); (2) AP: - 4.0 mm, ML: + 0.7 mm, DV: - 8.0 mm (from skull) (2 µl) (Cenci *et al.* 1998).

Induction and rating of dyskinesia

Three weeks after 6-OHDA lesion rats were treated chronically with L-DOPA methyl ester, in the following called L-DOPA (6 mg/kg, p.o.), plus benserazide (15 mg/kg, p.o.) once daily for 3 x 5 days (Monday - Friday between 9.00 a.m. to 5.00 p.m., within three weeks, without treatments at weekends, administration volume 2 ml/kg).

This treatment led to an induction of stable dyskinetic movements, which were quantified using a previously described rating scale (Cenci *et al.* 1998) by an "experimentally blinded" observer not aware of the experimental group alignment every 20 minutes following administration of L-DOPA or vehicle.

The scoring of abnormal involuntary movement behaviour involves (1) axial subtype: axial motor response complications indicated by dystonic posturing or choreiform twisting of the neck and upper body, (2) limb subtype: abnormal, purposeless movement of the forelimb and digits, (3) orolingual subtype: orolingual dyskinesia indicated by empty jaw movements and contralateral tongue protrusion. For each observation period (1 min) the subtypes of these abnormal involuntary movements were rated individually on a severity scale from 0 to 4 based on their duration and severity: 0 = nonexistent; 1 = occasional < 50 % of observation time; 2 = frequent > 50% of observation time; 3 = continuous but can be interrupted; 4 = continuous, full blown and not interruptible. The sum of axial, limb and orolingual dyskinesia rating was expressed as a total dyskinesia score and used instead of the individual score if the treatments produced similar changes in the different subtype scores. Turning behaviour does not provide a specific measure of L-DOPA-induced abnormal involuntary movement (Lundblad *et al.* 2002) and was not included.

In vivo microdialysis surgery

Under ketamine/xylazine (70/6 mg/kg, i.p.) and isoflurane (0.4 – 2 % in N_2O/O_2 (70:30)) anaesthesia one or two intracerebral guide cannulae (striatum: MAB 4.9.IC; GP and SNr: MAB 4.15.IC, Microbiotech, Sweden) were implanted aiming at the left and right striata at the following coordinates relative to the bregma: striatum: AP: + 0.7 mm, ML: ± 3.0 mm, DV: - 3.0 mm (from skull); GP: AP: - 1.2 mm, ML: + 3.0 mm, DV: - 6.2 mm (from skull); SNr: AP: - 5.5 mm, ML: + 2.2 mm, DV: - 8.2 mm (from skull) according to the rat brain atlas of Paxinos and Watson (1998). Two holes were drilled for the placement of the guide cannulae, which were fixed to the skull with two stainless steel screws and methacrylic cement (Paladur, Heraeus Kulzer GmbH & Co. KG, Hanau, Germany) or dental cement (PermaCem, DMG Chemisch-Pharmazeutische Fabrik GmbH, Hamburg, Germany). Following surgery, rats were housed individually in perspex cages and allowed to recover for at least three days before performing the in vivo microdialysis procedure.

GP and SNr (MAB 4.15.IC, Microbiotech, Stockholm, Sweden) at the following coordinates relative to the bregma:

In vivo microdialysis procedure

The evening before the experiment concentric microdialysis probes (striatum: MAB 4.9.4.Cu, 4 mm membrane length; GP: MAB 4.15.2.Cu, 2 mm membrane length; SNr: MAB 4.15.1.Cu, 1 mm membrane length, Microbiotech, Sweden) were introduced and the rats were placed into a microdialysis system with a balanced arm for freely moving animals. The probes were connected through a dual channel swivel (Instech Laboratories, Inc. USA) to a pump (TSE Technical & Scientific Equipment GmbH, Bad Homburg, Germany) via FEP tubing (inner diameter 0.12 mm) and perfused with aCSF containing 147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$ and 1 mM Na_2HPO_4, pH 7.0-7.4, at a constant flow rate of 2 µl/min up to the end of the experiment. The following morning dialysis samples were collected every 20 minutes into a vial containing 10 µl of 0.1 M hydrochloric acid.

Dyskinetic behaviour was individually monitored for each rat every 20 minutes. The reported data was not corrected for the in vitro recovery, which was 12-14 % for DA and DA metabolites, 13 % for L-DOPA, 27 % for idazoxan and 8 % for PPX (for MAB 4.9.4.Cu). In vitro recovery for L-DOPA was 4 % for MAB 4.15.1.Cu and 6 % for MAB 4.15.2.Cu. Using MAB 4.9.2.Cu, the in vitro recovery was 9 % for GABA and 7 % for glutamate. To measure the in vitro recovery the probe was placed in a medium with a defined concentration of the analyte. After an equilibration period of 60 minutes three 20 minute samples were collected from the microdialysis probe effluent and analysed by high performance liquid chromatography (HPLC). The mean of the three samples was compared with a sample taken from the medium and expressed as % relative recovery of the medium. After the experiments the correct localisation of the probes was verified and only the rats with appropriate probe placement were included in the experiment.

Reverse in vivo microdialysis surgery

Two intracerebral guide cannulae (MAB 4.9.IC, Microbiotech, Sweden) were implanted as described above with minor modification of the coordinates for the striatum: AP: + 0.7 mm, ML: ± 3.0 mm, DV: - 4.0 mm (from skull). The coordinates for the GP and SNr have been used as above described.

Reverse in vivo microdialysis procedure

The reverse in vivo microdialysis procedure was similarly performed to the in vivo microdialysis procedure but with minor modifications. On the day of the experiment concentric microdialysis probes (striatum: MAB 4.9.2.PES, 2 mm membrane length, Microbiotech, Sweden) were introduced and the rats were placed into the microdialysis system. The probes were perfused with aCSF at a constant flow rate of 4 µl/min. Dyskinetic behaviour including axial, limb and orolingual dyskinesia was monitored during the whole experiment every 5 minutes. After the experiment the correct localisation of the probes was verified and only the rats with appropriate probe placement were included in the study.

CSF, brain and plasma sample collection procedure

The CSF was firstly collected via puncture of the cisterna magna followed by removal of the blood via punctuation of the heart. Blood samples were obtained using S-Monovette™ containing 1.6 mg EDTA/ml blood (Sarstedt AG & Co., Nümbrecht, Germany) and gently shaken. Subsequently, the rats were transcardially perfused with Ringer's solution for 3 minutes. Afterwards, the brain was removed, transferred into plastic tubes and weighed. Plasma was obtained by centrifugation of the blood for 10 minutes (3200 x g) at 4 °C. Plasma, brain and CSF samples were frozen at -20 °C or -80 °C prior to liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis.

Sample preparation procedure

Brain tissue was homogenised either using sonication or using Dispomix™ 25 tubes (Xiril AG, Hombrechtikon, Switzerland) in LC-MS grade water (1:7). 10 µl aliquots of CSF, brain and plasma samples were diluted with 10 µl of acetonitrile/water (80:20) and 80 µl of methanol/acetonitrile (50:50) in order to precipitate proteins. The samples were mixed and kept at -20 °C for at least 15 minutes to improve the precipitation. Subsequently, samples were centrifuged at 3200 x g for 10 minutes at 4 °C and the supernatants were analysed by LC-MS/MS.

HPLC system

The HPLC system consisted of an ASI-100T autosampler and P680 ISO isocratic pump system (Dionex, Idstein, Germany). The detector potential was set at + 650 mV using a glassy carbon electrode and an ISAAC Ag/AgCl reference electrode (Antec VT-03, Leyden, The Netherlands). Aliquots were injected by an autosampler with a cooling module set at 4°C. Data were calculated using an external five-point standard calibration by Chromeleon® chromatography data system software (Dionex, Idstein, Germany).

LC-MS/MS system

The HPLC system consisted of a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), Agilent 1200 Binary Pump, Agilent 1200 Micro Vacuum Degasser and Agilent 1200 Thermostatted Column Compartment (Agilent Technologies, Morges, Switzerland). Eluates were detected using an API 4000™ triple quadrupole mass spectrometer (MDS Sciex, Ontario, Canada) in the positive ESI mode. The ion spray voltage was set at 4500 V and the source temperature at 500°C. Data were acquired and analysed using the software Analyst® version 1.4 (MDS Sciex, Ontario, Canada).

Drugs and chemicals

All drugs were calculated as free bases. The injection volume was 1 ml/kg unless otherwise indicated L-DOPA methyl ester hydrochloride, benserazide hydrochloride, DA hydrochloride, DOPAC, HVA, 3-MT, GABA, glutamate, 6-OHDA hydrobromide, pargyline hydrochloride, desipramine hydrochloride, amantadine hydrochloride as well as HPLC and LC-MS/MS chemicals of the highest available purity were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ketamine hydrochloride (Ketavet®) was purchased from Pfizer Pharma GmbH (Berlin, Germany). Xylazine hydrochloride (Rompun®) was obtained from Bayer Vital GmbH (Leverkusen, Germany). Idazoxan hydrochloride, propranolol hydrochloride, HEAT hydrochloride and cirazoline hydrochloride were purchased from Biotrend Chemikalien GmbH (Köln, Germany). PPX dihydrochloride as well as [D_7]-PPX dihydrochloride was synthesised at Boehringer Ingelheim Pharma GmbH & Co. KG. [D_6]-GABA, [D_5]-glutamate and [D_3]-L-DOPA were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Statistical analysis

The statistical analysis was carried out using SAS version 8.2 (SAS Institute Inc., USA), S-PLUS®6.1 (Insightful Corp., USA) and GraphPad Prism version 4.00/5.01 for Windows (GraphPad software, USA). All values are expressed as mean ± SEM if not otherwise stated. $P < 0.05$ was considered as statistically significant.

CHAPTER III

Intrastriatal inhibition of aromatic amino acid decarboxylase prevents L-DOPA-induced dyskinesia

ABSTRACT

L-DOPA-induced dyskinesia consists of involuntary choreiform and dystonic movements. Here we report whether intrastriatal L-DOPA itself is able to trigger dyskinetic behaviour and which role the neurotransmitter DA and its metabolites play. Intrastriatal L-DOPA as well as DA administration at the 6-OHDA-lesioned side led to a significant appearance of dyskinetic behaviour, whereas DA metabolites were ineffective. Intrastriatal inhibition of the enzyme aromatic amino acid decarboxylase (AADC) by benserazide prevented the appearance of L-DOPA-induced dyskinetic movements at the lesioned side. Principle component analysis of DA and DA metabolite levels with dyskinesia scores after L-DOPA/benserazide (6/15 mg/kg) administration indicated a significant correlation only for DA, whereas DA metabolites did not show any significant correlation with the occurrence of dyskinetic behaviour.

We conclude that intrastriatal L-DOPA itself is not able to induce dyskinetic movements, whereas the increase of intrastriatal DA levels is instrumental for L-DOPA- and DA-induced dyskinetic behaviour.

INTRODUCTION

Initially, therapy with the gold standard L-DOPA dramatically alleviates the cardinal symptoms of PD namely bradykinesia, rigidity and tremor and improves the quality of life of patients suffering from PD (Schapira *et al.* 2006). However, within a few years, the L-DOPA therapy is commonly associated with side effects, most notably motor complications including dyskinesia, motor fluctuations and the wearing off phenomenon (Brotchie *et al.* 2005; Muller and Russ 2006). L-DOPA-induced dyskinesia involves involuntary movements such as chorea, dystonia and athetosis involving head, trunk and limbs (Schapira *et al.* 2006). L-DOPA-induced dyskinesia affects 50% of patients within 5 years and develops in almost 90 % of patients after 9 years of L-DOPA therapy (Obeso *et al.* 2000a; Rascol and Fabre 2001; Schapira *et al.* 2006; Ahlskog and Muenter 2001).

To date, no animal species develops dyskinesia during its lifetime without undergoing pharmacological manipulation. However, different species are used to evoke dyskinetic like behaviour, namely primates (Bezard *et al.* 2003; Jenner 2003) or rodents (Lundblad *et al.* 2002; Lundblad *et al.* 2005). A prerequisite for the development of L-DOPA-induced dyskinesia is a significant nigrostriatal neurodegeneration. In rodents unilateral intracerebral injection of 6-OHDA is used to generate degeneration of dopaminergic neurons projecting from the SNc to the striatum. This lesion is followed by chronic L-DOPA treatment to induce abnormal involuntary movements affecting the forelimb contralateral to the lesion, the trunk and the orofacial musculature (Lundblad *et al.* 2002).

The underlying molecular mechanisms of L-DOPA-induced dyskinesia are not resolved. Several factors such as disruption of presynaptic DA homeostasis, plasticity changes in the direct and indirect basal ganglia pathway, pathophysiological changes of the firing patterns in the basal ganglia output structures, cortical overactivation and several receptor mediated changes at dopaminergic, GABAergic and glutamatergic neurons contribute to the complex molecular and neuroanatomical dysfunction involved in L-DOPA-induced dyskinesia (Cenci 2007). Recently, Carta *et al.* (2006) reported that high levels of L-DOPA in the striatal extracellular fluid are necessary for the appearance of dyskinetic motor manifestations and that extracellular striatal L-DOPA levels paralleled the expression of dyskinetic behaviour. This finding highlights the role of the striatum and raises the question of the relevance of downstream events which arise after the metabolism of L-DOPA as well as after the metabolism of DA.

The objective of the present study is to investigate (1) whether intrastriatal L-DOPA itself triggers dyskinetic behaviour and (2) which role intrastriatal DA and its metabolites play in the manifestation of dyskinetic movements. Therefore, we administered L-DOPA in combination with benserazide via bilateral in vivo reverse microdialysis technique into the striata of dyskinetic rats. Benserazide is a peripheral inhibitor of the enzyme AADC, which converts L-DOPA to the neurotransmitter DA. If this conversion is blocked within the striatum, the effect of L-DOPA itself can be assessed. The method of intrastriatal administration via reverse in vivo microdialysis was used to evaluate the effect of DA and its metabolites on the occurrence of dyskinetic behaviour. Furthermore, we investigated the effect of systemic administration of L-DOPA/benserazide on extracellular levels of DA and its

metabolites paralleled by monitoring dyskinesia to underscore which of the neurochemical measurements correlates best with the behavioural readout.

MATERIALS AND METHODS

Animals, 6-OHDA lesion surgery, Induction and rating of dyskinesia

Please see chapter II “General methodology” page 22.

In vivo microdialysis surgery in the striatum

Please see chapter II “General methodology” page 23.

DA and DA metabolite measurement using bilateral in vivo microdialysis in the striatum

Please see chapter II “General methodology” (In vivo microdialysis surgery) page 24. Fractions 1 to 4 (0 - 80 min) were used for calculation of the basal levels which were regarded as 100 %. At the beginning of fraction 5, saline was administered (p.o.) and additional 4 fractions were sampled (80 - 160 min). At fraction 9, L-DOPA (6 mg/kg, p.o. (n = 8) or 24 mg/kg, p.o. (n = 7)) in combination with benserazide (15 mg/kg, p.o.) was administered and the sampling was continued up to fraction 23 (5 hours sampling after L-DOPA injection, 160 - 460 min). The experiment was performed using a cross-over design. Half of the rats were treated with L-DOPA 6 mg/kg or L-DOPA 24 mg/kg on the first day. On the second day of testing the treatment allocation was switched. Between the two treatment regimes a washout period of at least 2 days was included.

Post mortem tissue preparation

Dyskinetic rats were sacrificed by decapitation after anaesthesia with isoflurane (n = 7). Immediately afterwards, the left and right striata were dissected out on an ice-cooled plate, transferred separately in plastic tubes, weighed, homogenised by sonication for 10 s in 1000 µl perchloric acid (0.4 M) and centrifuged at 3200 x g for 20 minutes at 4°C. The supernatants were passed through a 0.2 µm filter (Minisart RC4, Sartorius AG, Goettingen, Germany) and subsequently frozen at - 80 °C until HPLC analysis.

HPLC analysis of microdialysis and post mortem samples

Please see chapter II “General methodology” (HPLC system) page 26. The samples were analysed for DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxy-phenylacetic acid (HVA) using HPLC combined with electrochemical detection (ECD) under isocratic conditions. Chromatographic separation was performed using a reversed-phase column (100 x 2.1 mm i.d. with pre-column 10 x 2.1 mm i.d., filled with ODS-AQ, 120 Å, 3 µm, YMC Europe GmbH, Dinslaken, Germany). The mobile phase consisted of 1.7 mM 1-octanesulfonic acid sodium salt, 1.0 mM Na_2EDTA x 2 H_20, 8.0 mM NaCl, 100.0 mM NaH_2PO_4 x 2 H_2O, adjusted to pH 3.80 with H_3PO_4, filtered through a 0.22 µm filter, mixed up with 9.3 % acetonitrile and was delivered at a flow rate of 0.4 ml/min.

Reverse in vivo microdialysis surgery

Please see chapter II “General methodology” page 24.

Intrastriatal administration of L-DOPA, L-DOPA/benserazide, DA and DA metabolites via reverse in vivo microdialysis

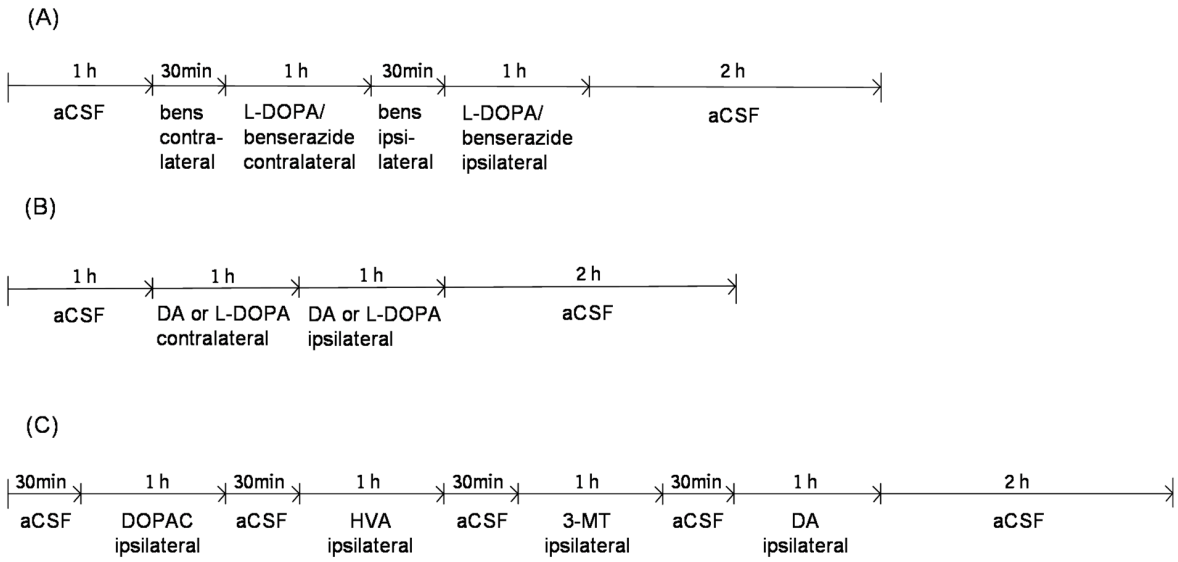


Fig. 2. Time flow chart of the intrastriatal administration of three independent experiments: (A) L-DOPA/benserazide, (B) L-DOPA and DA, (C) DOPAC, HVA, 3-MT and DA. The compounds were administered locally into the ipsilateral 6-OHDA-lesioned or contralateral non lesioned striatum. Dyskinetic behaviour, including axial, limb and orolingual movements, was monitored every 5 minutes during the whole experiment.

Please see chapter II “General methodology” (Reverse in vivo microdialysis procedure) page 25. This experiment was independently conducted from the measurement of DA and its metabolites. Following insertion of the probes, aCSF was perfused for 1 hour. In order to investigate the effect of L-DOPA itself, benserazide was administered locally for 30 minutes (0.2 nmol/min; n = 8) subsequently followed by L-DOPA/benserazide (0.4/0.2 nmol/min) for 1 additional hour on the contralateral side. Afterwards this procedure was repeated on the ipsilateral side (for experimental design please see Fig. 2A). L-DOPA (0.4 nmol/min; n = 6) or DA (0.4 nmol/min; n = 4) were perfused on the contralateral side followed by the ipsilateral side for 60 minutes each (Fig. 2B). In the second experiment (n = 8), DOPAC (0.4 nmol/min), HVA (0.4 nmol/min) and 3-methoxytyramine (3-MT) (0.4 nmol/min) were consecutively perfused on the ipsilateral side for 1 hour each. A 30 minutes aCSF perfusion was performed as washout between administration of the different metabolites. Afterwards a perfusion of DA (0.4 nmol/min) followed for 1 more hour (Fig. 2C). Drug concentration was chosen according to Carta *et al.* (2006), who demonstrated the maximum dyskinetic effect at L-DOPA 100mM (= 0.4 nmol/min).

Drugs and chemicals

Please see chapter II “General methodology” page 26.

Statistical analysis

Please see chapter II “General methodology” page 27. Rating scores of L-DOPA-induced dyskinesia were analysed by either one-way analysis of variance (ANOVA) with treatment as the independent factor or two-way ANOVA with time as the dependent factor and dose as the independent factor followed by the Bonferroni post hoc test. For comparison of dyskinesia scores with the theoretical mean which was defined as 0.0 (pre dyskinesia score) a one sample t-test was performed.

Post mortem neurochemical results were analysed with a paired t-test to compare the lesioned and the non lesioned striatum.

The cross-over microdialysis study was analysed by a four-way ANOVA for repeated measurements with 4 factors including lesion, treatment, group and test period within the cross-over design. Two-way ANOVA with lesion as the dependent factor and dose as the independent factor was used to compare basal values between the lesioned and non lesioned side. $P < 0.05$ was considered as statistically significant.

A principal component analysis (PCA) was performed to analyse the multivariate relations between dyskinetic movement scores and neurochemical parameters. PCA transforms the original variables into new variables called principal components. The first principal component is taken to be along the direction with the maximum variance. Each succeeding component accounts for as much of the remaining variability as possible. Hence, PCA and the respective biplot allow a global presentation of the high dimensional data within a two-dimensional space of the first and second principal component.

RESULTS

Effects of systemic L-DOPA administration on dyskinetic behaviour

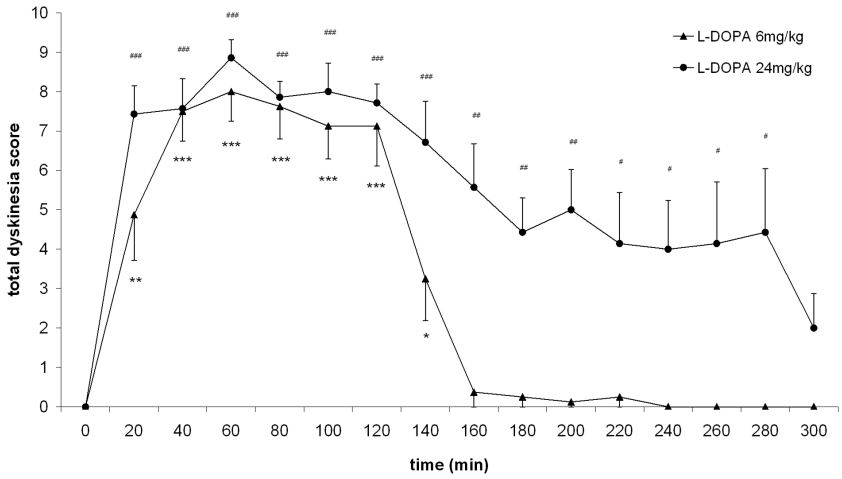


Fig. 3. L-DOPA-induced dyskinesia after administration of L-DOPA (6 mg/kg (n = 8) or 24 mg/kg (n = 7); p.o.) plus benserazide (15 mg/kg; p.o.). Monitoring of dyskinesia was carried out every 20 minutes throughout the experiment and expressed as total dyskinesia score. One sample t-test was performed for comparison with a theoretical mean, which was defined as 0 (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for L-DOPA 6 mg/kg; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ for L-DOPA 24 mg/kg). Data are presented as mean ± SEM.

Fig. 3 shows the time course of dyskinetic behaviour during the microdialysis procedure. Dyskinesia was monitored every 20 minutes for a time period of 5 hours following administration (p.o.) of L-DOPA plus benserazide. Administration of vehicle did not show any dyskinetic behaviour. Two-way ANOVA for repeated measurements yielded a significant interaction of time × dose of L-DOPA ($F(15;195) = 3,81$; $P < 0.001$). Both doses of L-DOPA (6 mg/kg and 24 mg/kg) led to a significant increase of dyskinetic behaviour including axial, limb and orolingual movements ($P < 0.001$). Statistical comparison of maximum values revealed no significant differences

between the L-DOPA doses (sum of total dyskinesia score at 40 - 120 min: 6 mg/kg and 24 mg/kg 37.4 ± 2.8 and 40 ± 2.40, respectively). In contrast, L-DOPA 24 mg/kg induced a significantly longer lasting dyskinetic behaviour indicated by a significant increase of the total dyskinesia score (20 - 280 min) whereas dyskinesia returned to basal levels after 160 minutes following L-DOPA 6 mg/kg.

Effects of systemic L-DOPA administration on striatal DA, DOPAC and HVA levels

	Lesioned 6 mg/kg (n=8)	Non lesioned 6 mg/kg (n=8)	Lesioned 24 mg/kg (n=7)	Non lesioned 24 mg/kg (n=7)
DA (nmol/l)	0.40 ± 0.04	2.86 ± 0.28	0.35 ± 0.02	3.70 ± 0.24
DOPAC (nmol/l)	8.75 ± 1.07	621.23 ± 42.36	7.34 ± 1.25	622.5 ± 66.64
HVA (nmol/l)	6.63 ± 0.61	351.86 ± 14.76	5.37 ± 0.50	362.27 ± 29.85

Table 2. Basal values (fraction 1 - 4) of extracellular levels of DA, DOPAC and HVA. Two-way ANOVA for repeated measurements revealed a significant difference between lesioned and non lesioned side (*** $P < 0.001$). Data are presented as mean ± SEM. Animals per group are given in parenthesis.

Basal levels of DA, DOPAC and HVA of all experimental groups are presented in table 2. Statistical analysis revealed a significant difference in basal levels between the lesioned and the non lesioned side of DA ($F(1;14) = 79.27$; $P < 0.001$), DOPAC ($F(1;14) = 81.86$; $P < 0.001$) and HVA ($F(1;14) = 114.70$; $P < 0.001$).

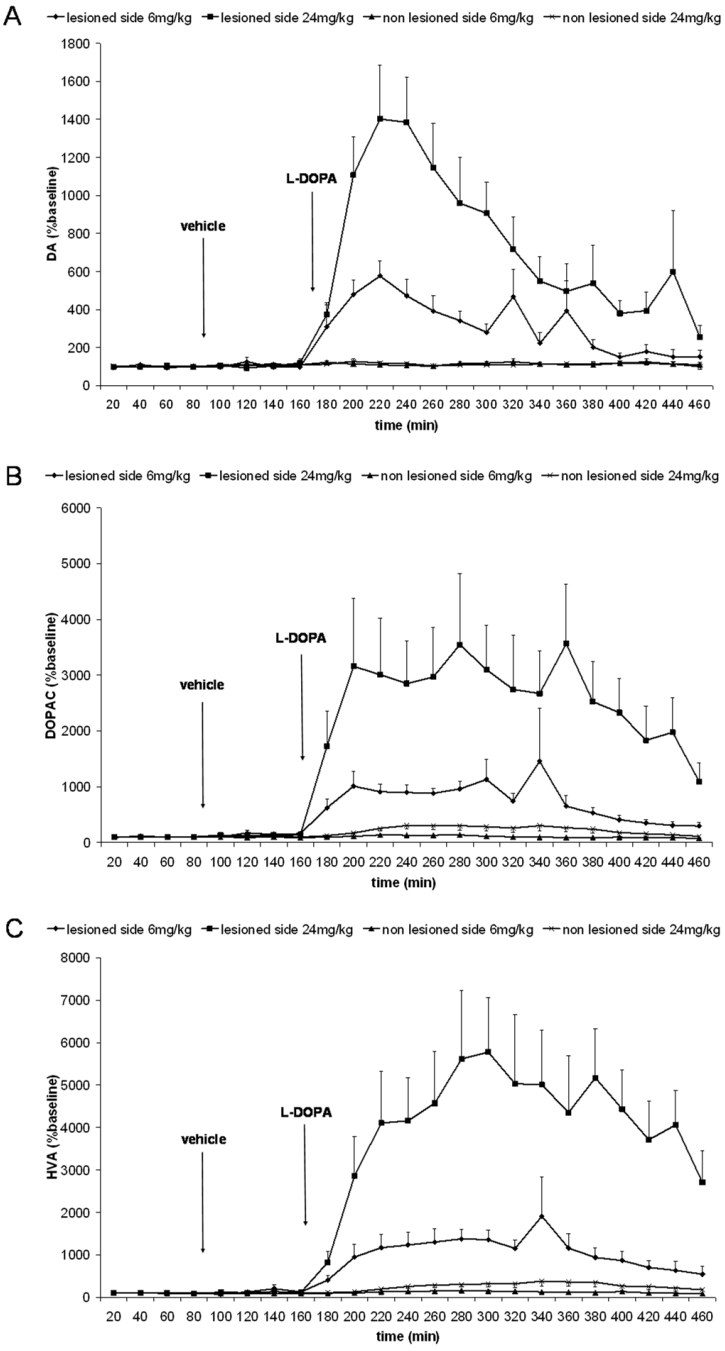


Fig. 4. Effects of systemic L-DOPA (6 mg/kg (n = 8) or 24 mg/kg (n = 7); p.o.) plus benserazide administration (15 mg/kg; p.o.) on extracellular striatal levels of DA (A), DOPAC (B) and HVA (C). A four-way ANOVA yielded a significant interaction of lesion × treatment (DA: $P < 0.01$; DOPAC: $P < 0.01$; HVA: $P < 0.001$). Data are presented as mean ± SEM. Please note the different scale of the Y-axes.

In the cross-over study a significant effect of group or test period was neither observed for DA nor DOPAC or HVA, indicating no carry-over effects. A four-way ANOVA to investigate extracellular DA, DOPAC and HVA levels yielded a significant interaction of lesion × treatment (DA: $F(1;16) = 12.33$; $P < 0.01$; DOPAC: $F(1;16) = 12.54$; $P < 0.01$; HVA: $F(1;16) = 22.82$; $P < 0.001$), indicating different treatment effects for the lesioned and the non lesioned side. Significant differences on the lesioned side between L-DOPA 6 mg/kg and 24 mg/kg were obtained in extracellular levels of DA ($P < 0.001$), DOPAC ($P < 0.001$) and HVA ($P < 0.001$). L-DOPA revealed a significant dose related increase (expressed as mean % values after L-DOPA administration, 180 – 460 min) compared to vehicle administration in extracellular levels of DA (6 mg/kg: 324% ($P < 0.01$); 24 mg/kg: 680% ($P < 0.001$)) (Fig. 4A), DOPAC (6 mg/kg: 754% ($P < 0.05$); 24 mg/kg: 2380% ($P < 0.001$)) (Fig. 4B) and HVA (6 mg/kg: 1044% ($P < 0.05$); 24 mg/kg: 3770% ($P < 0.001$)) (Fig. 4C) on the lesioned side. In contrast, the non

lesioned side did not show any significant effects in DA and its metabolites after treatment with L-DOPA.

Correlation analysis between dyskinetic behaviour and neurochemical parameters

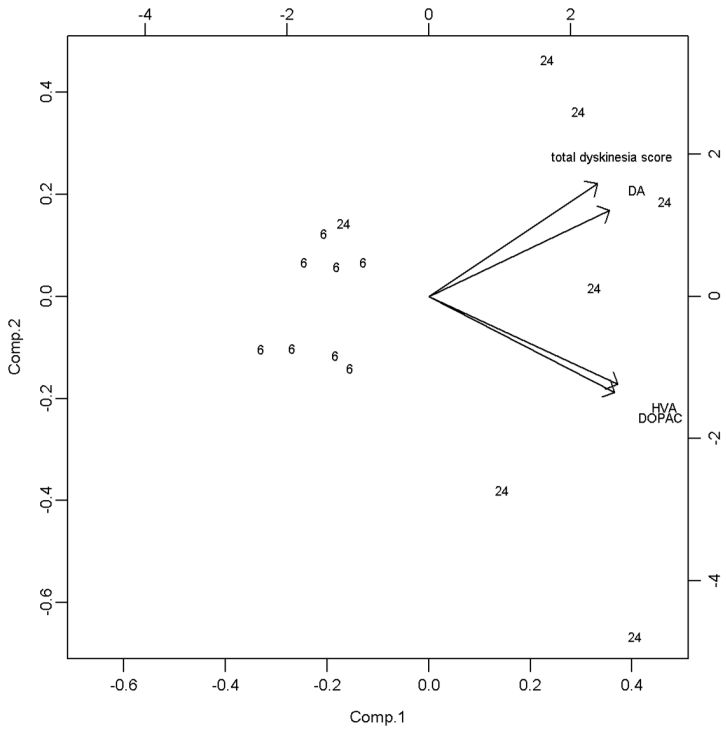


Fig. 5. Biplot of PCA for parameters DA, DOPAC, HVA and total dyskinesia scores. The x- and y-axis represent the scores for the first and second principal component, respectively.
For DA and total dyskinesia score a significant partial correlation was obtained (r = 0.73, P < 0.01), whereas for DOPAC and HVA no correlation was found. DOPAC and HVA are highly correlated to each other (r = 0.92, P < 0.001).The single animals are marked by their dose group (different L-DOPA doses are indicated by 6 and 24, respectively).

The PCA is visualized in a biplot (Fig. 5) representing both the original variables and the transformed observations regarding to the principal component axes (Gabriel and Odoroff 1990). The original variables are represented by arrows from the point of origin showing the direction of positive changes of the parameters. The length of the arrows represents the standard deviation of the variables. The correlation between two parameters is represented by the angle between the corresponding arrows, thereby a small angle indicates a strong positive correlation. For each pair of variables the Pearson partial correlation coefficient was calculated to measure the strength of the relationship between these two variables, while controlling the effect of the remaining two variables. For DA and total dyskinesia score a significant partial correlation was obtained (r = 0.73, P < 0.01), whereas for DOPAC (r = - 0.22, P = 0.46) as well as for HVA (r = 0.25, P = 0.41) no correlation was found. As expected, DOPAC and HVA are highly correlated to each other (r = 0.92, P < 0.001).

Post mortem neurochemistry

	Lesioned striatum (n=7)	Non lesioned striatum (n=7)
DA (ng/mg)	0.026 ± 0.008 ***	10.70 ± 1.78
DOPAC (ng/mg)	0.008 ± 0.003 ***	1.47 ± 0.24
HVA (ng/mg)	0.009 ± 0.003 ***	0.76 ± 0.12

Table 3. Post mortem neurochemistry results of the lesioned and the non lesioned striatum of dyskinetic rats (n = 7). Values are expressed as ng/mg wet tissue weight. Data are expressed as mean ± SEM and analysed by paired t-test (*** P < 0.001).

To verify the extent of the 6-OHDA lesion a post mortem analysis of DA, DOPAC and HVA levels of the lesioned and the non lesioned striatum was performed. Statistical analysis yielded a significant depletion of DA of 99.75 % (P < 0.001), DOPAC of 99.42 % (P < 0.001) and HVA of 98.80 % (P < 0.001) of the lesioned striatum in comparison to the non lesioned striatum (Table 3).

Effects of intrastriatal administration of L-DOPA, L-DOPA/benserazide, DA, DOPAC, HVA and 3-MT on dyskinetic behaviour

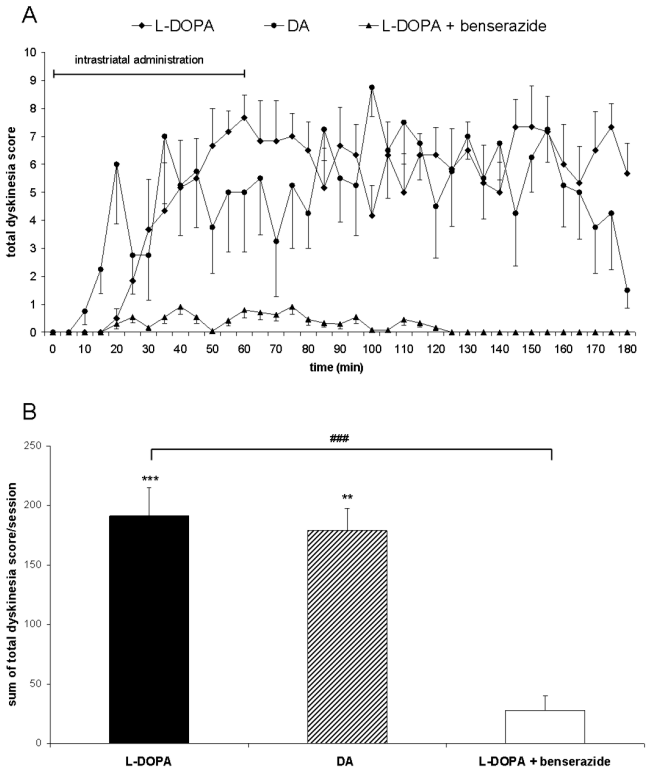


Fig. 6. Effects of intrastriatal administration of L-DOPA (0.4 nmol/min; n = 6), DA (0.4 nmol/min; n = 4) and L-DOPA/benserazide (0.2/0.4 nmol/min; n = 8). Part (A) shows the time course of total dyskinesia score, which was monitored every 5 minutes over 180 minutes. In part (B) the sum of total dyskinesia score over a period of 180 minutes is displayed. Data are mean ± SEM and analysed by one sample t-test for comparison with the theoretical mean (** P < 0.01, *** P < 0.001) and one-way ANOVA and Bonferroni post hoc test were performed for statistical comparison of the treatments (### P < 0.001).

Neither the striatal administration of L-DOPA (0.4 nmol/min), DA (0.4 nmol/min) nor L-DOPA/benserazide (0.4/0.2 nmol/min) on the contralateral non lesioned side produced any dyskinetic behaviour (total dyskinesia score = 0). In contrast, intrastriatal DA ($P < 0.01$) as well as L-DOPA administration on the ipsilateral 6-OHDA-lesioned side led to a pronounced increase in dyskinesia ($P < 0.001$) (Fig. 6). In addition, L-DOPA- and DA-induced dyskinesia were not significantly different (179 ± 18.7 and 191.3 ± 23.5 total dyskinesia score, respectively). However, the DA-induced dyskinesia appeared earlier and was of shorter duration than after L-DOPA treatment. For comparison of intrastriatal L-DOPA- and DA-induced dyskinetic behaviour please see supplementary video clips.

After intrastriatal administration of L-DOPA a similar pattern of axial, orolingual and limb dyskinesia could be observed compared to dyskinetic behaviour following systemic administration of L-DOPA/benserazide. However, more fluctuations in dyskinetic movements were obtained after intrastriatal administration. Due to the suboptimal testing environment in microdialysis cylinders a higher degree of rotational behaviour was produced than in rectangular home cages.

In order to investigate the effect of L-DOPA itself on the appearance of dyskinesia, the enzyme AADC was blocked by intrastriatal benserazide administration. Intrastriatal L-DOPA in combination with benserazide at the lesioned side did not increase dyskinetic behaviour. Notably, a significant difference in total dyskinesia scores after striatal administration of L-DOPA in comparison to L-DOPA/benserazide treatment was obtained ($P < 0.001$).

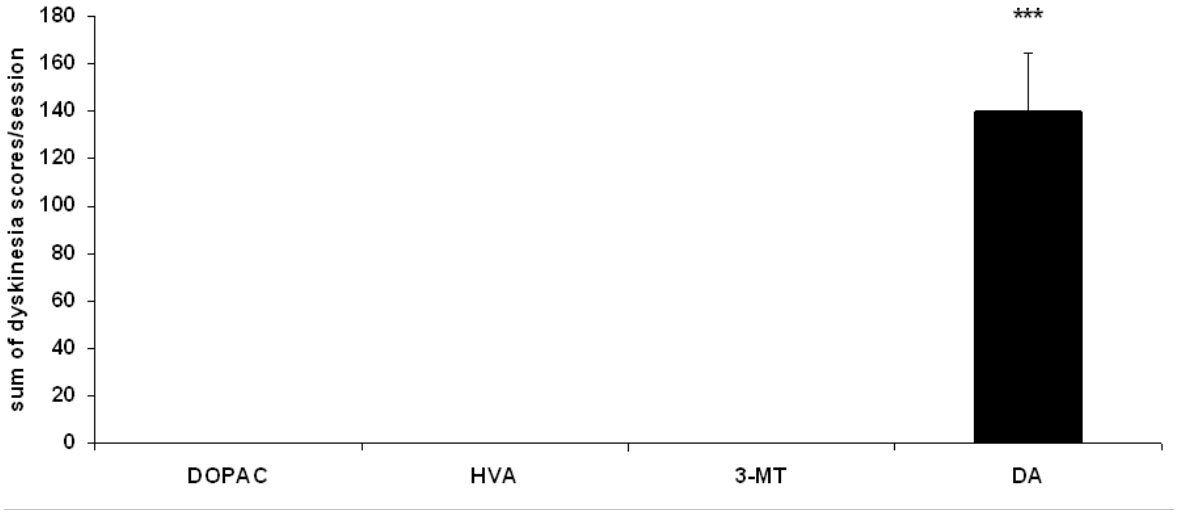


Fig. 7. Effects of intrastriatal administration of DOPAC (0.4 nmol/min; n = 8), HVA (0.4 nmol/min; n = 8), 3-MT (0.4 nmol/min; n = 8) and DA (0.4 nmol/min; n = 8). These compounds were perfused consecutively on the ipsilateral side for 1 hour each (30 minutes aCSF between the individual compounds). Dyskinetic behaviour was monitored during the whole experiment every 5 minutes up to 120 minutes after the administration of the last drug. The sum of total dyskinesia score over 90 minutes for DOPAC (total dyskinesia score = 0), HVA (total dyskinesia score = 0) and 3-MT (total dyskinesia score = 0) and over 180 minutes for DA is presented. Data are expressed as mean ± SEM and analysed by one sample t-test for comparison with theoretical mean (*** $P < 0.001$).

The results of intrastriatal DA and DA metabolite administration are displayed in Fig. 7. Neither DOPAC (0.4 nmol/min) nor HVA (0.4 nmol/min) nor 3-MT (0.4 nmol/min) led to an induction of dyskinesia (total dyskinesia score = 0). 3-MT produced a slight enhancement of motor activity, which was mainly attributed to an increase in rearing and sniffing behaviour but not to dyskinetic behaviour. In contrast, intrastriatal DA administration showed a significant increase in dyskinesia ($P < 0.001$).

DISCUSSION

The present study demonstrated that striatal L-DOPA administration itself is not able to induce dyskinetic behaviour in a rat model of dyskinesia. This result was obtained by striatal administration of benserazide blocking the conversion of L-DOPA to DA.

Accumulating evidence shows that in addition to being a precursor of DA L-DOPA may be a neurotransmitter in its own right (Ueda *et al.* 1995; Misu *et al.* 2003; Misu *et*

al. 2002; Hornykiewicz 2002). Misu *et al.* (2003) proposed that L-DOPA itself fulfils the criteria for neurotransmitters (synthesis, metabolism, active transport, presence, physiological release, competitive antagonism, physiological or pharmacological responses).

The question raised whether L-DOPA could produce dyskinetic behaviour in its own right without metabolism to DA, because L-DOPA mimics some of the biological effects such as agonism at DA D2 receptors (Hume *et al.* 1995).

In the present study we demonstrated that L-DOPA was not able to induce dyskinesia suggesting that the expression of dyskinesia is dependent on the conversion of L-DOPA to DA or downstream processes. Indeed, after perfusion of DA via the microdialysis probe into the lesioned striatum dyskinetic movements were observed which were similar in their maximum level to L-DOPA-induced dyskinesia. However, the DA-induced dyskinesia appeared earlier and was of shorter duration than after L-DOPA treatment, which suggests that L-DOPA has to be converted to DA first before it produces dyskinetic behaviour.

Neither L-DOPA nor DA administered at the non lesioned striatum produced any dyskinetic behaviour suggesting that the degeneration of dopaminergic neurons is necessary for the manifestation of dyskinetic movements. This result is in agreement with a recent study reporting that systemic L-DOPA treatment in sham lesioned animals did not display any dyskinetic behaviour (Meissner *et al.* 2006) and that more than 80% of nigrostriatal degeneration is necessary to induce dyskinetic movements at therapeutic L-DOPA doses (Winkler *et al.* 2002).

Moreover, in MPTP-treated macaques a pronounced nigrostriatal neurodegeneration is a necessary but not sufficient condition for the occurrence of L-DOPA-induced dyskinesia implicating that neither the extent nor the pattern of the neurotoxin-induced lesion can predict the manifestation of dyskinetic behaviour (Guigoni *et al.* 2005).

The striatum is the largest dopaminoceptive component of the basal ganglia. The DA denervated striatum plays a key role, if not the most important role in L-DOPA-induced dyskinesia. Dyskinetic behaviour can be prevented or even reversed by different strategies targeting the striatum including continuous L-DOPA delivery using recombinant adeno-associated viruses (rAAV) vectors applied into the striatum (Carlsson *et al.* 2006) or intrastriatal infusion of fosB antisense (Andersson *et al.* 1999).

The striatum projects to the output regions of the basal ganglia, GPi as well as to the SNr by either the “direct” pathway or the “indirect” pathway involving the globus pallidus externus (GPe) and the STN (Alexander and Crutcher 1990).

Systemic L-DOPA administration eliciting dyskinetic movements led to an increase of extracellular gamma aminobutyric acid (GABA) overflow in the SNr but not in the GPe indicating that the direct pathway may play a more important role in the manifestation of dyskinesia than the indirect pathway (Mela *et al.* 2007). This notion is confirmed by molecular alterations in DA D_1 receptor-rich striatal efferent neurons of the direct pathway, which are closely associated with L-DOPA-induced dyskinesia including an upregulation of transcription factors and plasticity gene in dynorphinergic (direct pathway) but not in enkephalinergic (indirect pathway) striatal neurons (Andersson *et al.* 1999; Carta *et al.* 2005; Mela *et al.* 2007).

DBS of the STN which reduces the glutamatergic overactivity in the indirect pathway, ameliorates L-DOPA-induced dyskinesia in patients suffering from PD. Currently it is debated whether this effect may simply result from a reduction of the daily L-DOPA dose (Follett 2004) or if the indirect pathway is involved in L-DOPA induced dyskinetic movements. In a case report, Figueiras-Mendez *et al.* (1999) demonstrated that dyskinetic behaviour improved markedly after bilateral STN stimulation without reduction of L-DOPA dose. On the other hand, high frequency stimulation of the STN did not affect dyskinetic movement in the rat dyskinesia model, which led to the conclusion that at least in the rat dykinesia model the indirect pathway seems to be of minor importance (Oueslati *et al.* 2007)

Severe nigrostriatal neurodegeneration alters the main pharmacokinetic processes of L-DOPA such as uptake and metabolism in the striatum (Cenci and Lundblad 2006). After a lesion of the nigrostriatal pathway, L-DOPA processing in dopaminergic neurons is impaired. In the parkinsonian brain it has been proposed that L-DOPA is taken up, decarboxylated and released as DA by non dopaminergic cells containing AADC such as serotonergic neurons or astrocytes (Carta *et al.* 2007; Maeda *et al.* 2005). This provides a source of unregulated DA efflux into the extracellular space due to the lack of high affinity reuptake by the DA plasmamembrane transporter and by missing autoreceptors for regulation of synthesis and release of DA in these cells. According to these alterations a degeneration of the nigrostriatal projection neurons predisposes to L-DOPA induced dyskinesia because a physiological balance of DA transmission could not be achieved leading to high fluctuations of extracellular DA

levels. We simulated this high non physiological concentration of DA in the extracellular space of the striatum by delivering DA directly into the striatum via the microdialysis probe and postulate that intrastriatal administration of DA is a well-suited model for L-DOPA- and DA-induced dyskinesia.

Following the DA metabolism pathway we investigated whether the DA metabolites DOPAC, HVA and 3-MT contribute to the manifestation of dyskinesia as well.

In the plasma of dyskinetic patients high levels of 3-O-methyldopa, the methylmetabolite of L-DOPA, were detected (Feuerstein *et al.* 1977). Possibly the methylmetabolites of DA as e. g. 3-MT or some other metabolites may also play a role in the appearance of dyskinetic movements. In this respect, Charlton and Crowell, Jr. (2000) investigated the effect of intraventricular injected 3-MT and 3,4-dimethoxyphenylethylamine (DIMPEA) on locomotive behaviour of rats as well as their effect on DA binding. They showed an increase in locomotor activity and DA binding by DIMPEA, whereas administration of 3-MT decreased the activity as well as the DA binding.

In the rat brain DOPAC is the major metabolite of DA. Released DA is metabolized to DOPAC after intraneuronal reuptake by the enzyme MAO. Released DA is also metabolized by the sequential action of COMT and MAO. Additionally, DA is metabolized to 3-MT by COMT to a lesser extent (Cooper *et al.* 2003). The DA metabolites have to be directly administered into the striatum, because they are unable to cross the blood-brain-barrier. Neither DOPAC nor HVA or 3-MT were able to induce dyskinetic movements, whereas 3-MT caused a slight enhancement of activity resembling a dopamimetic effect, which may be due to the conversion to DIMPEA, which has been shown to increase locomotor activity (Charlton and Crowell, Jr. 2000).

Several in vivo microdialysis studies investigating the effect of L-DOPA on extracellular DA and DA metabolite levels in 6-OHDA-lesioned rats have been reported (Abercrombie *et al.* 1990; Meissner *et al.* 2006; Miller and Abercrombie 1999; Rodriguez *et al.* 2007; Tanaka *et al.* 1999; Wachtel and Abercrombie 1994). This is the first study to monitor dyskinetic movements and measure the neurochemical effects of L-DOPA on extracellular DA and DA metabolite levels simultaneously.

Systemic administration of L-DOPA/benserazide led to an induction of dyskinetic behaviour which was dose related. The maximum level of dyskinesia was similar for

both doses indicating a ceiling effect. The neurochemical alterations were most pronounced in extracellular levels of HVA on the lesioned side (6 mg/kg, 24 mg/kg: 1044 %, 3770 %, respectively), followed by DOPAC (6 mg/kg, 24 mg/kg 754 %,: 2380 %, respectively). Extracellular DA levels increased to a lesser extent (6 mg/kg, 24 mg/kg 324 %, 680 %, respectively).

Rodriguez *et al.* (2007) demonstrated in a microdialysis study that the L-DOPA-induced increase of extracellular DOPAC levels is more pronounced than that of DA. Indeed, the increase of DA (600 %) and DOPAC (10,000 %) as well as the dose of L-DOPA (99 mg/kg) was much higher than in the present study. Recently, Meissner *et al.* (2006) performed a microdialysis study in 6-OHDA lesioned rats primed with L-DOPA and found an increase of DA (20-fold). DOPAC and HVA levels were enhanced 11- and 9-fold, respectively. The peak DA levels were slightly higher in the study of Meissner *et al.* (2006) compared to the present study and the changes in DA metabolite were lower, which might be explained by the 4-fold higher dose of L-DOPA and that the experiment was carried out under anaesthesia.

As mentioned above, L-DOPA produced a dose-related significant increase in DA on the lesioned side, whereas extracellular levels of DA of the non lesioned side were not affected. This is in line with the results of many other groups (Abercrombie *et al.* 1990; Miller and Abercrombie 1999; Rodriguez *et al.* 2007; Wachtel and Abercrombie 1994). The low response of DA on the intact side indicates that under physiological conditions dopaminergic neurons are able to handle extracellular DA very efficiently thus keeping physiological homeostasis.

In a principle component analysis the dyskinetic behaviour was correlated to extracellular levels of DA but not to DOPAC and HVA. Indeed, the reverse in vivo microdialysis experiment showed that the intrastriatal DOPAC and HVA administration did not produce any dyskinetic behaviour, whereas intrastriatal DA administration significantly induced dyskinesia.

In conclusion, the present study underpins that intrastriatal L-DOPA has no function in its own right in the L-DOPA-induced dyskinesia model of rats. Rather we demonstrated that the neurotransmitter DA is able to induce dyskinetic movements when administered locally into the lesioned striatum, whereas the metabolites DOPAC, HVA and 3-MT did not produce any dyskinetic movements. This data is in line with the alterations of DA, DOPAC and HVA after systemic administration of L-DOPA/benserazide. Alteration in DA levels highly correlates to the occurrence of

dyskinesia, whereas DOPAC and HVA do not show any correlation to dyskinetic movements.

CHAPTER IV

Comparison of intrastriatal administration of noradrenaline and L-DOPA on dyskinetic movements

ABSTRACT

L-DOPA-induced dyskinesia is known as involuntary debilitating movement, which limits quality of life in patients suffering from Parkinson's disease. The present study focuses on the role of the neurotransmitter noradrenaline (NA) on dyskinetic movements in comparison to the effect of L-DOPA.

Rats were unilaterally lesioned with 6-OHDA and treated with L-DOPA/benserazide (6/15 mg/kg, p.o.) to induce stable dyskinetic movements. On the day of the experiment, NA (0.04 nmol/min, 0.4 nmol/min) as well as L-DOPA (0.04 nmol/min, 0.4 nmol/min) were perfused into the lesioned and non lesioned striatum of dyskinetic rats using the reverse in vivo microdialysis technique. Neither NA nor L-DOPA treatment of the non lesioned striatum produced any dyskinetic behaviour. In contrast, administration of L-DOPA 0.4 nmol/min into the lesioned striatum led to a significant increase in dyskinesia indicated by abnormal axial, limb and orolingual movements. Notably, perfusion with NA 0.4 nmol/min into the lesioned striatum revealed a highly significant induction of dyskinetic movements, which are similar to the dyskinesia subtype profile of L-DOPA. In conclusion, NA is as potent as L-DOPA to express dyskinetic movements in L-DOPA primed rats.

INTRODUCTION

In PD the long term use of the gold standard L-DOPA can result in major adverse effects to the motor functions, namely dyskinesia, which comprises choreatic and dystonic movements. There is pharmacological evidence that noradrenergic neurotransmission is involved in the pathogenesis of L-DOPA-induced dyskinesia. The α_2 adrenoceptor antagonist idazoxan has shown antidyskinetic efficacy in monkey studies (Fox *et al.* 2001; Grondin *et al.* 2000). Moreover, idazoxan reduced L-DOPA-induced dyskinesia without affecting the L-DOPA mediated relief of symptoms in clinical trials (Henry *et al.* 1999; Rascol *et al.* 2001). The question arises, whether an increase in noradrenergic transmission by local administration of NA can induce dyskinetic behaviour. Previously, we found that DA as well as L-

DOPA delivered directly into the lesioned striatum of dyskinetic rats can produce dyskinetic movements (Buck and Ferger 2008). In the present study we demonstrated that NA is as potent as L-DOPA to provoke dyskinetic movements when administered locally into the striatum of L-DOPA primed rats, which previously received a unilateral 6-OHDA lesion of the MFB.

MATERIALS AND METHODS

Animals, 6-OHDA lesion surgery, Induction and rating of dyskinesia

Please see chapter II “General methodology” page 22.

Reverse in vivo microdialysis surgery in the striatum

Please see chapter II “General methodology” page 24.

Reverse in vivo microdialysis procedure in the striatum

Please see chapter II “General methodology” page 25. Following insertion of the probes, aCSF was perfused for 1 hour. L-DOPA (0.04 nmol/min, n = 4; 0.4 nmol/min, n = 6) or NA (as bitartrate salt) (0.04 nmol/min, n = 4; 0.4 nmol/min, n = 6) were perfused on the contralateral side followed by the ipsilateral side for 60 minutes each.

Drugs and chemicals

Please see chapter II “General methodology” page 26.

Statistical analysis

Please see chapter II “General methodology” page 27. A two-factorial ANOVA was performed with the factors treatment and dose and the interaction term treatment x dose. The ANOVA was followed by pairwise t-tests and a one sample t-test (comparison of dyskinesia scores with the theoretical mean which was defined as 0.0) for each experimental group taking into account the error term of the ANOVA as an estimate for the common variation. $P < 0.05$ was considered as statistically significant.

RESULTS

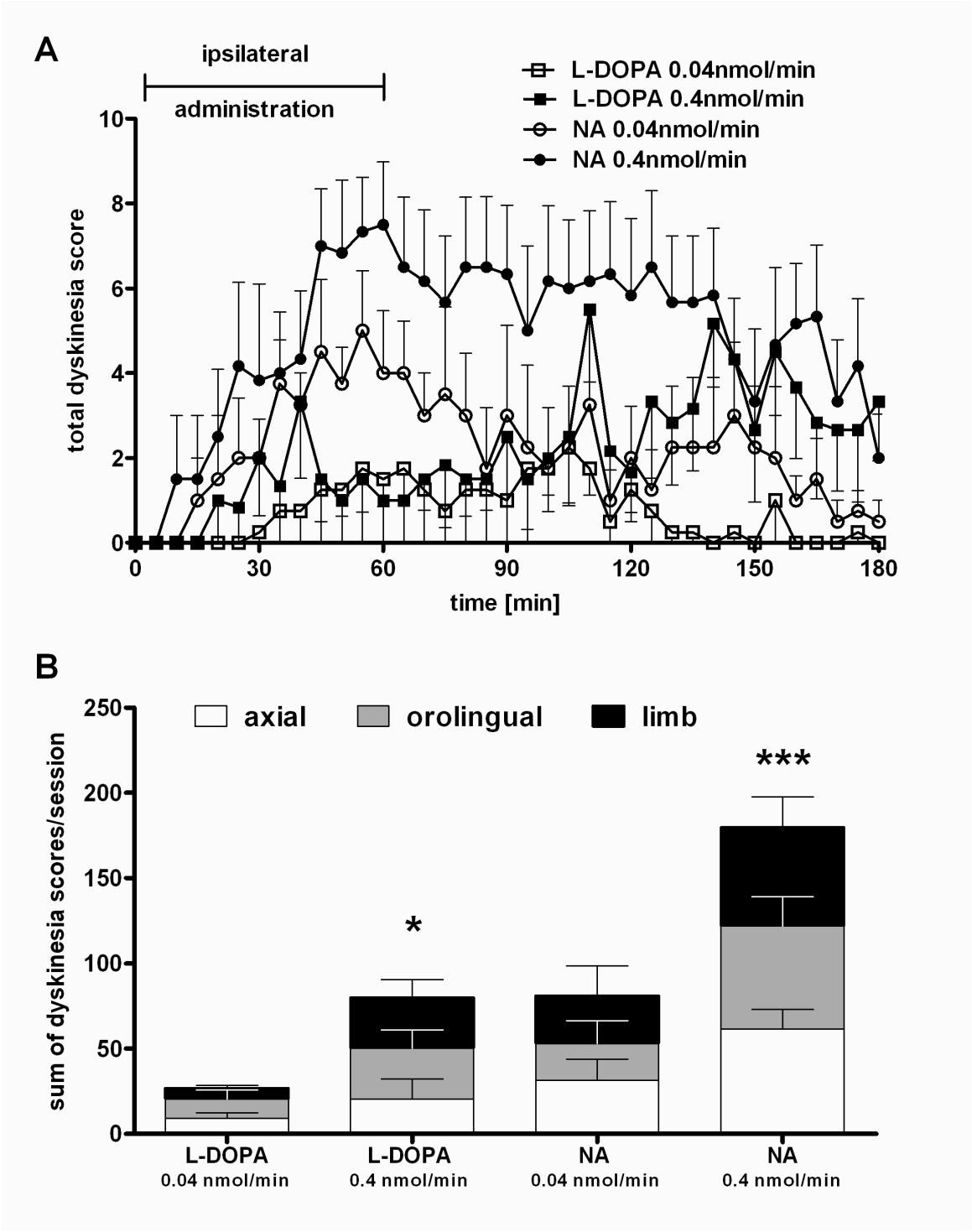


Fig. 8. (A) Time course of total dyskinesia score of the intrastriatal ipsilateral administration of L-DOPA ((0.04 nmol/min (n = 4), 0.4 nmol/min (n = 6)) and NA (0.04 nmol/min (n = 4), 0.4 nmol/min (n = 6)) for 60 min. Monitoring of dyskinetic behaviour was continued up for 120 min. (B) Sum of axial, limb and orolingual dyskinesia scores after intrastriatal ipsilateral administration over a period of 180 min. Data are expressed as mean ± SEM and analysed by one sample t-test within the ANOVA model for determination of the increase in dyskinesia (* $P < 0.05$,*** $P < 0.001$ for comparison with the theoretical mean = 0).

L-DOPA as well as NA were administered intrastriatally to assess their effects on dyskinetic behaviour of rats. Neither administration of L-DOPA nor NA on the non lesioned side produced any behavioural effects (total dyskinesia score = 0). In contrast, statistical analysis yielded a significant induction of dyskinesia after administration of L-DOPA 0.4 nmol/min on the lesioned side (79.8 ± 32.5 total dyskinesia score, $P < 0.05$) (Fig. 8A and 8B). Ipsilateral administration of NA 0.4 nmol/min revealed a highly significant increase of dyskinetic movements (179.7 ± 44.1 total dyskinesia score, $P < 0.001$). L-DOPA 0.04 nmol/min produced low dyskinesia, which was not statistically significant (26.8 ± 3.6 total dyskinesia score). NA 0.04 nmol/min showed an increase in dyskinetic behaviour, which was not statistically significant but yielded a trend (81.0 ± 40.3 total dyskinesia score, $P = 0.068$). Considering the subtype dyskinesia scores, axial dyskinesia was significantly evoked after administration of NA 0.04 nmol/min (31.3 ± 12.6, $P < 0.05$) as well as NA 0.4 nmol/min (61.5 ± 11.4, $P < 0.001$). Orolingual dyskinesia was significantly produced after treatment with L-DOPA 0.4 nmol/min (29.7 ± 10.8, $P < 0.05$) and NA 0.4 nmol/min (60.3 ± 17.2, $P < 0.001$). Limb dyskinesia increased significantly after administration of L-DOPA 0.4 nmol/min (29.7 ± 10.7, $P < 0.05$) and NA 0.4 nmol/min (57.8 ± 17.8, $P < 0.001$). The ANOVA revealed a significant difference in treatment for axial dyskinesia scores ($F(1,16) = 7.62$, $P < 0.05$), followed by a pair wise t-test which showed a significant difference in axial dyskinesia scores between L-DOPA 0.4 nmol/min (20.5 ± 11.6) and NA 0.4 nmol/min (61.5 ± 11.4) administration ($P < 0.05$). Furthermore, the NA-induced dyskinesia appeared earlier than the dyskinesia evoked by intrastriatal administration of L-DOPA.

DISCUSSION

This is the first study which demonstrates that local administration of NA into the lesioned striatum can induce dyskinetic movements in rats in a similar manner to intrastriatal L-DOPA treatment.

Most of the studies investigating dyskinetic behaviour were performed after systemic administration of L-DOPA (Dekundy *et al.* 2007; Lundblad *et al.* 2002). Recently, it was demonstrated that L-DOPA induced dyskinetic movements in rats after local administration into the 6-OHDA-lesioned striatum, suggesting that the striatum plays

a key role in L-DOPA-induced dyskinesia (Buck and Ferger 2008; Carta *et al.* 2006). In a subsequent experiment, the effect of striatal administration was abolished by blocking the conversion of L-DOPA to DA (Buck and Ferger 2008), which highlights the importance of DA in the striatum of dyskinetic rats.

However, it has become increasingly apparent that PD and L-DOPA-induced dyskinesia are multisystemic disorders in which neurotransmitter systems other than DA are involved. There is evidence that NA in fact plays a major role in PD pathology and symptomatology as well as in motor complications (Brotchie 2005; Colosimo and Craus 2003; Fornai *et al.* 2007; Linazasoro *et al.* 2008; Nagatsu and Sawada 2007; Rommelfanger and Weinshenker 2007; Schapira 2005).

Previous experimental studies have demonstrated that α_2 adrenoceptor antagonists such as yohimbine reduce L-DOPA-induced dyskinesia in rodent (Dekundy *et al.* 2007; Lundblad *et al.* 2002) as well as primate models (Gomez-Mancilla and Bedard 1993). Moreover, some α_2 adrenoceptor antagonists like idazoxan and fipamezole have shown antidyskinetic efficacy without compromising the anti-parkinsonian action of L-DOPA in monkey studies (Fox *et al.* 2001; Grondin *et al.* 2000; Savola *et al.* 2003) and clinical trials. However, the mechanism of the alleviation of L-DOPA-induced dyskinesia by these "anti-adrenergic" drugs remains elusive. The blockade of the action of NA synthesised from L-DOPA is thought to be involved (Fox *et al.* 2001), because dyskinesia evoked by L-DOPA, but not by DA agonists like apomorphine, can be reduced by an α_2 adrenoceptor antagonist.

In situ hybridisation studies revealed a poor distribution of α_{1B}, α_{1D}, α_{2A}, α_{2B}, β_1 and β_2 adrenoceptor mRNA in the rat striatum, whilst the α_{2C} adrenoceptor mRNA was found to be highly expressed in this brain area (McCune *et al.* 1993; Nicholas *et al.* 1996). More precisely, the α_{2C} adrenoceptor is located on GABAergic medium spiny projection neurons of the striatum (Holmberg *et al.* 1999) and modulates GABA outflow from the striatum to GP and the SN (Zhang and Ordway 2003). Mela *et al.* (2007) demonstrated an increase in extracellular GABA release after administration of L-DOPA in dyskinetic rats in the SNr. Conversely, the GABA overflow was not altered in the GPe. This finding indicates that the direct pathway seems to be more important for the manifestation of dyskinetic movements in the L-DOPA rat dyskinesia model. This result may also be relevant for the NA-induced dyskinesia, because stimulation of α_{2C} adrenoceptors by NA in the striatum is a key modulator of GABA release within the SNr (Alachkar *et al.* 2006).

Another explanation could be that NA mediates its dyskinesiogenic effect by acting on dopaminergic receptors. NA was found to act as a D_1 dopaminergic agonist in the embryonic avian retina (Kubrusly *et al.* 2007) and mimics the effect of DA on the DA D_2 receptor indicated by the inhibition of adenylate cyclase activity in the rat striatum (Onali *et al.* 1985). Furthermore, it was demonstrated that NA binds to the human DA D_4 receptor with high affinity (Lanau *et al.* 1997; Newman-Tancredi *et al.* 1997) and 10% of total D_2-like receptors are of the DA D_4 receptor located in the caudate putamen (Tarazi *et al.* 1997). Hence, NA may act also on dopaminergic receptors in the striatum to induce dyskinetic movements. Pharmacological studies to test this hypothesis are necessary.

Additionally to a direct DA receptor mediated effect, NA indirectly may enhance striatal DA levels by a presynaptic mechanism. However, the loss of terminals and the level of DA depletion in the striatum are almost complete in dyskinetic rats under the conditions used in the present study (Buck and Ferger, 2008) and it is unlikely that NA releases DA from striatal terminals.

In conclusion, this experiment provides the first evidence that NA produces dyskinetic movements resembling those induced by local or systemic L-DOPA administration, which underscores the relevance to investigate anti-adrenergic mechanisms for the treatment of dyskinetic movements in PD.

CHAPTER V

The selective α_1 adrenoceptor antagonist HEAT reduces L-DOPA-induced dyskinesia in a rat model of Parkinson's disease

ABSTRACT

In PD, the long term use of L-DOPA results in major adverse effects including dyskinesia or abnormal involuntary movements. The present study focuses on the effect of the selective α_1 adrenoceptor antagonist HEAT (2-[[β-(-4-hydroxyphenyl)ethyl]aminomethyl]-1-tetralone) in the 6-OHDA rat model of L-DOPA-induced dyskinesia. We demonstrate that the selective α_1 adrenoceptor antagonist HEAT (1 and 2mg/kg), the α_2 adrenoceptor antagonist idazoxan (9mg/kg) and the non-selective β_1/β_2 adrenoceptor antagonist propranolol (20mg/kg) alleviate dyskinetic movements induced by L-DOPA. Furthermore, the adrenoceptor antagonists at the doses used did not influence exploratory behaviour in the open field system indicating that the antidyskinetic effect is not due to a reduction in general motor activity. Intrastriatal administration of the selective α_1 adrenoceptor agonist cirazoline via reverse in vivo microdialysis did not induce dyskinesia. Additionally, we measured plasma, brain and cerebrospinal fluid (CSF) levels of HEAT. HEAT is a CNS active compound with a brain/plasma and CSF/plasma ratio of 4.29 and 0.15, respectively, which is appropriate for the investigation of α_1–mediated mechanisms in CNS disorders. In conclusion, these results demonstrate for the first time that a α_1 adrenoceptor antagonist reduced L-DOPA-induced dyskinesia in a rat model. Further studies assessing the risk benefit in comparison to existing therapies are needed before considering α_1 adrenoceptor antagonists as a target for the development of new antidyskinetic compounds.

INTRODUCTION

L-DOPA-induced dyskinesia eventually develops as serious treatment-limiting motor complication in the majority of patients suffering from PD (Jenner 2008a). Dyskinesia comprises abnormal involuntary movements such as chorea and dystonia and develops in up to 40% of PD patients within 4-6 years of L-DOPA therapy (Ahlskog and Muenter 2001).

The underlying pathophysiological mechanisms of L-DOPA-induced dyskinesia are not well understood. Several factors such as the dysregulation of DA release and clearance resulting in a large DA surge, upregulation of striatal transcription factors and plasticity genes, abnormal corticostriatal plasticity and altered firing patterns in basal ganglia output neurons are thought to play a role (Cenci 2007; Jenner 2008a). The main risk factors for the generation of dyskinetic movements are the degree of dopaminergic neurodegeneration within the basal ganglia (Obeso *et al.* 2000b), the onset and dose of L-DOPA therapy (Rascol 2000) as well as the young onset of the disease (Kumar *et al.* 2005).

Alternative pharmacological approaches to L-DOPA to alleviate the cardinal symptoms of PD involve the treatment with synthetic DA agonists as well as compounds influencing the enzymatic metabolism of DA such as MAO-B or COMT inhibitors. However, none of these drugs has surpassed the clinical benefit derived from L-DOPA. Additionally, monotherapy with DA agonists in PD patients can induce dyskinetic movements, albeit with a reduced incidence of motor complications (Rascol *et al.* 2000).

To date the weak non-competitive N-methyl-D-aspartate (NMDA) antagonist amantadine is the only drug which is clinically used to alleviate L-DOPA-induced dyskinesia. However, amantadine is not tolerated well because of cognitive side effects. There is a controversy in the literature regarding the duration of amantadine's antidyskinetic effect. Stocchi *et al.* (2008) reported that the effect of amantadine is often transient and lost within 1 year, whereas Verhagen Metman *et al.* (1999) showed that amantadine's effect on motor response complications is maintained for at least 1 year after treatment initiation. Nevertheless, there is an unmet medical need to identify novel mechanisms for the treatment of dyskinesia in PD. New treatment strategies would therefore target non-dopaminergic pathways to alleviate motor complications associated with L-DOPA therapy, whilst maintaining the anti-parkinsonian effect of L-DOPA. Currently, several approaches are evaluated in clinical trials such as adenosine A_{2a} antagonists, 5-HT_{1A} agonists, mGluR5 antagonists, NMDA antagonists, MAO inhibitors and α_2 adrenoceptor antagonists which are aiming to treat symptoms of PD and L-DOPA-induced dyskinesia or at least try to avoid dyskinesia.

Indeed, there is evidence that drugs influencing noradrenergic neurotransmission can be used as an adjunct therapy to L-DOPA in order to reduce motor complications.

Alleviation of established dyskinesia was achieved by treatment with α_2 adrenoceptor antagonists in rodents (Dekundy *et al.* 2007; Lundblad *et al.* 2002) and MPTP-treated monkeys (Fox *et al.* 2001; Gomez-Mancilla and Bedard 1993; Grondin *et al.* 2000; Henry *et al.* 1999; Savola *et al.* 2003) as well as in parkinsonian patients (Rascol *et al.* 2001). Additionally, the non-selective β_1/β_2 adrenoceptor antagonist propranolol was found to show antidyskinetic properties in the rat dyskinesia model (Dekundy *et al.* 2007), in monkeys (Gomez-Mancilla and Bedard 1993) and PD patients (Carpentier *et al.* 1996). Recently, the α_1 adrenoceptor antagonist prazosin demonstrated a reduction of L-DOPA-induced hyperactivity in MPTP-treated macaques. However, dyskinesia provoked by L-DOPA was not affected (Visanji *et al.* 2009).

The aim of the present study was to investigate the antidyskinetic properties of the selective α_1 adrenoceptor antagonist 2-[[β-(-4-hydroxyphenyl)ethyl]aminomethyl]-1-tetralone (HEAT) in hemiparkinsonian rats. Additionally, the selective α_1 adrenoceptor agonist cirazoline was administered locally into the lesioned striatum to study if α_1 adrenoceptor activation is able to provoke dyskinesia.

MATERIALS AND METHODS

Animals, 6-OHDA lesion surgery, Induction and rating of dyskinesia

Please see chapter II “General methodology” page 22.

Pharmacological antagonism of L-DOPA-induced dyskinesia

The α_1 adrenoceptor antagonist HEAT, the α_2 adrenoceptor antagonist idazoxan and the non-selective β_1/β_2 adrenoceptor antagonist propranolol were investigated for their effects on L-DOPA-induced dyskinesia. The compounds were tested in a blind, randomised, repeated measurement design. Between the different days of testing, a washout period of four days was included. The rats were treated with L-DOPA/benserazide (6/15 mg/kg, p.o.) once daily to maintain stable dyskinetic behaviour. Before each testing day, the rats were matched according to their total dyskinesia score to exclude compound effects from the last testing day. Each group of rats exhibits approximately the same mean of the total dyskinesia score as well as the same SEM to make sure that the initial conditions were the same. All compounds

were dissolved in saline and administered 30 minutes (i.p.) prior to L-DOPA/benserazide (6/15 mg/kg, p.o.). Amantadine (40 mg/kg, i.p.) served as a positive control (n = 8). HEAT was given at doses of 0.5 mg/kg (n = 8), 1 mg/kg (n = 8) and 2 mg/kg (n = 8). Idazoxan was administered at doses of 6 mg/kg (n = 8) and 9 mg/kg (n = 8). Propranolol was injected at doses of 10 mg/kg (n = 8) and 20 mg/kg (n = 8). Dyskinesia including axial, limb and orolingual subtype was monitored every 20 minutes for 240 minutes following administration of L-DOPA/benserazide by an "experimentally blinded" observer not aware of the experimental group alignment.

Assessment of motor side effects of adrenoceptor antagonists and amantadine

The measurement of the exploratory behaviour in rats was used to determine potential motor side effects of the different adrenoceptor antagonists. Exploratory behaviour was detected in the open field system Actimot™ (TSE Systems GmbH, Bad Homburg, Germany) in the dark with a hole board inserted in order to increase exploratory activity. Rats were placed individually in the centre of the activity box (46.5 cm x 46.5 cm) and horizontal motor activity (s) was determined at 1 minute intervals by infrared sensor pairs (interspace 1.4 cm) with a sampling rate of 100 Hz.
A total number of 96 naive rats was used in this experiment. The following compounds and doses were used: amantadine at 20 mg/kg (n = 6) and 40 mg/kg (n = 6); HEAT at 0.5 mg/kg (n = 6), 1 mg/kg (n = 6) and 2 mg/kg (n = 6); idazoxan at 6 mg/kg (n = 6) and 9 mg/kg (n = 6); propranolol at 10 mg/kg (n = 6) and 20 mg/kg (n = 6). The drugs were administered (i.p.) 30 minutes prior to the rats being placed individually in the open field system. Horizontal activity (s) was measured immediately afterwards for 20 minutes.

Reverse in vivo microdialysis surgery in the striatum

Please see chapter II "General methodology" page 24.

Intrastriatal administration of the α_1 adrenoceptor agonist cirazoline via reverse in vivo microdialysis in the striatum

Please see chapter II "General methodology" (Reverse in vivo microdialysis procedure) page 25. Following insertion of the probes and an equilibration period, the selective α_1 adrenoceptor agonist cirazoline hydrochloride (0.4 nmol/min, n = 4) was

perfused in the lesioned striatum for 60 minutes followed by an aCSF perfusion for 2 hours.

Sample collection procedure

Please see chapter II “General methodology” (CSF, plasma and brain sample collection procedure) page 25. The α_1 adrenoceptor antagonist HEAT (2 mg/kg, i.p.) was administered to naive rats. At time points 0.5 h (n = 6), 1 h (n = 6), 2 h (n = 6), 4 h (n = 6) and 8 h (n = 6), samples of CSF, blood and brain tissue were taken.

Sample preparation procedure

Brain tissue was homogenised using Dispomix™ 25 tubes and processed as described in chapter II “General methodology” page 25.

LC-MS/MS analysis of plasma, brain and CSF samples

Please see chapter II “General methodology” (LC-MS/MS system) page 26. Samples were analysed for HEAT using LC-MS/MS. Mobile phase “A” and “B” consisted of 0.1 % formic acid in LC-MS grade water and acetonitrile, respectively. The gradient was chosen as follows: 0.00 min: 95 % A, 0.10 min 95 % A, 1.00 min 0 % A, 1.40 min 0 % A, 1.50 min 95 % A, 2.00 min 95 % A and delivered at 0.5 ml/min onto a reversed-phase column (YMC-Pack ProC18, 50 x 2.1 mm i. d., 5 µm particles, YMC Europe GmbH, Dinslaken, Germany) at 20 °C. The column switching valve was set at 0.00 min to the waste, at 0.80 min to the mass spectrometer and at 1.40 min to the waste again.

Three transitions were chosen: 296-150 (declustering potential (DP) 61 V, collision energy (CE) 21 V, cell exit potential (CXP) 10 V), 296-121 (DP 61 V, CE 35 V, CXP 8 V), 296-77 (DP 61 V, CE 89 V, CXP 6 V) and transition 296-150 was used for the quantification of HEAT.

Drugs and chemicals

Please see chapter II “General methodology” page 26.

Statistical analysis

Please see chapter II “General methodology” page 27. To compare rating scores of L-DOPA-induced dyskinesia, a Kruskal-Wallis test was carried out followed by a Mann Whitney test.

In order to show not only a comparison between the dyskinesia scores of different treatment groups, but also the existence or absence of dyskinesia, a theoretical mean was assumed. The theoretical mean was defined as 0.0, which is equivalent to the pre dyskinesia score. A one-sample t-test was performed to compare dyskinesia scores with the theoretical mean.

The measurement of exploratory behaviour was analysed by two-way ANOVA with time as a dependent factor and dose as an independent factor followed by a Bonferroni post hoc test.

RESULTS

Effects of adrenoceptor antagonists on L-DOPA-induced dyskinesia

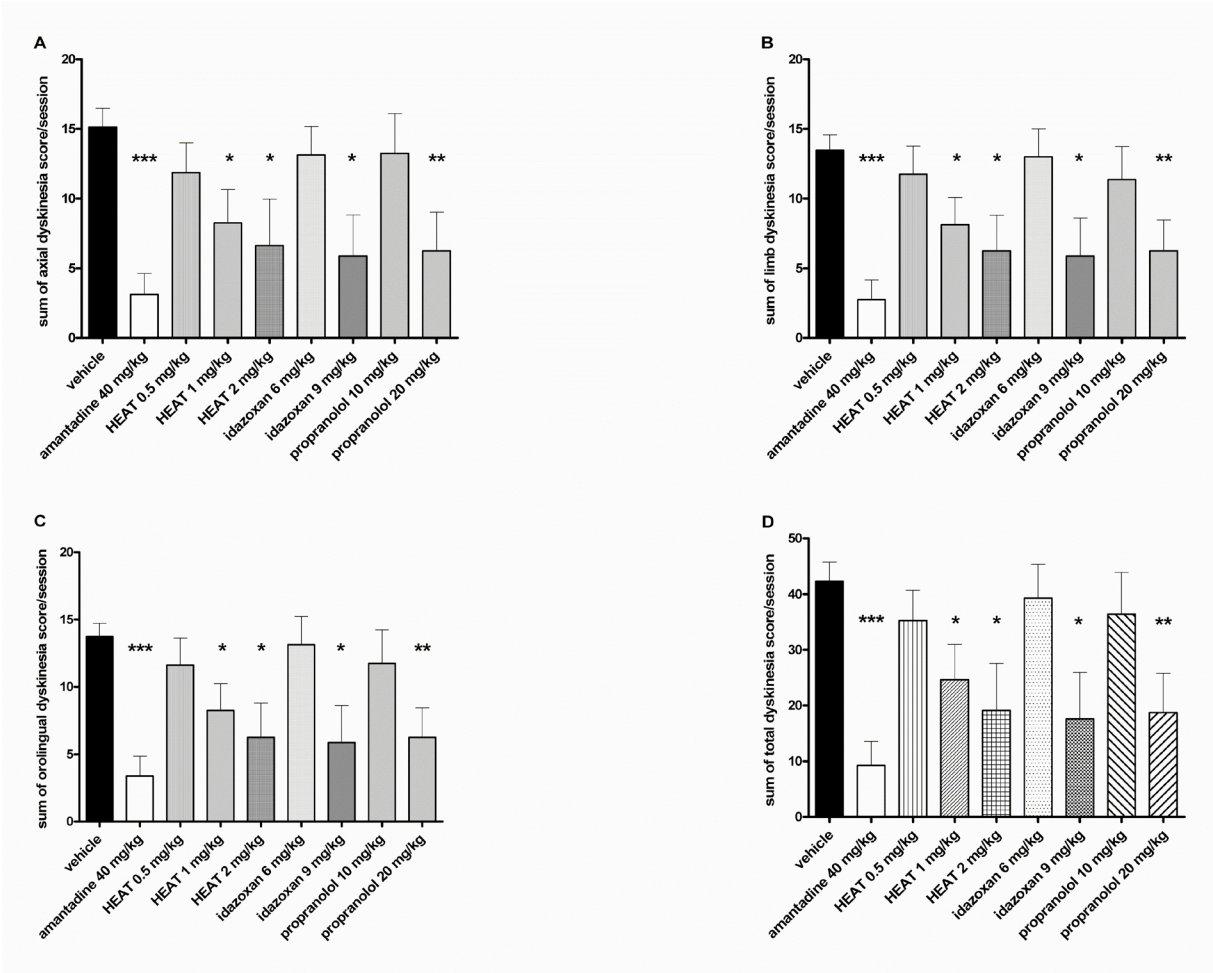


Fig. 9. Effects of adrenoceptor antagonists on L-DOPA-induced dyskinesia. The compounds were tested in a blind, randomised, cross-over design. Vehicle (n = 15), amantadine (40 mg/kg (n = 8)), HEAT (0.5 mg/kg (n = 8), 1 mg/kg (n = 8), 2 mg/kg (n = 8)), idazoxan (6 mg/kg (n = 8), 9 mg/kg (n = 8)) and propranolol (10 mg/kg (n = 8), 20 mg/kg (n = 8)) were administered 30 minutes (i.p.) prior to L-DOPA/benserazide (6/15 mg/kg, p.o.). Data are expressed as mean ± SEM and were analysed by a Kruskal-Wallis test followed by a Mann Whitney test .for statistical comparison of the treatments (***P < 0.001, **P < 0.01, *P < 0.05).

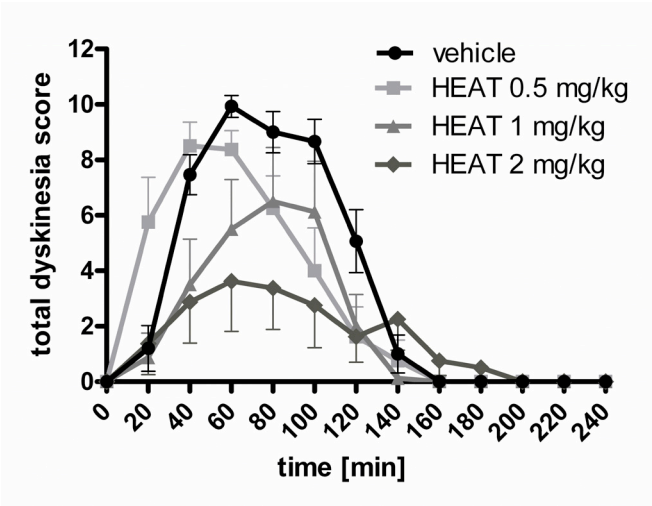


Fig. 10. Time course of total dyskinesia score following administration of HEAT (0.5 mg/kg (n = 8), 1 mg/kg (n = 8), 2 mg/kg (n = 8)) and vehicle (n = 15) 30 minutes (i.p.) prior to L-DOPA/benserazide (6/15 mg/kg, p.o.). Data are expressed as mean ± SEM.

Different adrenoceptor antagonists were investigated for their effects on L-DOPA-induced dyskinesia (Fig. 9 and 10). Statistical analysis displayed a significant effect of treatment on the axial ($P < 0.01$), limb ($P < 0.01$) and orolingual ($P < 0.01$) as well as on the total dyskinesia score ($P < 0.01$). Rats injected with vehicle plus L-DOPA/benserazide (6/15 mg/kg; p.o.) showed a significant induction ($P < 0.001$) of axial (15.1 ± 1.4), limb (13.5 ± 1.1) and orolingual (13.7 ± 1.0) dyskinesia, expressed as total dyskinesia score (42.3 ± 3.4). Amantadine 40 mg/kg revealed a significant reduction in the single dyskinesia scores indicated by axial (3.1 ± 1.5, $P < 0.001$), limb (2.8 ± 1.4, $P < 0.001$) and orolingual (3.4 ± 1.5, $P < 0.001$) dyskinesia as well as in the total dyskinesia score (9.3 ± 4.3, $P < 0.001$) in comparison to vehicle. Furthermore, the α_1 adrenoceptor antagonist HEAT at a dose of 1 mg/kg and 2 mg/kg reduced axial (1 mg/kg: 8.3 ± 2.4, $P < 0.05$; 2 mg/kg: 6.6 ± 3.3, $P < 0.05$), limb (1 mg/kg: 8.1 ± 2.0, $P < 0.05$; 2 mg/kg: 6.3 ± 2.6, $P < 0.05$) and orolingual (1 mg/kg: 8.3 ± 2.0, $P < 0.05$; 2 mg/kg: 6.3 ± 2.6, $P < 0.05$) dyskinetic movements significantly as well as the total dyskinesia score (1 mg/kg: 24.6 ± 6.3, $P < 0.05$; 2 mg/kg: 19.1 ± 8.4, $P < 0.05$). Additionally, treatment with the α_2 adrenoceptor antagonist idazoxan at a dose of 9 mg/kg alleviated axial (5.9 ± 2.9, $P < 0.05$), limb (5.9 ± 2.7, $P < 0.05$) and orolingual (5.9 ± 2.7, $P < 0.05$) dyskinesia significantly as well as the total dyskinesia score (17.6 ± 8.4, $P < 0.05$). Administration of the non selective β_1/ β_2 adrenoceptor antagonist propranolol at a dose of 20 mg/kg showed a significant reduction in the single dyskinesia subtype scores indicated by axial (6.3 ± 2.8, $P < 0.05$), limb (6.3 ± 2.2, $P < 0.05$) and orolingual (6.3 ± 2.2, $P < 0.05$) dyskinesia as well as in total dyskinesia score (18.8 ± 7.1, $P < 0.05$) compared to the vehicle group. The

lowest dose of HEAT (0.5 mg/kg), idazoxan (6 mg/kg) and propranolol (10 mg/kg) did not show a significant alleviation in dyskinesia.

Effects of adrenoceptor antagonists on motor activity

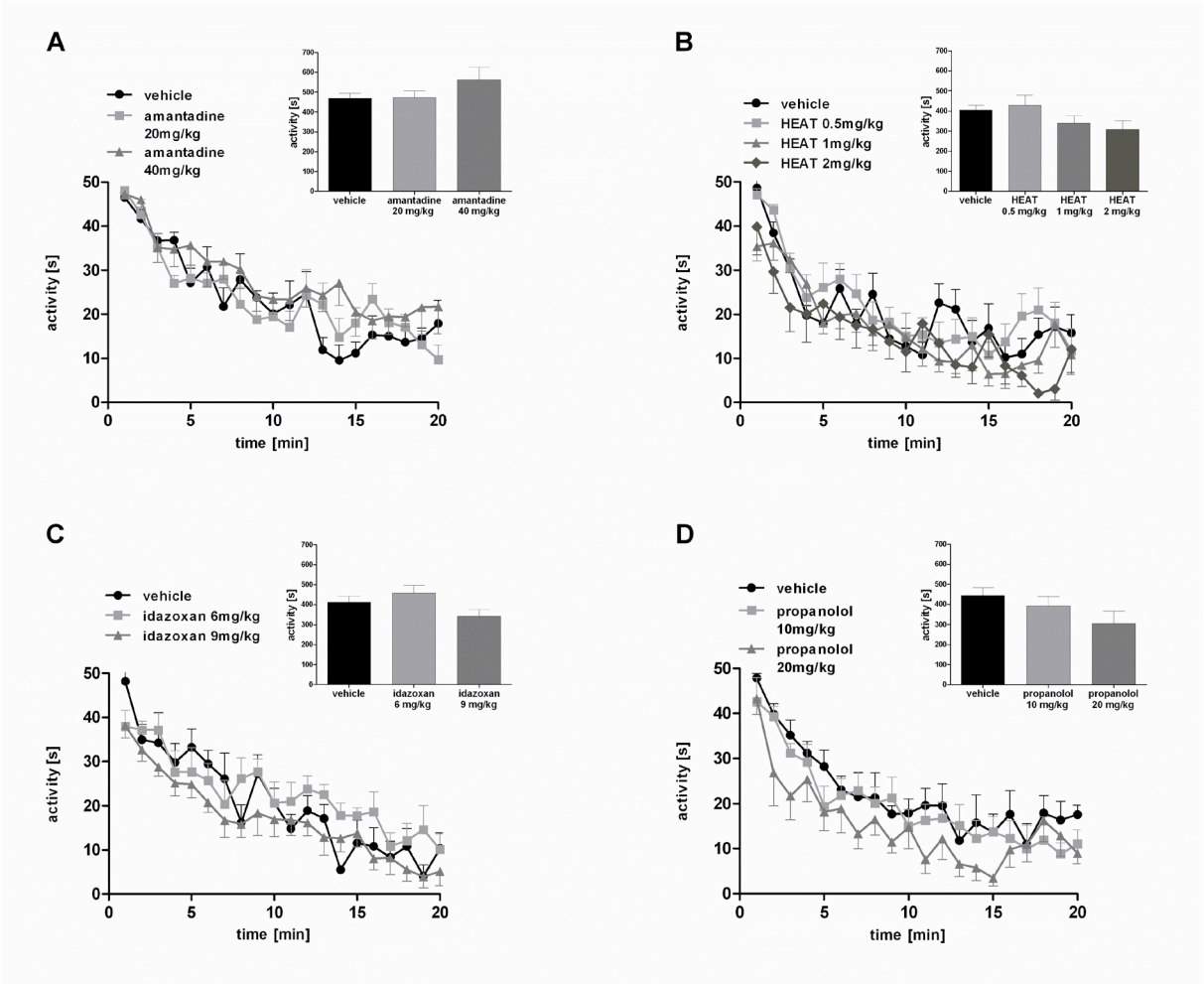


Fig. 11. Effects of the NMDA antagonist amantadine (A), the α_1 adrenoceptor antagonist HEAT (B), the α_2 adrenoceptor antagonist idazoxan (C) and the nonselective β_1/β_2 adrenoceptor antagonist propranolol (D) on exploratory behaviour in naive rats. Rats were injected with amantadine (vehicle (n = 6), 20 mg/kg (n = 6), 40 mg/kg (n = 6)), HEAT (vehicle (n = 6), 0.5 mg/kg (n = 6), 1 mg/kg (n = 6) and 2 mg/kg (n = 6)), idazoxan (vehicle (n = 6), 6 mg/kg (n = 6), 9 mg/kg (n = 6)) and propranolol (vehicle (n = 6), 10 mg/kg (n = 6), 20 mg/kg (n = 6)) 30 minutes (i.p.) prior to being placed into the open field system. The sum of activity over 20 minutes is displayed in a bar graph as an insert to each time course figure. Data are expressed as mean ± SEM and were analysed by two-way ANOVA and the Bonferroni post hoc test. No significant differences between the treatment groups were observed.

Adrenoceptor antagonists were investigated for their effects on exploratory behaviour in naive rats (Fig. 11). None of the adrenoceptor antagonists HEAT, idazoxan or propranolol showed any significant differences in exploratory behaviour compared to the vehicle group ($P > 0.05$) indicating that the compounds do not influence the motor activity of the rats. However, propranolol 20 mg/kg displayed a trend towards decreased exploratory activity, which did not reach statistical significance.

Effects of intrastriatal administration of cirazoline on dyskinetic behaviour

The selective α_1 adrenoceptor agonist cirazoline was perfused into the lesioned striatum of dyskinetic rats via reverse in vivo microdialysis. Intrastriatal treatment with cirazoline did not produce dyskinetic movements (data not shown). However, hypersalivation a α_1-related effect was observed.

Exposure to HEAT in plasma, brain and CSF

time	Plasma ng/ml (nmol/l)	Brain ng/g (nmol/kg)	CSF ng/ml (nmol/l)
0.5h	71.10 ± 39.79 (240.70 ± 134.71)	304.89 ± 101.49 (1032.20 ± 343.59)	10.94 ± 3.34 (37.03 ± 11.30)
1h	56.16 ± 74.59 (190.13 ± 252.51)	229.57 ± 216.79 (777.20 ± 733.93)	6.44 ± 5.96 (21.79 ± 20.18)
2h	6.99 ± 6.65 (23.68 ± 22.52)	31.35 ± 25.16 (106.12 ± 85.18)	0.73 ± 0.65 (2.48 ± 2.20)
4h	2.86 ± 1.79 (9.67 ± 6.06)	17.36 ± 11.94 (58.76 ± 40.41)	0.27 ± 0.49 (0.90 ± 1.67)
8h	1.02 ± 0.41 (3.47 ± 1.37)	6.54 ± 2.49 (22.14 ± 8.43)	0.10 ± 0.24 (0.33 ± 0.80)

Table 4. Plasma, brain and CSF levels of HEAT (2 mg/kg) at 0.5 h (n = 6), 1 h (n = 6), 2 h (n = 6), 4 h (n = 6) and 8 h (n = 6) following administration (i.p.) in naive rats. Data are expressed as mean ± SD.

The selective α_1 adrenoceptor antagonist HEAT was injected into naive rats. The concentration of HEAT was determined in plasma, brain and CSF at 0.5 h, 1 h, 2 h, 4

h and 8 h post administration (Table 4). Plasma, brain as well as CSF t_{max} values were observed at the 0.5 h time point. C_{max} values for plasma, brain and CSF were 71.10 ± 39.79 ng/ml, 304.89 ± 101.49 ng/g and 10.94 ± 3.34 ng/ml, respectively. The brain/plasma and CSF/plasma ratio at t_{max} was found to be 4.29 and 0.15, respectively.

DISCUSSION

The present study suggests that α_1 adrenoceptor antagonists play a role in L-DOPA-induced dyskinesia. We demonstrated that the selective α_1 adrenoceptor antagonist HEAT alleviated dyskinetic movements as well as the α_2 adrenoceptor antagonist idazoxan and the non-selective β_1/β_2 antagonist propranolol, whilst showing no confounding motor depressant effects.

During the preparation of this manuscript, Visanji *et al.* (2009) investigated the α_1 adrenoceptor antagonist prazosin in MPTP-lesioned macaques on motor behaviour. Assessment of L-DOPA-induced hyperactivity resulted in a 1.5-fold increase in L-DOPA-treated MPTP monkeys in comparison to normal, non MPTP-lesioned animals. Dyskinesia was scored by a well established rating scale (Hill *et al.* 2004). L-DOPA led to only a mild to moderate level of dyskinesia comprising predominantly of dystonia, but was in the absence of the choreic form of dyskinesia. Prazosin reduced L-DOPA-induced hyperactivity, whilst not significantly affecting dyskinetic movements. However, the authors discussed that due to the limited group size of the monkey study and the diversity of the phenomenology, an effect on one form of dyskinesia cannot be excluded (Visanji *et al.*, 2009). Our results are in contrast to the conclusion of Visanji *et al.* (2009) that α_1 adrenoceptors plays no major role in dyskinetic effects of L-DOPA per se.

Recently, Paquette *et al.* (2009) observed no effect of prazosin (0.1 mg/kg) on L-DOPA-induced dyskinesia in the 6-OHDA rat model. However, using a single dose of prazosin an effect on dyskinesia cannot be entirely excluded since a higher dose may show antidyskinetic efficacy. HEAT (0.5 mg/kg) was found not to be effective whereas HEAT (1 and 2 mg/kg) significantly reduced dyskinesia scores. Additionally, Hill and Brotchie (1999) demonstrated that prazosin at higher doses (1, 3 mg/kg) potentiated the antiparkinsonian effect of a non-dopaminergic drug.

Prazosin and HEAT are both potent inhibitors of the α_1 adrenoceptor, showing a K_i value of 0.1 nM with equal affinity for the α_{1A}, α_{1B} and α_{1D} subtype and their radio ligands are widely used in α_1 binding studies (Alexander *et al.* 2005). At high doses, prazosin may also act on other receptors such as the α_{2B} and α_{2C} adrenoceptor (Bylund *et al.* 1994) as well as the melatonin MT_3 receptor (Paul *et al.* 1999).

Most of the research concerning α_1 adrenoceptor antagonists has been performed in peripheral organs, particularly in the cardiovascular system. Here we identified that HEAT is appropriate for the investigation of central α_1-dependent mechanisms. We clearly demonstrated that HEAT crosses the blood brain barrier as indicated by a brain/plasma ratio of 4.29. Notably, HEAT achieves a high CSF exposure. CSF levels appear to be reasonably accurate to predict unbound drug concentration in the brain, which has been proven by preclinical and clinical studies. Hence, CSF exposure is a relevant surrogate for the in vivo assessment of CNS druggability (Lin 2008). This is of relevance for the assumption that the free drug level is sufficient for interaction with its receptor target.

In the present study, we demonstrated that HEAT reduced dyskinetic movements provoked by treatment with L-DOPA in 6-OHDA-lesioned rats. HEAT (1 and 2 mg/kg) significantly decreased axial, limb as well as orolingual dyskinetic movements, whereas no reduction in exploratory behaviour was observed indicating that the antidyskinetic action of HEAT is not due to a general motor depressant effect. We used exploratory behaviour to detect potential motor depressant effects and found that the automated open field system equipped with hole boards make the system more sensitive for monitoring alterations within the first 20 minutes. To reduce variability, we carried out this test in the dark. In order to increase the predictive validity of the dyskinesia model, it is necessary to control confounding factors. One relevant confounding factor may be alterations in motor activity. Recently, Jenner (2008b) pointed out that drugs causing sedation or altering cardiovascular parameters or altering muscle tone along with many others can worsen the effects of motor activity and alter the expression of dyskinesia in the MPTP-treated primate model. Indeed, the same factors are also relevant in the rat dyskinesia model. HEAT was free of motor depressant effects at the doses used as indicated by measurement of exploratory activity. In a preliminary study HEAT (2mg/kg, i.p.) administered 30 minutes prior to L-DOPA/benserazide (6/15 mg/kg, p.o.) reduced the total dyskinesia score in rats which received for the first time L-DOPA/benserazide. This effect was

accompanied by a reduction of automated motor activity measured by light beam interruptions reflecting both hyperactivity and dyskinetic movements. Although HEAT did not reduce exploratory behaviour in non lesioned rats (Fig. 11) we cannot rule out that in 6-OHDA-lesioned rats part of the antidyskinetic affect might be mediated by a reduction of L-DOPA-induced hyperactivity which is more prominent in lesioned than in intact rats. Additionally, α_1 adrenoceptor antagonists are clinically used for treatment of arterial hypertension suggesting that a higher dose of HEAT may provoke side effects such as sedation and hypotension. Further studies are needed to clarify the evaluation of α_1 adrenoceptor antagonists in animal models of cardiovascular dysfunction including measurement of blood pressure and heart rates and to investigate a potential interaction of α_1 adrenoceptor antagonists with the beneficial effects of L-DOPA.

Recently, local administration of NA in the DA-denervated striatum has been found to produce dyskinetic movements in rats (Buck and Ferger 2009a). In dyskinesia, the neurotransmitter NA may derive from exogenously applied L-DOPA and binds to α_1 adrenoceptors. Thus, the α_1 adrenoceptor antagonist HEAT may act via competitive antagonism of the neurotransmitter NA. To investigate if a direct α_1-mediated mechanism is underlying the appearance of dyskinesia, the effect of the selective α_1 adrenoceptor agonist cirazoline on dyskinetic movements was assessed by local perfusion into the lesioned striatum of rats. The model of administration of potentially dyskinesiogenic compounds into the 6-OHDA-lesioned striatum via reverse in vivo microdialysis has been validated using several compounds. It has been demonstrated that L-DOPA, DA as well as NA induces dyskinetic behaviour after perfusion into the lesioned striatum (Buck and Ferger 2008; Buck and Ferger 2009a; Carta *et al.* 2006). Administration of the selective α_1 adrenoceptor agonist cirazoline into the lesioned striatum did not produce any dyskinetic movements suggesting that activation of α_1 adrenoceptors itself does not play a role in the appearance of dyskinesia. Recently, the role of noradrenergic α_1 and α_2 receptors on subthalamic neuron firing rate in intact and 6-OHDA-lesioned rats has been elaborated. Belujon *et al.* (2007) demonstrated that at least in part motor activity is affected by presynaptic α_2 and postsynaptic α_1 adrenoceptor receptors in the STN. Thus, we cannot exclude that cirazoline may provoke its dyskinesiogenic effect via α_1 adrenoceptors brain structures in other than the striatum. Theoretically, HEAT may exert its antidyskinetic effect via modulation of noradrenergic activity in the STN.

It was previously shown that the α_1 adrenoceptor antagonist prazosin attenuated DA release after striatal administration, whereas systemic prazosin did not result in a decrease of striatal DA release (Sommermeyer *et al.* 1995). Conversely, evoked DA release after stimulation with NA (Pan *et al.* 2004), MK-801 (Mathe *et al.* 1996) and morphine (Auclair *et al.* 2004) was reduced by prazosin as measured by in vivo microdialysis. We hypothesize that HEAT attenuates the stimulated DA overflow derived from exogenously applied L-DOPA in the striatum. This mechanism may contribute to the alleviation of dyskinesia by decreasing pulsatile DA receptor stimulation which is regarded as an important factor in dyskinesia.

Previous experimental studies have demonstrated that α_2 adrenoceptor antagonists such as the traditional drug yohimbine reduced L-DOPA-induced dyskinesia in rodent (Dekundy *et al.* 2007; Lundblad *et al.* 2002) as well as primate models (Gomez-Mancilla and Bedard 1993). The present study showed that the more selective α_2 adrenoceptor antagonist idazoxan alleviated dyskinetic movements in 6-OHDA-lesioned rats in a similar manner to HEAT, without reducing exploratory activity. Idazoxan has not been previously tested in the 6-OHDA-lesioned rat model of L-DOPA-induced dyskinesia. However, the drug was investigated in a simple rodent assay for the in vivo identification of agents with potential to reduce L-DOPA-induced dyskinesia (Johnston *et al.* 2005). Idazoxan was found to selectively reduce L-DOPA-induced vertical activity in reserpinised rats, which was indicated to be a surrogate marker for putative antidyskinetic compounds. Idazoxan was effective at a dose of 3 mg/kg, which is 3-fold lower than the antidyskinetic dose in the present study. This discrepancy may be due to the different model and/or rat strain differences. Additionally, idazoxan alleviated dyskinetic movements in the MPTP-lesioned primate model without compromising the anti-parkinsonian response to L-DOPA (Fox *et al.* 2001; Grondin *et al.* 2000; Henry *et al.* 1999). However, the dyskinetic behaviour induced by treatment with the DA agonist apomorphine was not affected by idazoxan suggesting that the apomorphine-induced dyskinesia does not involve α_2 adrenoceptors (Fox *et al.* 2001). This discrepancy may be explained by the fact that L-DOPA, in contrast to apomorphine, can be metabolised to NA. Thus, it can be hypothesized that NA derived from exogenous L-DOPA may contribute to L-DOPA-induced dyskinesia. Concordantly, it was recently shown, that intrastriatal NA can induce dyskinetic movements by itself in a similar manner to L-DOPA in primed 6-OHDA-lesioned rats (Buck and Ferger 2009a). Moreover, the clinical effects of

idazoxan were investigated in patients with advanced PD and it was shown that dyskinesia severity improved without compromising the beneficial response to L-DOPA. However, the treatment with idazoxan resulted in adverse side effects including hypertension, tachycardia, flushing and headache (Rascol *et al.* 2001).

In the present study, the non selective β_1/β_2 adrenoceptor antagonist propranolol was found to be antidyskinetic at the dose of 20 mg/kg. Our results are in agreement with a previous study of Dekundy *et al.* (2007), which reported that propranolol reduced dyskinetic movements in the 6-OHDA rat model. Dekundy *et al.* (2007) additionally showed a trend towards a reduction in the motor performance of rats in the rotarod test at the highest dose of propranolol (20 mg/kg), which is again in agreement with results in the exploratory behaviour test of our study. Furthermore, propranolol was found to be effective against L-DOPA-induced dyskinesia in PD patients (Carpentier et al., 1996) and in MPTP-treated monkeys (Gomez-Mancilla and Bedard, 1993).

Amantadine, which was included as a positive control, reduced dyskinesia scores in a similar manner as previously shown by other groups (Dekundy *et al.* 2007; Lundblad *et al.* 2002).

In conclusion, the present study provides the first evidence that α_1 adrenoceptor antagonists play a role in L-DOPA-induced dyskinesia, which is in contrast to Visanji *et al.* (2009), who did not show an effect of the α_1 adrenoceptor antagonist prazosin on dyskinesia. The selective α_1 adrenoceptor antagonist HEAT reduced axial, limb and orolingual dyskinesia in 6-OHDA-lesioned rats, whilst showing no confounding motor depressant effects. HEAT is qualified for the investigation of α_1–mediated mechanisms in CNS disorders as demonstrated by measurement of plasma, brain and CSF HEAT levels.

CHAPTER VI

The α_2 adrenoceptor antagonist idazoxan alleviates L-DOPA-induced dyskinesia by reduction of striatal dopamine levels

ABSTRACT

L-DOPA-induced dyskinesia is characterised by involuntary debilitating movement, which limits quality of life in patients suffering from Parkinson's disease. Here we investigate effects of the α_2 adrenoceptor antagonist idazoxan on L-DOPA-induced dyskinesia as well as on alterations of extracellular L-DOPA and DA levels in the striatum in dyskinetic rats.

Male Wistar rats were unilaterally lesioned with 6-OHDA and subsequently treated with L-DOPA/benserazide to induce stable dyskinetic movements. Administration of idazoxan (9mg/kg, i.p.) significantly alleviated L-DOPA-induced dyskinesia, whereas idazoxan (3mg/kg, i.p.) did not affect dyskinetic behaviour. Bilateral in vivo microdialysis revealed that idazoxan 9mg/kg reduces extracellular peak L-DOPA levels in the lesioned and intact striatum as well as DA levels in the lesioned striatum. In parallel, the exposure to idazoxan in the striatum was monitored. Furthermore, no idazoxan and L-DOPA drug-drug interaction was found in plasma, brain tissue and CSF.

In conclusion, the decrease of L-DOPA-derived extracellular DA levels in the lesioned striatum significantly contributes to the antidyskinetic effect of idazoxan.

INTRODUCTION

Motor impairments associated with PD are the result of a massive depletion of striatal DA due to a progressive loss of dopaminergic neurons within the SNc (Ehringer and Hornykiewicz 1960; Riederer and Wuketich 1976). Consequently, the primary therapeutic treatment strategy involves the restoration of dopaminergic neurotransmission in the basal ganglia. Many dopaminergic drugs are currently available but none have surpassed the clinical efficacy derived from the DA precursor L-DOPA, which remains the most effective anti-parkinsonian compound since its introduction in the late 1960s (Cotzias *et al.* 1969). However, long term use of L-DOPA can eventually result in serious treatment-limiting motor complications, namely dyskinesia which comprises mainly of choreic and dystonic movements. Dyskinesia

develops progressively over time and up to 40% of PD patients suffer from dyskinesia within 4-6 years of L-DOPA therapy (Ahlskog and Muenter 2001).

At present the non-competitive NMDA glutamatergic antagonist amantadine is the only compound which is clinically used to alleviate L-DOPA-induced dyskinesia but its success has been limited (Stocchi *et al.* 2008). Thus, there is an unmet medical need to identify novel mechanisms for the treatment of dyskinesia in PD. Additionally, the predictive validity of preclinical animal models of L-DOPA-induced dyskinesia has to be further improved to promote drug discovery. During the progression of PD, the buffering capacity of DA, released from exogenously applied L-DOPA, disappears. As a consequence peak DA levels, which are suspected to cause peak dyskinesia, evolve. However, reduction of peak DA levels by simply reducing the L-DOPA dose is not the ultimate solution since a sufficient DA receptor stimulation has to be maintained. Indeed, achieving the balance between high extracellular DA levels which prone dyskinesia and low DA levels which cause the cardinal symptoms of PD is a major challenge in late stage PD.

There is pharmacological evidence that noradrenergic neurotransmission is involved in the pathogenesis of L-DOPA-induced dyskinesia and antiadrenergic compounds may positively affect PD symptomatology. The α_2 adrenoceptor antagonist idazoxan has shown antidyskinetic efficacy in monkey studies (Fox *et al.* 2001; Grondin *et al.* 2000). Moreover, in clinical trials idazoxan reduced L-DOPA-induced dyskinesia without affecting the L-DOPA mediated relief of symptoms (Henry *et al.* 1999; Rascol *et al.* 2001). Furthermore, fipamezole, another α_2 adrenoceptor antagonist targeting $\alpha_{2A,}$ α_{2B} and α_{2C} receptors, has been investigated in a phase II study in PD patients suffering from L-DOPA-induced dyskinesia. Fipamezole, as idazoxan, was able to reduce dyskinesia scores without compromising the anti-parkinsonian action of L-DOPA.

The mechanism of alleviation of L-DOPA-induced dyskinesia by these anti-adrenergic drugs remains elusive. The reduction of NA synthesised from L-DOPA is thought to be involved (Fox *et al.* 2001). This hypothesis is due to the fact that dyskinesia evoked by DA agonists like apomorphine cannot be reduced by a α_2 adrenoceptor antagonist as DA agonists cannot be metabolised to NA. Moreover, the anti-parkinsonian action of L-DOPA was prolonged by idazoxan whilst the combination of idazoxan and apomorphine did not result in an extension of the anti-parkinsonian effect (Fox *et al.* 2001). Recently, we studied the role of the α_1

adrenoceptor antagonist HEAT in the rat dyskinesia model and found an antidyskinetic effect in the range of other antiadrenergic drugs (Buck and Ferger 2009).

The present study was performed to investigate the effect of the α_2 adrenoceptor antagonist idazoxan on dyskinetic behaviour and neurochemical alterations of L-DOPA and DA levels following administration of L-DOPA/benserazide in the rat L-DOPA dyskinesia model. Extracellular levels of L-DOPA and DA were assessed using bilateral in vivo microdialysis technique in the lesioned as well the intact striatum using HPLC-ECD. In parallel, levels of idazoxan were monitored using LC-MS/MS. Additionally, the exposure to idazoxan plus L-DOPA was investigated in plasma, brain tissue and CSF to exclude a drug-drug interaction which might give false positive receptor mediated effects.

MATERIALS AND METHODS

Animals, 6-OHDA lesion surgery, Induction and rating of dyskinesia

Please see chapter II “General methodology” page 22.

In vivo microdialysis surgery in the striatum

Please see chapter II “General methodology” page 23.

In vivo microdialysis procedure in the striatum

Please see chapter II “General methodology” page 24. Fractions 1 to 4 (0 - 80 min) were used for calculation of the basal levels which were regarded as 100 %. At 90 minutes, idazoxan hydrochloride (9 mg/kg, i.p. (n = 8) and 3 mg/kg, i.p. (n = 8)) and vehicle (saline, i.p. (n = 8)) was administered, respectively. Thirty minutes later (120 min), L-DOPA (6 mg/kg, p.o.) in combination with benserazide (15 mg/kg, p.o.) was administered and the sampling was continued for 5 hours (up to 420 min).

Assessment of pharmacokinetics of L-DOPA and idazoxan

Please see chapter II “General methodology” (CSF, plasma and brain sample collection procedure) page 25. Idazoxan hydrochloride (9 mg/kg, i.p. (n = 4)) and vehicle (i.p. (n = 4)) were administered 30 minutes prior to the treatment with L-

DOPA/benserazide (6/15 mg/kg, p.o.) to naive rats. Forty minutes following L-DOPA administration samples of CSF, blood and brain tissue were taken.
One hemisphere of the brain was homogenised by sonication and processed as previously described in chapter II “General methodology” (Sample preparation procedure) page 25.

HPLC analysis of microdialysis samples (L-DOPA and DA)

Please see chapter II “General methodology” (HPLC system) page 26. Samples were splitted for HPLC (45 µl) and LC-MS/MS (5 µl) analysis. Microdialysis samples were analysed for L-DOPA as well as DA using HPLC combined with ECD under isocratic conditions. Chromatographic separation was performed using a reversed-phase column (Grom-Sil 120 ODS-4 HE, 250 x 4.0 mm i.d., 5 µm, Grace Davison Discovery Sciences™, Lokeren, Belgium). The mobile phase consisted of 1.85 mM 1-octanesulfonic acid sodium salt, 0.13 mM $Na_2EDTA \times 2\ H_2O$, 8.00 mM NaCl, 57.51 mM NaH_2PO_4, 10 % acetonitrile, adjusted to pH 2.50 with H_3PO_4, filtered through a 0.22 µm filter, and was delivered at a flow rate of 1 ml/min.

LC-MS/MS analysis of microdialysis and plasma, brain and CSF samples (idazoxan)

Please see chapter II “General methodology” (LC-MS/MS system) page 26. Microdialysis as well as plasma, brain and CSF samples were analysed for idazoxan using LC-MS/MS.). Mobile phase “A” and “B” consisted of 0.1 % formic acid in water and acetonitrile (LC-MS grade), respectively. The gradient elution profile was chosen as follows: 0 min: 15 % A (1000 µl/min), 0.10 min: 15 % A (200 µl/min), 1.00 min: 80 % A (200 µl/min), 1.50 min: 80 % A (200 µl/min), 1.60 min: 15 % A (200 µl/min), 2.40 min: 15 % A (200 µl/min), 2.50 min: 15% A (1000 µl/min), 3.00 min: 15 % A (1000 µl/min). Chromatographic retention was obtained using a hydrophilic interaction liquid chromatography (HILIC) column (ZIC®-HILIC, 20 x 2.1 mm i.d., 3.5 µm, SeQuant AB, Umeå, Sweden) with a pre-microfilter (0.5 µm, Upchurch Scientific, Oak Harbor, WA, USA) at 20°C. The column switching valve was set at 2.50 minutes to the waste.
Two transitions were chosen: 205-97 (DP 71 V, CE 33 V, CXP 6 V) and 205-54 (DP 71 V, CE 71 V, CXP 10 V). Transition 205-97 was used for the quantification of idazoxan.

LC-MS/MS analysis of plasma, brain and CSF samples (L-DOPA)

Please see chapter II “General methodology” (LC-MS/MS system) page 26. Plasma, brain and CSF samples were analysed for L-DOPA using LC-MS/MS. Mobile phase “A” and “B” consisted of 0.1 % formic acid in water (LC-MS grade) and methanol (LC-MS grade), respectively, and was delivered at 300 µl/min. The gradient elution profile was chosen as follows: 0 min: 95 % A, 0.50 min: 95 % A, 2.00 min: 5 % A, 2.40 min: 5 % A, 2.60 min: 95 % A, 9.00 min: 95 % A. Chromatographic retention was obtained using a reversed-phase column (Zorbax Eclipse XDB-C18, 150 x 4.6 mm i.d., 5 µm, Agilent Technologies, Morges, Switzerland) with a pre-microfilter (0.5 µm, Upchurch Scientific, Oak Harbor, WA, USA) at 20°C.

Three transitions were chosen: 198-181 (DP 51 V, CE 15 V, CXP 12 V), 198-107 (DP 51 V, CE 37 V, CXP 8 V) and 198-135 (DP 51 V, CE 27 V, CXP 8 V). Transition 198-181 was used for the quantification of L-DOPA. [D_3]-L-DOPA was used as internal standard and analysed using the transition 201-154 (DP 56 V, CE 19 V, CXP 12 V).

Drug and chemicals

Please see chapter II “General methodology” page 26.

Statistical analysis

Please see chapter II “General methodology” page 27. The time course of total dyskinesia was analysed by a two-way ANOVA for repeated measurements with time as dependent factor and dose as independent factor followed by a Bonferroni post hoc test. A one-way ANOVA with treatment as an independent factor was carried out to compare the sum of total dyskinesia scores followed by a two-tailed t-test.

In general, behavioural scores are regarded to be analysed using non-parametric statistics due the ordinal scaling associated with the scores. However, dyskinesia scores reflect a proportion of time in which a specific kind of behaviour is observed. Therefore, he dyskinesia scores can be treated as almost proportional scoring data, in which the score 4 can be assumed to be twice as large as the score 2. Thus, we analysed dyskinesia score using parametric statistics (Cenci and Lundblad 2007). Additionally, this the advantage that lower animal numbers are needed for parametric statistics which is in agreement with animal welfare and for the purpose of the ethical guidelines of AAALAC.

A two-way ANOVA with lesion as the dependent factor and treatment as the independent factor was used to compare basal values between the lesioned and the intact striatum. Statistical analysis of the in vivo microdialysis data was carried out by calculating the area under the curve (AUC) and logarithmising the AUC. A one-way ANOVA with treatment as the independent factor was used to analyse the logarithmised AUCs followed by a two-tailed t-test. For the idazoxan data, the limit of quantitation (LOQ) (10^{-12} M) was used in case of idazoxan levels which were not detectable. The correlation analysis between extracellular idazoxan and L-DOPA levels measured with in vivo microdialysis was performed with the nonparametric Spearman test using the logarithmised AUCs of idazoxan as well as L-DOPA.
The comparison of L-DOPA and idazoxan levels in plasma, brain and CSF was carried out using an unpaired t-test.

RESULTS

Effects of idazoxan on L-DOPA-induced dyskinesia

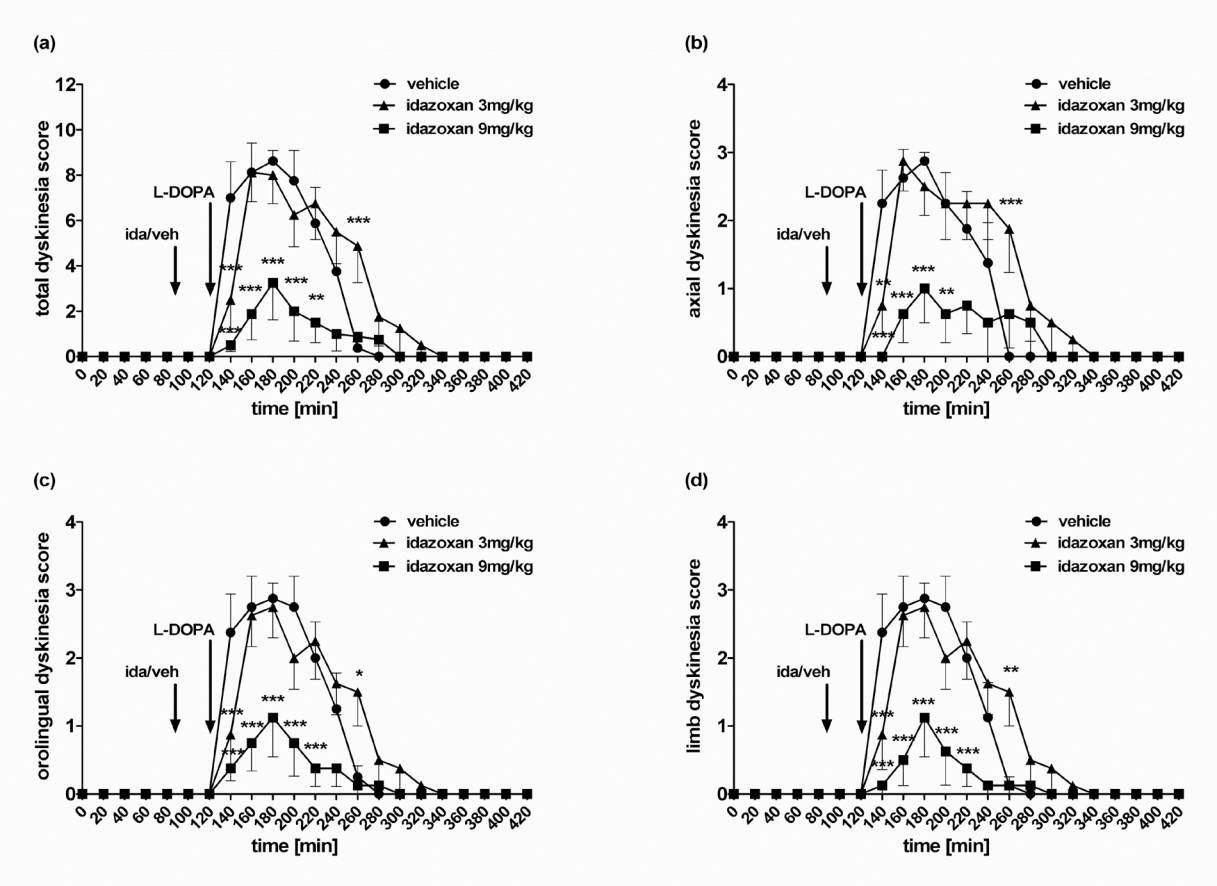


Fig. 12. Effects of idazoxan on total dyskinesia score (a), axial dyskinesia (b), orolingual dyskinesia (c) and limb dyskinesia (d). Idazoxan (9 mg/kg, i.p. (n = 8) and 3 mg/kg, i.p. (n = 8)) and vehicle (saline, i.p. (n = 8)) was administered 30 minutes prior to L-DOPA/benserazide (6/15 mg/kg, p.o.). Dyskinesia was monitored every 20 minutes. Data are expressed as mean ± SEM and were analysed by a two-way repeated measurement ANOVA followed by a Bonferroni post hoc test for statistical comparison of the treatments (***P < 0.001, **P < 0.01, *P < 0.05).

The dyskinesia scores following administration of vehicle and idazoxan 3 and 9 mg/kg in combination with L-DOPA are displayed in Fig. 12. Dyskinetic movements were monitored during the microdialysis experiment every 20 minutes up to 5 hours following treatment with L-DOPA. Statistical analysis of the total dyskinesia time course yielded a significant interaction of time x treatment ($F_{(42,441)}$ = 4.303, P < 0.001) as well as a significant effect on time ($F_{(21,441)}$ = 30.30, P < 0.001) and treatment ($F_{(2,441)}$ = 9.840, p = 0.001) (Fig. 12a). Time interval 140 – 220 minutes showed a significant decrease in total dyskinesia score comparing idazoxan 9 mg/kg

and vehicle (140 – 200 minutes: $P < 0.001$, 220 minutes: $P < 0.01$) indicating an antidyskinetic effect of the α_2 adrenoceptor antagonist idazoxan at dose 9 mg/kg. Statistical comparison of idazoxan 3 mg/kg and vehicle showed a significant effect in total dyskinesia score at time points 140 minutes ($P < 0.001$) and at 260 minutes ($P < 0.001$).

Statistical analysis of the axial dyskinesia time course revealed a significant interaction of time x treatment ($F_{(42,441)} = 3.791$, $P < 0.001$) as well as a significant effect on time ($F_{(21,441)} = 24.78$, $P < 0.001$) and treatment ($F_{(2,441)} = 6.449$, $P < 0.01$) (Fig. 12b). The orolingual dyskinesia time course showed a significant interaction of time x treatment ($F_{(42,441)} = 3.798$, $P < 0.001$) as well as a significant effect on time ($F_{(21,441)} = 29.86$, $P < 0.001$) and treatment ($F_{(2,441)} = 9.988$, $P < 0.001$) (Fig. 12c). The limb dyskinesia time course demonstrated a significant interaction of time x treatment ($F_{(42,441)} = 4.462$, $P < 0.001$) as well as a significant effect on time ($F_{(21,441)} = 29.18$, $P < 0.001$) and treatment ($F_{(2,441)} = 11.77$, $P < 0.001$) (Fig. 12d). Axial dyskinesia score following administration of idazoxan 9 mg/kg was reduced during the time interval 140 – 200 minutes and orolingual as well as limb dyskinesia scores were alleviated during the time interval 140 – 220 minutes using idazoxan 9 mg/kg. A significant effect in axial, orolingual and limb dyskinesia subtypes was observed at time point 140 minutes as well as 260 minutes using idazoxan 3 mg/kg.

In conclusion, idazoxan 9 mg/kg demonstrated a significant alleviation of dyskinesia in contrast to idazoxan 3 mg/kg.

Effects of administration of idazoxan plus L-DOPA on extracellular L-DOPA and DA levels in the striatum

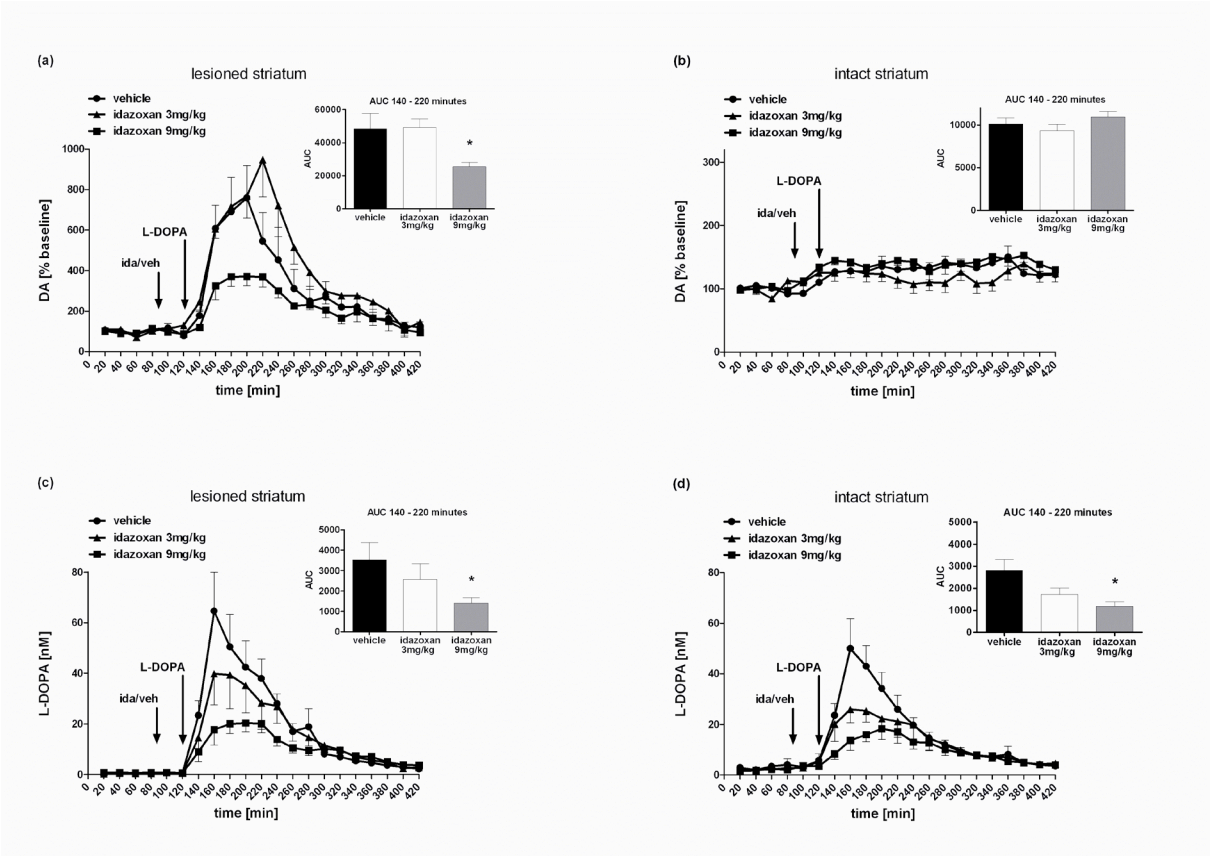


Fig. 13. In vivo microdialysis. Effects of idazoxan (9 mg/kg, i.p. (n = 8) and 3 mg/kg, i.p. (n = 8)) and vehicle (saline, i.p. (n = 8)) on extracellular levels of DA (a, b) and L-DOPA (c, d) in the lesioned (a, c) and intact striatum (b, d) of dyskinetic rats. The AUC of the time interval 140 – 220 minutes is displayed in a bar graph as an insert to each time course figure. Data are expressed as mean ± SEM. Please note the different scales of the y-axes.

In vivo microdialysis samples were analysed for extracellular DA and L-DOPA levels using HPLC-ECD (Fig. 13). In the lesioned striatum, basal levels of DA were 0.24 ± 0.07 nM in the vehicle group, 0.15 ± 0.02 nM in the idazoxan 3 mg/kg group and 0.20 ± 0.04 nM in the idazoxan 9 mg/kg group. In the intact striatum, basal levels of DA were 2.07 ± 0.27 nM in the vehicle group, 2.09 ± 0.16 nM in the idazoxan 3 mg/kg group and 2.48 ± 0.27 nM in the idazoxan 9 mg/kg group. Basal levels on the lesioned as well as on the intact side did not differ significantly between the treatment groups ($F_{(2,21)}$ = 0.9132, p = 0.4166). Statistical comparison demonstrated a significant difference in basal levels of DA between the lesioned and the intact striatum in all groups ($F_{(1,21)}$ = 224.26, P < 0.001).

According to the behavioural data, statistical analysis of the AUC was carried out at the time interval 140 - 220 minutes. Statistical analysis of DA levels in the lesioned striatum yielded a significant treatment effect ($F_{(2,16)}$ = 4.762, P < 0.05) (Fig. 13a). The AUC of DA levels was significantly decreased in the idazoxan 9 mg/kg group compared to the vehicle group (P < 0.05), whereas idazoxan 3 mg/kg showed no significant difference in DA levels in comparison to vehicle (p = 0.6712). Statistical analysis of DA levels on the intact side yielded no significant effect ($F_{(2,21)}$ = 1.382, p = 0.2730) (Fig. 13b) as well as comparison between idazoxan 9 mg/kg and vehicle (p = 0.3768) and idazoxan 3 mg/kg and vehicle (p = 0.4529). Post hoc tests showed a significant decrease of L-DOPA levels between idazoxan 9 mg/kg and vehicle (P < 0.05) on the lesioned side (Fig. 13c), whilst no effect between idazoxan 3 mg/kg and vehicle was observed (p = 0.2722). A significant treatment effect of L-DOPA levels was observed in the intact striatum ($F_{(2,21)}$ = 4.954, P < 0.05) (Fig. 13d). Comparison between idazoxan 9 mg/kg and vehicle demonstrated a significant decrease of L-DOPA levels in the idazoxan group (P < 0.05), whilst idazoxan 3 mg/kg did not show an effect on L-DOPA levels (p = 0.0661).

In conclusion, idazoxan 9 mg/kg revealed a significant decrease of extracellular L-DOPA levels in the lesioned and intact striatum as well as a significant reduction of extracellular DA levels in the lesioned striatum.

Measurement of extracellular idazoxan levels

In vivo microdialysis samples were measured on extracellular idazoxan levels using LC-MS/MS (Fig. 14). Statistical analysis of the AUCs from time point 0 to 420 minutes showed a significant treatment effect on the lesioned side ($F_{(2,21)}$ = 45.77, P < 0.001) as well as on the intact side ($F_{(2,21)}$ = 34.26, P < 0.001). Idazoxan levels in both the idazoxan 3mg/kg group (P < 0.001) and the idazoxan 9mg/kg group (P < 0.001) were significantly increased in comparison to the vehicle group in the lesioned as well as in the intact striatum.

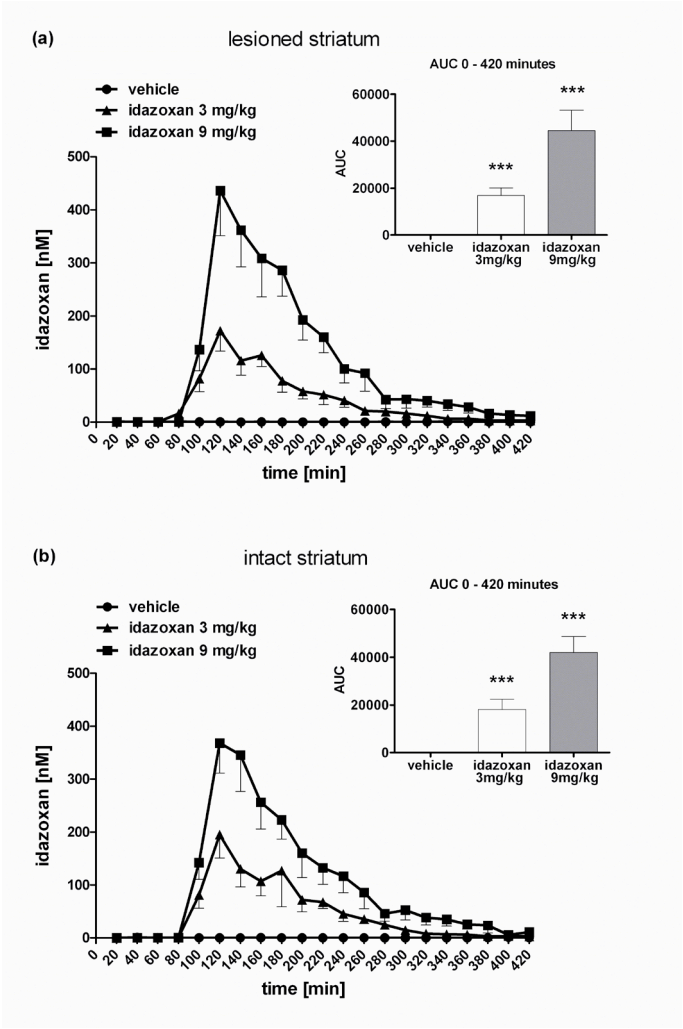


Fig. 14. In vivo microdialysis. Measurement of extracellular idazoxan levels in the lesioned (a) and intact (b) striatum following administration of idazoxan (9 mg/kg, i.p. (n = 8) and 3 mg/kg, i.p. (n = 8)) and vehicle (saline, i.p. (n = 8)). The AUC is displayed in a bar graph as an insert to each time course figure. Data are expressed as mean ± SEM.

A significant negative correlation was observed between idazoxan and L-DOPA levels for the time interval 140 – 220 minutes confirming that treatment with idazoxan reduces L-DOPA levels (data not shown). On the lesioned side, the correlation coefficient was found to be -0.4652 ($P < 0.05$), on the intact side -0.6057 ($P < 0.01$).

Pharmacokinetic drug-drug interaction of L-DOPA and idazoxan

Plasma, brain and CSF levels in rats treated with vehicle + L-DOPA and idazoxan 9 mg/kg + L-DOPA were measured to assess a drug-drug interaction between L-DOPA and idazoxan (Table 5). L-DOPA levels in plasma ($p = 0.9865$), brain ($p = 0.6318$) as well as CSF ($p = 0.9935$) did not differ significantly between rats receiving only L-DOPA and rats treated with idazoxan + L-DOPA indicating that the reduction of extracellular L-DOPA levels by idazoxan is not due to a general drug-drug interaction.

	Vehicle + L-DOPA	Idazoxan + L-DOPA
L-DOPA plasma levels [nmol/L]	4160.0 ± 540.7	4147.5 ± 461.4
L-DOPA brain levels [nmol/kg]	514.8 ± 220.3	366.6 ± 153.5
L-DOPA CSF levels [nmol/L]	260.5 ± 47.8	261.0 ± 34.7
Idazoxan plasma levels [nmol/L]	0 ± 0	373.3 ± 71.2
Idazoxan brain levels [nmol/kg]	0 ± 0	2212.5 ± 425.5
Idazoxan CSF levels [nmol/L]	1.1 ± 0.4	232.0 ± 56.0

Table 5. Plasma, brain and CSF levels of L-DOPA and idazoxan. Idazoxan (9 mg/kg, i.p. (n = 4)) and vehicle (i.p. (n = 4)) were administered 30 minutes prior to the treatment with L-DOPA/benserazide (6/15 mg/kg, p.o.) to naive rats. Forty minutes following L-DOPA administration samples of CSF, blood and brain tissue were taken and analysed using LC-MS/MS. Data are expressed as mean ± SEM.

DISCUSSION

The present study demonstrated that the α_2 adrenoceptor antagonist idazoxan attenuates extracellular peak L-DOPA levels in the lesioned and intact striatum as well as extracellular DA levels in the lesioned striatum which contribute to the antidyskinetic action of idazoxan.

The antidyskinetic effect of idazoxan is in agreement with studies using other α_2 adrenoceptor antagonists as well as idazoxan itself. Previous experimental studies have demonstrated that α_2 adrenoceptor antagonists such as the traditional drug yohimbine reduced L-DOPA-induced dyskinesia in rodent (Dekundy *et al.* 2007; Lundblad *et al.* 2002) as well as in primate models (Gomez-Mancilla and Bedard 1993). Yohimbine offers a broad pharmacological profile: it mainly acts as a α_2 adrenoceptor antagonist, but it also binds to DA D_2 receptors and serotonergic 5-HT_{1A}, 5-HT_{1D} and 5-HT_{2B} receptors, thus, it cannot be ruled out that the antidyskinetic

effect of yohimbine is due to the unspecific action at receptors other than the α_2 adrenoceptor. Compared to yohimbine, the α_2 adrenoceptor antagonist idazoxan shows improved selectivity but the compound was also found to interact with I_2 imidazoline binding sites (Miralles *et al.* 1993) as well as with moderate affinity to 5-HT_{1A} receptors (Fozard *et al.* 1987). Idazoxan has no selectivity for α_2 adrenoceptor subtypes and binds to the $\alpha_{2A,}$ α_{2B} and α_{2C} adrenoceptor with K_i values of 10 nM, 37 nM and 20 nM, respectively. The distribution of α_2 adrenoceptor subtypes varies between different brain regions. The α_{2A} adrenoceptor is widely distributed within the cerebral cortex, locus coeruleus, hippocampus and cerebellum (MacDonald and Scheinin 1995; McCune *et al.* 1993; Nicholas *et al.* 1993). The α_{2B} adrenoceptor is not highly expressed in the CNS but a relatively low signal was detected in the thalamus (MacDonald and Scheinin 1995; Nicholas *et al.* 1993). α_{2C} adrenoceptors are present in many brain regions, but the highest expression was shown in the basal ganglia (MacDonald and Scheinin 1995; Nicholas *et al.* 1993) suggesting that the α_{2C} adrenoceptor is the most likely subtype at which idazoxan mediates its antidyskinetic action.

L-DOPA-induced dyskinesia was not reduced by treatment with idazoxan 3 mg/kg, whereas idazoxan 9 mg/kg significantly alleviated dyskinetic movements. Bilateral in vivo microdialysis revealed that L-DOPA injection led to a pronounced increase in extracellular L-DOPA levels, which did not differ in the lesioned and in the intact striatum. As previously demonstrated, administration of L-DOPA induced a significant increase of extracellular DA levels in the lesioned striatum. In contrast, DA levels on the contralateral side did not increase indicating that DA can be handled very efficiently under physiological conditions (Buck and Ferger 2008). Treatment with idazoxan 3 mg/kg showed a trend towards a reduction of peak L-DOPA levels on both sides but this did not reach statistical significance. DA levels were not affected by treatment with idazoxan 3 mg/kg at all, correlating with its lack of antidyskinetic effect. The higher dose of idazoxan 9 mg/kg significantly attenuated extracellular peak L-DOPA levels in the lesioned as well as intact striatum. In addition, extracellular peak DA levels in the lesioned striatum were significantly reduced which we consider to be the reason for the antidyskinetic action of idazoxan. Extracellular DA levels in the intact striatum were not affected either by L-DOPA treatment as demonstrated previously (Buck and Ferger 2008) or by idazoxan. Furthermore, a significant correlation between idazoxan and L-DOPA levels was obtained confirming

that idazoxan affects extracellular L-DOPA levels. Thus, the higher the concentration of idazoxan in the striatum, the lower L-DOPA levels were observed in individual rats. In order to clarify if a drug-drug interaction of idazoxan and L-DOPA is responsible for this effect, the combination of L-DOPA and idazoxan was investigated on L-DOPA levels in plasma, CSF and brain. Idazoxan did not alter plasma, brain and CSF L-DOPA levels indicating that idazoxan acts via modulation of extracellular L-DOPA levels rather than by binding to L-DOPA. The antidyskinetic effect of idazoxan and the correlation with the reduction of L-DOPA peak levels in striatal microdialysates is a key finding. Notably, only the high dose led to a decrease in L-DOPA as well as DA microdialysate levels and importantly this correlates well with the behavioural data.

Idazoxan improved parkinsonian symptoms in different PD rat models such as in the haloperidol-induced catalepsy test (Invernizzi *et al.* 2003; Kleven *et al.* 2005), in the reserpine-induced L-DOPA hyperactivity model (Johnston *et al.* 2005), in the 6-OHDA-induced bar and grid test (Srinivasan and Schmidt 2004) as well as in the methylphenidate- and apomorphine-induced rotational test (Chopin *et al.* 1999). Idazoxan (0.3 and 2.5 mg/kg) reversed haloperidol-induced catalepsy significantly but did not modify the DA overflow in the striatum and had no effect on haloperidol-induced increase of DA measured by in vivo microdialysis (Invernizzi *et al.* 2003). In line with these results, the present study revealed no effect of idazoxan 3 mg/kg on extracellular DA levels derived from exogenously applied L-DOPA in the striatum.

Idazoxan exhibits moderate 5-HT_{1A} agonist properties (Kleven *et al.* 2005) which might contribute to the effect on dyskinesia as well as on extracellular DA levels. Recently, the role of DA released from serotonergic neurons as an important presynaptic factor of L-DOPA-induced dyskinesia in the rat PD model has been demonstrated (Carta *et al.* 2008). Indeed, the removal of 5-HT afferents by the serotonergic neurotoxin 5,7-dihydroxytryptamine, or by reducing 5-HT neuron activity by 5-HT_{1A} and 5-HT_{1B} drugs, resulted in an almost complete reversal of L-DOPA-induced dyskinesia (Carta *et al.* 2007). The concept of DA as a ‘false transmitter’ released from 5-HT terminals has also been proposed by Kannari *et al.* (2001), who showed that 5-HT_{1A} agonists reduced the L-DOPA-induced DA overflow in the rat striatum. Thus, the effect of idazoxan might also be mediated by agonism at 5-HT_{1A} receptors taking into account that idazoxan reduced dyskinesia at a relatively high dose of 9 mg/kg. Moreover, idazoxan decreased DOPA synthesis in the striatum measured by post mortem neurochemistry in rats (Sastre-Coll *et al.* 1999). It was

found that the idazoxan-induced reduction of DOPA was abolished by pre-treatment with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, an alkylating compound used to irreversibly inactivate α_2 adrenoceptors. This suggests that the effect of idazoxan in the striatum is mediated by α_2 adrenoceptors. In addition to the effect on extracellular L-DOPA levels, idazoxan reduced spontaneous activity of STN neurons resulting in enhanced motor activity of rats which was postulated to account for the behavioural effects in animal models and PD patients (Belujon *et al.* 2007).

A widely used predictive animal model of L-DOPA-induced dyskinesia is the MPTP-lesioned monkey treated with L-DOPA. This model not only provides data on the antidyskinetic efficacy of a compound but also gives insight into "on-time" regulation by L-DOPA. Idazoxan alleviated dyskinetic movements provoked by L-DOPA in MPTP-lesioned primates. Additionally, idazoxan did not compromise the anti-parkinsonian response to L-DOPA, rather the combination of idazoxan and L-DOPA extended the "on-time" of L-DOPA (Fox *et al.* 2001; Grondin *et al.* 2000; Henry *et al.* 1999). These effects are largely in line with dampening L-DOPA/DA peak levels. Furthermore, the dyskinetic behaviour induced by treatment with the DA agonist apomorphine was not affected by idazoxan in the monkey dyskinesia model (Fox *et al.* 2001). This observation argues for the fact that idazoxan alleviates dyskinesia due to reduction of extracellular L-DOPA and DA levels. The effect of apomorphine is mediated by postsynaptic DA D_2 receptor stimulation and not by an increase of extracellular DA levels explaining that idazoxan does not relieve apomorphine-induced dyskinesia.

The intention of this study was to investigate a potential mechanism of idazoxan's mode of action rather to promote idazoxan for the treatment of PD patients suffering from disabling dyskinesia. Eventually, idazoxan failed to be an acceptable treatment in clinical trials. Indeed, the clinical effects of idazoxan were investigated in patients with advanced PD and it was shown that dyskinesia severity improved without compromising the beneficial response to L-DOPA. However, treatment with idazoxan caused adverse side events such as hypertension, tachycardia, flushing and headache (Rascol *et al.* 2001) and precludes its clinical use.

In conclusion, the present study showed for the first time that idazoxan reduces extracellular L-DOPA levels in the striatum. In addition, extracellular DA levels in the lesioned striatum are attenuated which contribute to the alleviation of L-DOPA-induced dyskinesia by idazoxan. We postulate that it is important when evaluating

prospective targets aiming at the alleviation of L-DOPA-induced dyskinesia to investigate extracellular levels of L-DOPA and DA under conditions which closely resemble the disease state.

This study was conducted with the help of Patrizia Voehringer, who measured L-DOPA and idazoxan levels.

CHAPTER VII

Striatal L-DOPA but not GABA and glutamate is the crucial trigger to evoke dyskinetic movements in 6-OHDA-lesioned rats

ABSTRACT

Dyskinesia eventually develops in the majority of PD patients treated with L-DOPA. Here, we investigate the role of the striatum, GP and SNr associated with GABA, glutamate and L-DOPA to provoke dyskinetic movements.

We demonstrated that GABA and glutamate did not trigger the manifestation of dyskinesia in the striatum, the GP nor the SNr using the model of reverse in vivo microdialysis. As expected, L-DOPA administration into the lesioned striatum was a highly effective trigger to switch on dyskinesia. Notably, local L-DOPA perfusion in the ipsilateral GP and SNr did not provoke dyskinetic behaviour.

We conclude that an acute and local increase of neither GABA nor glutamate provokes dyskinesia. Since L-DOPA in the striatum but not in the GP or SNr switched on dyskinetic behaviour, we postulate a site specific action of L-DOPA at least for the evocation of already established dyskinesia.

INTRODUCTION

The DA precursor L-DOPA was the first antiparkinsonian drug to be introduced and still remains the cornerstone of symptomatic pharmacotherapy for patients suffering from PD. However, there are major limitations to L-DOPA therapy including the development of motor fluctuations and dyskinesia. Dyskinesia consists of debilitating abnormal involuntary movements such as chorea and dystonia. Eventually up to 40% of PD patients develop dyskinesia within 4-6 years of L-DOPA therapy (Ahlskog and Muenter 2001), which are perceived as disabling and non disabling dyskinesia. The prevalence of dyskinesia escalates with duration and severity of the disease (Obeso *et al.* 2000b) as well as with duration and dosage of L-DOPA treatment (Rascol 2000).

The induction and manifestation of dyskinetic movements involves multiple factors including disruption of presynaptic DA homeostasis, pathophysiological changes of firing patterns within the basal ganglia nuclei, cortical overactivation, several receptor

mediated changes at dopaminergic, GABAergic and glutamatergic neurons affecting neurotransmission within the direct and indirect pathway of the basal ganglia motor loops (Cenci 2007). The classical view for the onset of dyskinesia involves neurotransmitter changes within the basal ganglia opposite from that assumed for the parkinsonian state following DA depletion. However, there is much discussion regarding the contribution of the direct versus the indirect pathway of the basal ganglia loops to the mechanisms of L-DOPA-induced dyskinesia. The weaknesses of some of the proposed basal ganglia models arise questions to elucidate the complexity of dyskinesia (Jenner 2008b). Moreover, the interpretation of data obtained from animal models mimicking L-DOPA-induced dyskinesia becomes increasingly complex when considering that alterations during the expression of dyskinesia may be either a consequence of drug treatment or simply the abnormal involuntary movements themselves (Jenner 2008a).

Several animal models mimicking L-DOPA-induced dyskinesia are available including non-human primates and rodents. For practical reasons the 6-OHDA rat dyskinesia model (Cenci *et al.* 1998) is preferred to study mechanisms whenever using invasive techniques such as in vivo microdialysis and when higher animal numbers are required. Dyskinesia can be induced systemically by treatment with L-DOPA, originally described by Cenci *et al.* (1998), or locally by administration of L-DOPA into the 6-OHDA-lesioned striatum via reverse in vivo microdialysis (Buck and Ferger 2008; Buck and Ferger 2009a; Carta *et al.* 2006). The model of reverse in vivo microdialysis offers three main advantages for mechanistic studies: 1) target specific delivery of compounds, 2) compounds which do not cross the blood brain barrier, such as most neurotransmitters, can be investigated on alterations of dyskinetic movements, 3) differentiation between a trigger for the expression of dyskinesia or a consequence of the abnormal involuntary movement and drug treatment, respectively.

The present study focuses on the role of GABA, glutamate as well as L-DOPA in the striatum, GP and SNr for evocation of L-DOPA-induced dyskinesia in 6-OHDA-lesioned rats with already established dyskinesia by chronic L-DOPA priming procedure.

MATERIALS AND METHODS

Animals and 6-OHDA lesion surgery

Please see chapter II “General methodology” page 22.

Induction and rating of dyskinesia

Please see chapter II “General methodology” page 22. The sum of axial, limb and orolingual dyskinesia ratings was expressed as a total dyskinesia score and used instead of the individual score because the dyskinesia scores did not differ from each other. For the subsequent experiments rats were divided into two groups, dyskinetic and non dyskinetic rats, according to their total dyskinesia score at the day of the experiment. The history of both groups (6-OHDA lesion, L-DOPA treatment) was identical.

In vivo microdialysis surgery in the striatum, GP and SNr

Please see chapter II “General methodology” page 23. The study was divided in two experiments: one series of rats was implanted with one probe in the GP and one probe in the SNr; another series of rats was implanted with one probe in the striatum. In the first experiment, two intracerebral guide cannulae were implanted aiming at the GP and SNr. In the second experiment, one intracerebral guide cannula was implanted aiming at the striatum.

Reverse in vivo microdialysis procedure in the striatum, GP and SNr

Please see chapter II “General methodology” page 25. Following insertion of the probes, aCSF was perfused for 1 hour. GABA (0.4 nmol/min), glutamate (0.4 nmol/min) and L-DOPA (0.4 nmol/min) were consecutively perfused on the ipsilateral side (GP: n = 3; SNr: n = 4; striatum: n = 7) for 1 hour each, except L-DOPA in the striatum, which was perfused for 90 minutes, since slight dyskinesia appeared 30 minutes after beginning of L-DOPA treatment and increased very slowly. A 30 minutes aCSF perfusion was performed as washout between perfusion of the different compounds. Dyskinetic behaviour was monitored during the whole experiment every 5 minutes up to 150 minutes after the end of the L-DOPA treatment. In the GP and SNr monitoring of dyskinesia was stopped 30 minutes after the end of L-DOPA treatment since the rats did not show any dyskinetic movements.

In vivo microdialysis procedure in the striatum, GP and SNr

Please see chapter II “General methodology” page 24. For the measurement of L-DOPA in SNr and GP experiment, dyskinetic (n = 9) and non dyskinetic (n = 9) rats were used. For the determination of L-DOPA in the striatum, which was performed in a parallel study, dyskinetic (n = 7) and non dyskinetic (n = 5) rats were included.

Fractions 1 to 4 (0 – 80 min) were used for calculation of the basal levels which were regarded as 100 %. At time point 80 minutes, L-DOPA/benserazide (6/15 mg/kg, p.o.) was administered and the sampling was continued up for 5 hours (80 – 380 min). After the experiments the correct localisation of the probes was verified and only the rats with appropriate probe placement were included in the experiment (please see Fig. 15).

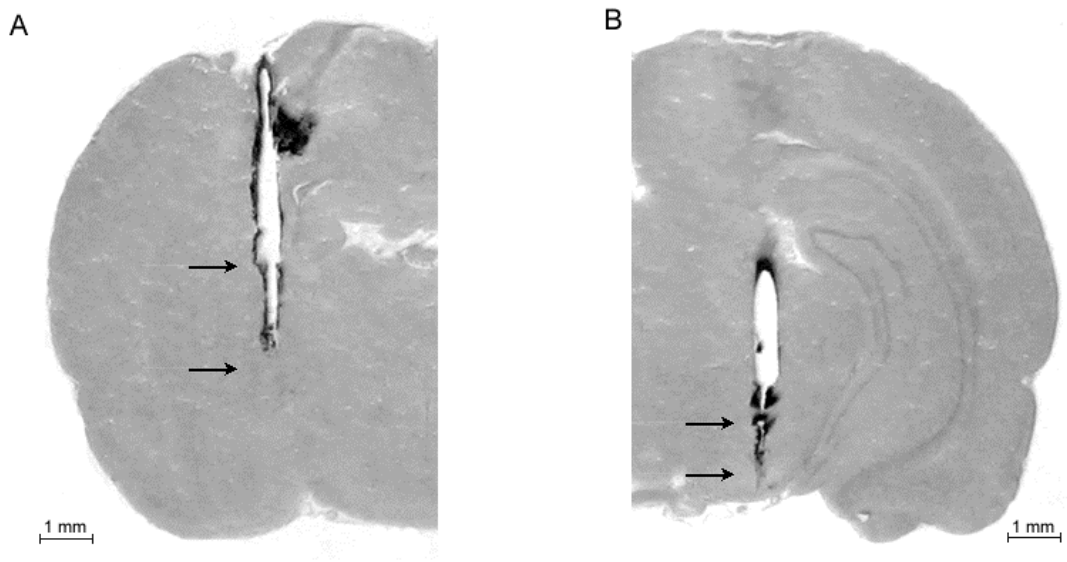


Fig. 15. Probe localisation in the GP (A) and the SNr (B). Placement of the probes was verified using Indian Ink. The arrows indicate the origin and the end of the membrane of the probe.

Sample preparation procedure

Ten µl of the microdialysis sample were diluted with 10 µl of internal standard solution ([D_3]-L-DOPA in acetonitrile). Ten µl of this solution were injected into the LC-MS/MS.

LC-MS/MS analysis of microdialysis samples

Please see chapter II “General methodology” (LC-MS/MS system) page 26. Samples from the GP, SNr and striatum were analysed for L-DOPA using LC-MS/MS. Mobile phase “A” and “B” consisted of 0.1 % formic acid in water and acetonitrile (LC-MS grade), respectively. The gradient elution profile was chosen as follows: 0 min: 15 % A (1000 µl/min), 0.10 min: 15 % A (200 µl/min), 1.00 min: 80 % A (200 µl/min), 1.50 min: 80 % A (200 µl/min), 1.60 min: 15 % A (200 µl/min), 2.40 min: 15 % A (200 µl/min), 2.50 min: 15% A (1000 µl/min), 3.00 min: 15 % A (1000 µl/min). Chromatographic retention was obtained using a HILIC column (ZIC®-HILIC, 20 x 2.1 mm i.d., 3.5 µm, SeQuant AB, Umeå, Sweden) with a pre-microfilter (0.5 µm, Upchurch Scientific, Oak Harbor, WA, USA) at 20°C. The column switching valve was set at 0.00 minutes to the waste, at 0.70 minutes to the mass spectrometer and at 2.00 minutes to the waste again.

Three transitions were chosen for L-DOPA: 198-181 (DP 51 V, CE 15 V, CXP 12 V), 198-107 (DP 51 V, CE 37 V, CXP 8 V) and 198-135 (DP 51 V, CE 27 V, CXP 8 V). Transition 198-181 was used for the quantification of L-DOPA. [D_3]-L-DOPA was used as internal standard and analysed using the transition 201-154 (DP 56 V, CE 19 V, CXP 12 V).

Drugs and chemicals

Please see chapter II “General methodology” page 26.

Statistical analysis

Please see chapter II “General methodology” page 27. L-DOPA levels in the microdialysis experiment are expressed as mean ± SEM and analysed by parametric statistics. Scores of dyskinesia are expressed as median ± interquartile range and analysed by non parametric statistics. $P < 0.05$ was considered as statistically significant.

For comparison of dyskinesia scores with the theoretical median which was defined as 0.0 (pre dyskinesia score), a Wilcoxon signed rank test was performed. Statistical comparison between dyskinetic and non dyskinetic animals was carried out using a Mann Whitney U test.

Statistical analysis of the in vivo microdialysis data (L-DOPA measurement) was performed by calculating the AUC and transformed by logarithmising the AUC to

obtain Gaussian data distribution. A two-tailed t-test was used to compare the logarithmised AUCs of the 2 groups.

RESULTS

Effects of local administration of GABA, glutamate and L-DOPA in the GP, SNr and striatum on dyskinesia

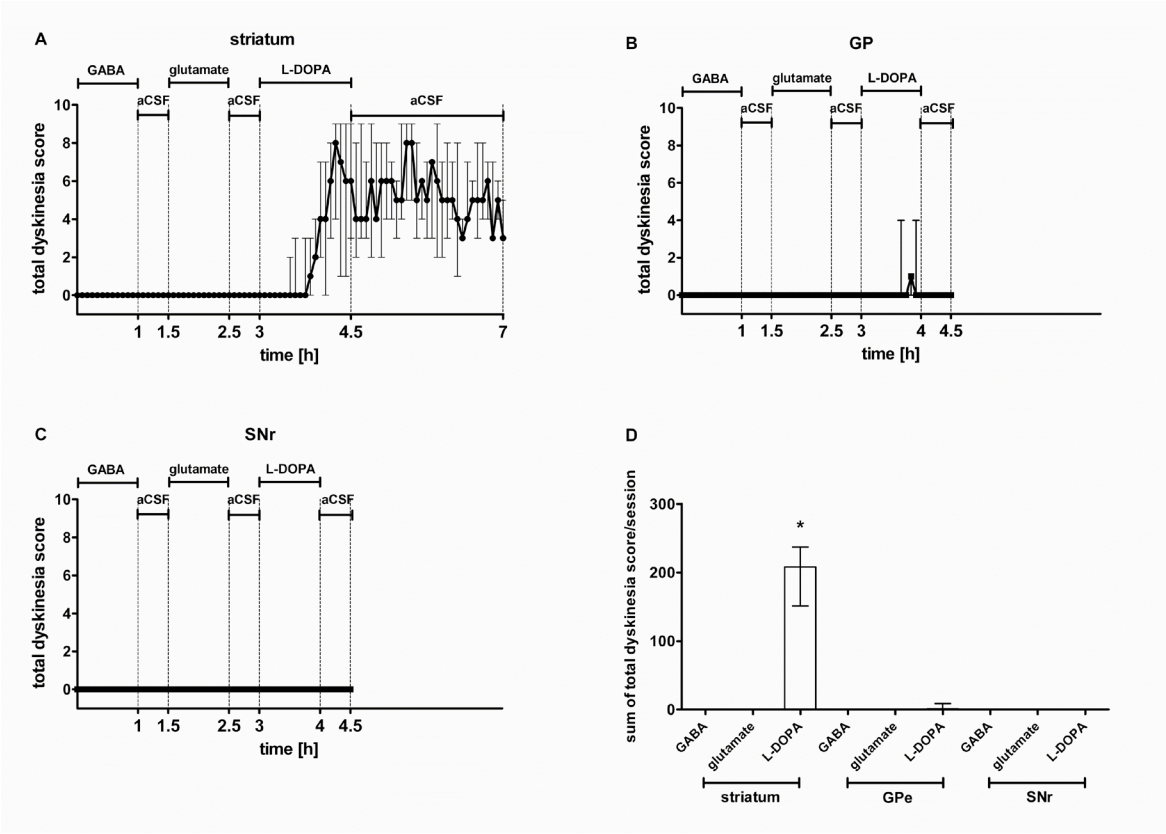


Fig. 16. Reverse in vivo microdialysis. Effects of administration of GABA (0.4 nmol/min), glutamate (0.4 nmol/min) and L-DOPA (0.4 nmol/min) into the ipsilateral striatum (A), GP (B) and SNr (C). Panel A-C show the time course of total dyskinesia score monitored every 5 minutes. In panel D, the sum of total dyskinesia score/session is displayed. Data are presented as median ± interquartile range. Cumulative scores were analysed by a Wilcoxon signed rank test for comparison to the theoretical median, which was defined as 0.0 (*P < 0.05).

GABA, glutamate and L-DOPA were administered into GP, SNr and striatum on the side ipsilateral to the lesion using reverse in vivo microdialysis (Fig. 16). Dyskinesia was monitored every 5 minutes during the experiment. Administration of GABA as well as glutamate into the GP, SNr and striatum did not induce dyskinetic behaviour

(sum of total dyskinesia score = 0). Treatment with L-DOPA in the GP led to sporadic, very slight and not significant dyskinetic movements (sum of total dyskinesia score = 3), whilst L-DOPA administration in the SNr did not evoke dyskinesia (sum of total dyskinesia score = 0). In contrast, perfusion of L-DOPA in the lesioned striatum led to a significant increase in dyskinetic movements (sum of total dyskinesia score = 204 ± 19, $P < 0.05$).

Thirty minutes after onset of L-DOPA administration dyskinesia developed. Fifty-five minutes after starting the L-DOPA perfusion dyskinetic movements significantly increased ($P < 0.05$) to a plateau and persisted over the whole observation period until 240 minutes following onset of L-DOPA administration (except time point 65 minutes).

Effects of systemic L-DOPA administration on dyskinetic behaviour

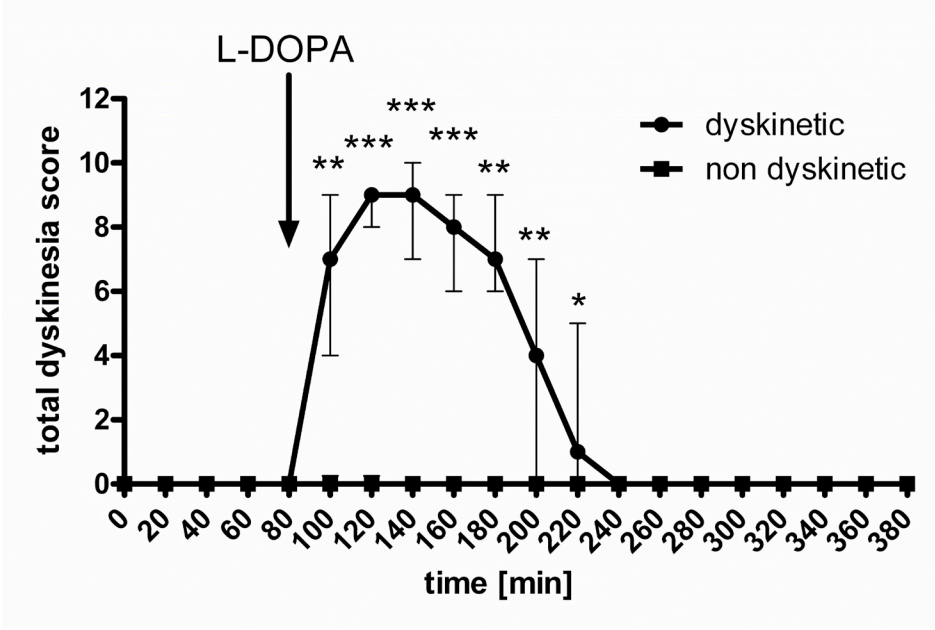


Fig. 17. In vivo microdialysis. Effects of systemic administration of L-DOPA/benserazide (6/15 mg/kg; p.o.) on extracellular L-DOPA levels in the striatum (A), GP (B) and SNr (C). Statistical analysis revealed no differences between dyskinetic and non dyskinetic rats. Data are presented as mean ± SEM.

The time course of total dyskinesia during the in vivo microdialysis experiment is displayed in Fig. 17. Dyskinesia was monitored every 20 minutes during the experiment. Rats of the dyskinesia group significantly developed dyskinetic movements 20 minutes following L-DOPA which lasted for 140 minutes. Rats of the

non dyskinetic groups did not show dyskinetic behaviour during the whole observation period. Comparison of the sum of total dyskinesia score between dyskinetic (sum of total dyskinesia score = 43.6 ± 2.6) and non dyskinetic rats (sum of total dyskinesia score = 1.4 ± 0.7) revealed a significant difference ($P < 0.001$).

Measurement of extracellular L-DOPA levels

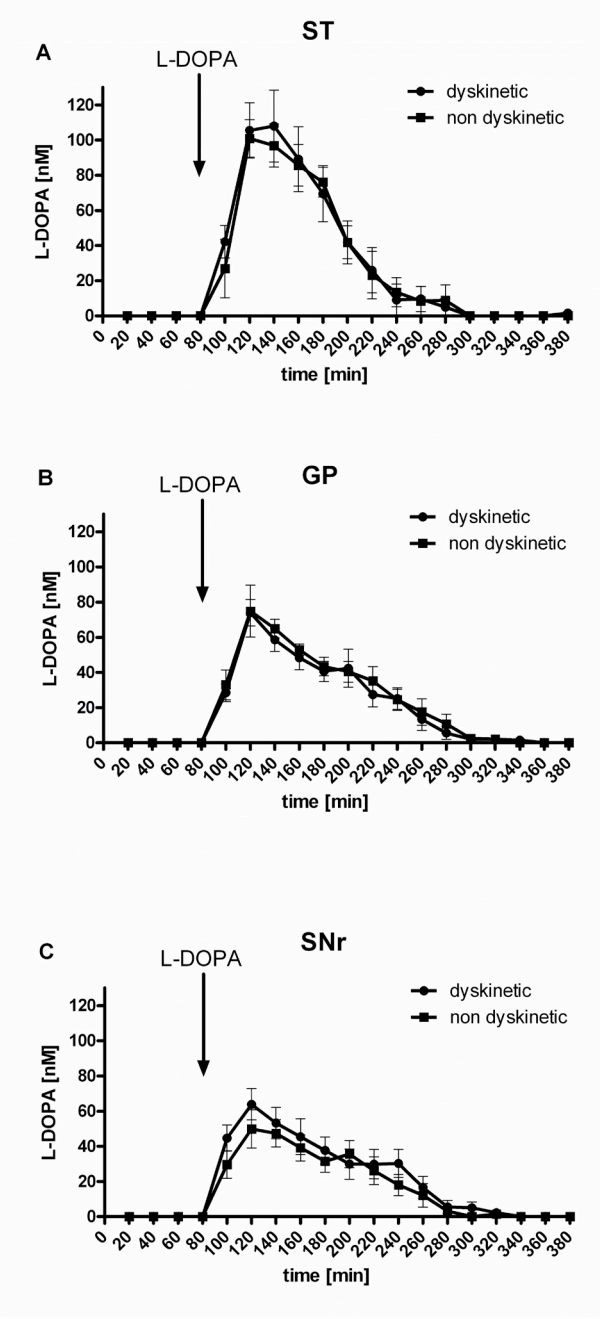


Fig. 18. Time course of L-DOPA-induced dyskinesia in dyskinetic and non dyskinetic rats. Monitoring of dyskinesia was carried out every 20 minutes throughout the experiment and expressed as a total dyskinesia score. Data are presented as median ± interquartile range and analysed by a Mann Whitney U test for statistical comparison of the groups (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

In order to investigate differences regarding the exposure to L-DOPA between dyskinetic and non dyskinetic rats, extracellular L-DOPA levels in the GP, SNr as well as striatum were analysed using in vivo microdialysis (Fig. 18). Basal levels of L-DOPA were not detectable. Time course of extracellular L-DOPA levels paralleled in all 3 brain areas. Maximum levels were achieved 40 minutes following per oral administration of L-DOPA/benserazide (6/15 mg/kg) and declined within 3 hours. Statistical comparison of the AUC of L-DOPA levels after L-DOPA injection in the striatum did not show a significant difference between dyskinetic and non dyskinetic rats ($P = 0.7990$). Maximum L-DOPA levels were 106 nM and 101 nM in dyskinetic and non dyskinetic animals, respectively. In the GP, analysis of the AUC of L-DOPA levels following injection of L-

DOPA did not reveal a significant difference between dyskinetic and non dyskinetic rats (P = 0.5319). Maximum L-DOPA levels in the GP were found to be 74 and 75 nM in the dyskinetic and non dyskinetic rats, respectively. Considering the SNr, statistical analysis of the AUC of L-DOPA levels did not yield a significant difference between the dyskinetic and non dyskinetic group as well (P = 0.8136). In the SNr, maximum levels of L-DOPA achieved 64 and 50 nM in dyskinetic and non dyskinetic cases, respectively. Comparison of the maximum level of L-DOPA between the dyskinetic and non dyskinetic cases did not reveal a significant difference either in the GP (P = 0.5086), the SNr (P = 0.6453) or the striatum (P = 0.6431).

DISCUSSION

The present study demonstrated that an acute and local increase of neither GABA nor glutamate in the striatum, GP and SNr did evoke dyskinetic movements in rats with already established dyskinesia. In contrast, local L-DOPA administration into the DA denervated striatum was a highly effective trigger to switch on dyskinetic movements. Notably, local L-DOPA in the GP and SNr on the side ipsilateral to the lesion did not provoke dyskinetic movements indicating that the action of L-DOPA is site specific and that the striatum is the crucial brain area for the manifestation of dyskinesia.

To the best of our knowledge, no studies have been undertaken which investigate the ability of different compounds to evoke dyskinesia after local administration into different brain areas of rats with already established L-DOPA-induced dyskinesia. Local administration of drugs was used to antagonise L-DOPA-induced dyskinesia as shown by intrastriatal administration of the 5-HT_{1A} agonist (±)-8-OH-DPAT (Dupre *et al.* 2008) or local administration of the 5-HT_{1A} agonist sarizotan into the STN (Marin *et al.* 2009). As a matter of course, the rat dyskinesia model is an appropriate and acknowledged model to investigate various drugs on their ability to alleviate dyskinetic movements following systemic administration (Dekundy *et al.* 2007; Lundblad *et al.* 2002; Monville *et al.* 2005). However, the effect of dyskinesia evocation by local administration of different compounds has been more rarely investigated (Buck and Ferger 2008; Buck and Ferger 2009a; Carta *et al.* 2006). This model provides an excellent technique to study trigger factors for the expression of

dyskinesia as well as the involvement of certain brain areas within the manifestation of dyskinetic behaviour.

The reverse in vivo microdialysis technique to deliver potentially dyskinesiogenic compounds into the DA denervated striatum has been validated. Indeed, L-DOPA, DA as well as NA evoked dyskinesia in rats when given locally into the 6-OHDA-lesioned striatum (Buck and Ferger 2008; Buck and Ferger 2009a; Carta *et al.* 2006). In contrast, intrastriatal administration of DA metabolites, namely DOPAC, HVA and 3-MT did not produce dyskinetic movements (Buck and Ferger 2008). Thus, the present model of striatal reverse in vivo microdialysis is sensitive to differentiate between dyskinesiogenic and non dyskinesiogenic compounds. In comparison to other studies, we focused on the evocation of dyskinesia in rats with already established dyskinetic history rather than the development of dyskinesia. In fact, the model of reverse in vivo microdialysis to provoke dyskinetic movements can be clearly distinguished from mechanisms underlying the induction of dyskinesia (Nadjar *et al.* 2009). In the present study, we found that the amino acid neurotransmitters GABA and glutamate did not evoke dyskinetic movements following intrastriatal perfusion, whereas intrastriatal L-DOPA triggered the expression of dyskinesia.

Systemic injection of L-DOPA was found to increase extracellular glutamate levels in the DA denervated and intact striatum (Jonkers *et al.* 2002). Another study showed that chronic L-DOPA treatment increased basal glutamate levels in the lesioned striatum, but L-DOPA injection did not result in an additional alteration of extracellular glutamate levels (Robelet *et al.* 2004). In contrast, it was recently demonstrated that chronic L-DOPA induced an increased basal glutamate output in the striatum, but following L-DOPA injection a decline in extracellular glutamate levels in the 6-OHDA-lesioned striatum was observed (Morgese *et al.* 2009). Thus, there are ambivalent results whether L-DOPA increases, decreases or does not alter the extracellular striatal glutamate overflow. In the present study we observed that a local and acute increase of glutamate in the striatum is not a trigger for evocation of dyskinesia. This finding is not in contrast to the established role of glutamatergic receptors in the pathophysiology of L-DOPA-induced dyskinesia. For example, Samadi *et al.* (2008) demonstrated a markedly different pattern of mGluR5 receptor binding and mGluR5 negative allosteric modulators such as MTEP are effective to treat the development and manifestation of L-DOPA-induced abnormal involuntary movements (Dekundy *et al.* 2006; Mela *et al.* 2007).

Consistent with previous findings, local administration of L-DOPA into the 6-OHDA-lesioned striatum produced a significant increase in total dyskinesia scores suggesting that DA derived from exogenously applied L-DOPA is instrumental for the manifestation of dyskinesia. Recently, we demonstrated that local administration of DA into the lesioned striatum provokes dyskinetic behaviour in a similar manner as local L-DOPA (Buck and Ferger 2008). However, perfusion of L-DOPA plus benserazide locally in the lesioned striatum did not lead to dyskinetic movements indicating that L-DOPA itself is not able to evoke dyskinesia and that L-DOPA has to be converted to DA which is the dyskinesiogenic trigger. In contrast to the intrastriatal L-DOPA treatment, local perfusion of L-DOPA into the GP and SNr on the side ipsilateral to the lesion did not provoke dyskinetic behaviour. These results underpin the hypothesis that the striatum is the crucial brain area in L-DOPA-induced dyskinesia. In fact, it has been shown that dyskinesia can be prevented by various strategies targeting the striatum including continuous L-DOPA delivery using rAAV vectors applied into the striatum (Carlsson *et al.* 2006) or reversed by intrastriatal infusion of fosB antisense (Andersson *et al.* 1999). We postulate a site specific action of L-DOPA in the striatum since L-DOPA delivered locally into the striatum but not into the GP and SNr on the side ipsilateral to the lesion provoked dyskinetic movements.

The central pharmacokinetics of L-DOPA seems to play a major role in dyskinetic movements. Accordingly, we measured extracellular levels of L-DOPA in the striatum, GP and SNr of dyskinetic and non dyskinetic rats using in vivo microdialysis. The time course paralleled in all three brain areas reaching a maximum level 40 minutes following administration and declining within 3 hours. The maximum level and AUC of extracellular L-DOPA levels were almost identical in dyskinetic and non dyskinetic cases either in the striatum, GP or in the SNr. These results are in contrast to a study from Carta *et al.* (2006), who showed that the central pharmacokinetics following treatment with L-DOPA differs in the striatum of dyskinetic and non dyskinetic rats. In the latter study, extracellular levels of L-DOPA in the striatum reached peak levels within 40 minutes and were 5-fold elevated in dyskinetic (250 nM) compared to non dyskinetic rats (50 nM). In the present study we determined maximum L-DOPA levels in the striatum of 106 nM and 101 nM in dyskinetic and non dyskinetic animals, respectively. The underlying reasons for this discrepancy may involve some methodological differences including route of

administration, rat strain used as well as different time schedules for L-DOPA treatment. A study from Meissner *et al.* (2006) investigating extracellular L-DOPA levels in the striatum of primed and non-primed sham-operated rats and primed and non-primed 6-OHDA-lesioned rats showed that the maximum levels of L-DOPA are higher in 6-OHDA-lesioned animals compared to sham-operated rats. In this study, the maximum level of L-DOPA injection was reported to be 140 nM 50 minutes following L-DOPA which is in the same range as in the present study. For the first time extracellular L-DOPA levels in the GP and SNr following treatment with L-DOPA in dyskinetic rats were monitored. In agreement to the results of the striatum, extracellular L-DOPA levels in the GP as well as SNr increased to the same extent in both dyskinetic and non dyskinetic rats.

In conclusion, the present study provides evidence that enhancement of GABA and glutamate in different brain areas within the basal ganglia is not a trigger for the manifestation of dyskinesia. In fact, a high concentration of exogenously applied L-DOPA provokes dyskinesia in the striatum, but not in the GP and SNr. This data indicates a site specific effect of L-DOPA and encircles a potential drug targeting for antidyskinetic compounds in PD.

This study was performed with the help of Patrizia Voehringer, who measured L-DOPA levels.

CHAPTER VIII

Continuous dopaminergic stimulation by pramipexole is effective to treat early morning akinesia in animal models of Parkinson's disease: a pharmacokinetic-pharmacodynamic study

ABSTRACT

Short-acting DA agonists are usually administered several times a day resulting in fluctuating plasma and brain levels. DA agonists providing continuous dopaminergic stimulation (CDS) may achieve higher therapeutic benefit for example by alleviating nocturnal disturbances as well as early morning akinesia. In the present study, continuous release (CR) of pramipexole (PPX) was maintained by subcutaneous implantation of Alzet® minipumps, whereas subcutaneous PPX injections were used to mimic PPX immediate release (IR).

In the catalepsy bar test, PPX-CR (1 mg/kg/day) reversed the haloperidol-induced motor impairment in the morning and over the whole observation period of 12h. In contrast, PPX-IR (tid 1 mg/kg, pre-treatment the day before) was not effective in the morning but catalepsy was reduced for 6h after PPX-IR (1 mg/kg) injection. In the reserpine model, early morning akinesia indicated by the first motor activity measurement in the morning was significantly reversed by PPX-CR (2 mg/kg/day). Again, PPX-IR (tid 0.3 mg/kg, pre-treatment the day before) was not able to antagonise early morning akinesia. These results are in agreement with in vivo microdialysis measurements showing a continuous decrease of extracellular DA levels and a continuous PPX exposure in the PPX-CR (1 mg/kg/day) group. In contrast, PPX-IR (0.3 mg/kg) produced a transient decrease of extracellular DA levels over 6h and showed maximum PPX levels 2h after dosing which decreased over the following 6-8h.

The present study demonstrates that PPX-CR may offer a higher therapeutic benefit than PPX-IR on early morning akinesia and confirms earlier reports that PPX-IR reverses motor impairment for several hours.

INTRODUCTION

After discovery of L-DOPA and various DA receptor agonists, a new area of research started for the pharmacotherapy of PD. Indeed, not only the affinity, potency and selectivity for the distinct DA receptors of anti-parkinsonian drugs were regarded as important to improve the pharmacotherapy, but also the knowledge about the impact of pulsatile or CDS received more and more attention. DA agonists or L-DOPA formulations resulting in long plasma half-lives are aiming to achieve CDS which eventually should prevent unwanted effects related to fluctuations in brain and plasma drug levels.

The concept of CDS (Bezard *et al.* 2001; Jenner 2008b; Nutt *et al.* 2000; Olanow *et al.* 2006) postulates that it is desirable to avoid the non-physiological pulsatile DA receptor stimulation. This concept could translate into prolonged therapeutic efficacy and on top would result in lower propensity to develop motor fluctuations, dyskinesia and less nocturnal disturbances as well as early morning akinesia. PPX may be a candidate for CDS because of its good tolerability and favourable pharmacokinetic properties (high oral bioavailability, no significant interaction with hepatic cytochrome P450 enzymes, long half-life) in humans (Kvernmo *et al.* 2006). PPX is a non-ergoline full DA receptor agonist with selectivity for the DA D_3 receptor (Mierau and Schingnitz 1992; Mierau *et al.* 1995; Piercey *et al.* 1996; Piercey 1998) and has been approved by the FDA and other regulatory authorities in 1997 for the treatment of early and advanced stages of PD. Since this time, PPX has become one of the most widely used DA receptor agonists and is in use in monotherapy as well as in combination with other anti-parkinsonian medication such as L-DOPA for the treatment of PD. More recently, PPX is also registered for treatment of patients suffering from restless legs syndrome.

Preclinical data on CDS by PPX in animal models of PD are sparse. To date only one study using CDS by PPX has been published which aimed to investigate the neuroprotective effects of CDS against lipopolysaccharide (LPS)-induced dopaminergic cell death in vivo. Iravani *et al.* (2008) demonstrated in this study that continuous subcutaneous infusion of PPX over 4 weeks preserved tyrosine hydroxylase positive neurons in the nigrostriatal pathway against dopaminergic cell death induced by the supranigrally administered LPS in rats. This effect was absent in rats acutely injected with PPX arguing for the beneficial effects on neuroprotection of CDS over acute treatment.

The present study focuses on CDS by PPX targeting symptomatic effects in two animal models of PD. In addition, for the first time brain PPX pharmacokinetics was simultaneously investigated with levels of the biomarker DA after PPX immediate (PPX-IR) and continuous release (PPX-CR).

MATERIALS AND METHODS

Animals

Please see chapter II “General methodology” page 22.

Haloperidol-induced catalepsy

Haloperidol-induced catalepsy is used as an animal model of extrapyramidal side-effects and for screening anti-parkinsonian drugs. Cataleptic immobility is regarded as an animal equivalent of akinesia and is demonstrated by an animal allowing its body to be placed in and maintain abnormal or unusual postures (Sanberg *et al.* 1988; Schmidt *et al.* 1992). Catalepsy was induced by treatment of rats with haloperidol (0.5 mg/kg, i.p.) and maintained for 12 hours by administration of haloperidol (0.1 mg/kg, i.p.) every 4 hours. The rats were placed with their forelimbs on a horizontal bar elevated 6 cm from the floor. The time (s) during which the rats maintain in this unusual position was recorded up to 60 seconds (cut-off period 60 seconds). Catalepsy considered fulfilled when the rat moved its forelimbs and stepped down the bar or climbed upon the bar. Three treatment groups were chosen. In the PPX-CR group (n = 9), ALZET® osmotic minipumps (model 2004 or 1007D, DURECT Corporation, Cupertino, CA, USA) filled with PPX solution were implanted subcutaneously under isoflurane anaesthesia the day before the catalepsy experiment. PPX was delivered continuously at the dose of 1 mg/kg/day. The PPX-IR group (n = 9) was treated with PPX (1 mg/kg, s.c.) 3 times (morning, midday, evening) on the day before the catalepsy experiment. On the day of the experiment, the first measurement of catalepsy was performed 2 hours after the bolus injection of haloperidol. Subsequently, the PPX-IR and vehicle group (n = 9) were treated with PPX (1 mg/kg, s.c.) and vehicle, respectively. Catalepsy was measured 2, 4, 6, 8, 10 and 12 hours later.

Reserpine-induced akinesia

Reserpine-induced akinesia was measured in the open field system Actimot™ (TSE Systems GmbH, Bad Homburg, Germany) for 1 hour in the morning. Rats were placed individually in the centre of the activity box (46.5 cm x 46.5 cm) and horizontal motor activity (m) was determined in 10 minutes intervals by infrared sensor pairs (interspace 1.4 cm) with a sampling rate of 100 Hz.

Three treatment groups were chosen. In the PPX-CR group (n = 7), ALZET® osmotic minipumps (model 1007D, DURECT Corporation, Cupertino, CA, USA) filled with PPX solution were implanted subcutaneously under isoflurane anaesthesia 3 days before the measurement of akinesia. PPX was delivered continuously at the dose of 2 mg/kg/day. The PPX-IR (n = 6) group was treated with PPX (0.3 mg/kg, s.c.) 3 times (morning, midday, evening) on the day before the akinesia measurement. All rats were treated with reserpine (1 mg/kg, s.c.) in the afternoon the day before the experiment. Reserpine was first dissolved in 100% acetic acid and in a subsequent step diluted with water to a final concentration of 1% acetic acid. Seventeen hours later, motor activity was measured in the open field system for 60 minutes (early morning akinesia).

In vivo microdialysis surgery

Please see chapter II “General methodology” page 23. Subsequently, the ALZET® osmotic minipump (model 1007D, DURECT Corporation, Cupertino, CA, USA) filled with PPX solution was implanted subcutaneously in rats of the PPX-CR group. PPX was delivered continuously at a dose of 1 mg/kg/day. Following surgery, rats were allowed to recover for 2 days before performing the in vivo microdialysis procedure.

In vivo microdialysis procedure

Please see chapter II “General methodology” page 24. The in vivo microdialysis procedure was performed with minor modifications in this study. After an equilibration period of 2 hours, dialysis samples were collected every 30 minutes into a vial containing 10 µl of hydrochloric acid (0.1 M). During the night, the sampling interval was prolonged to 60 minutes (20 µl of hydrochloric acid). Fractions 1 to 4 (0 - 2 h) were used for calculation of the basal levels. After 2 hours, the PPX-IR and PPX-CR group were treated with PPX (0.3 mg/kg, s.c. (n = 4)) and vehicle (saline, s.c. (n =

4)), respectively. The sampling was then continued for 17.5 hours up to the next morning.

HPLC analysis of microdialysis samples

Please see chapter II “General methodology” (HPLC system) page 26. Microdialysis samples were splitted for HPLC-ECD (60 µl) and LC-MS/MS (10 µl) analysis. Samples were analysed for DA using HPLC-ECD under isocratic conditions. Chromatographic separation was achieved using a reversed-phase column (Grom-Sil 120 ODS-4 HE, 250 x 4.0 mm i.d., 5 µm particles, Grace Davison Discovery Sciences, Deerfield, IL, USA) at 35°C. The mobile phase consisted of 1.85 mM 1-octanesulfonic acid sodium salt, 0.13 mM $Na_2EDTA \times 2\ H_2O$, 8.00 mM NaCl, 57.51 mM NaH_2PO_4, adjusted to pH 2.50 with H_3PO_4, filtered through a 0.22 µm filter, mixed up with 5 % acetonitrile and was delivered at a flow rate of 1 ml/min.

LC-MS/MS analysis of microdialysis samples

Please see chapter II “General methodology” (LC-MS/MS system) page 26. Microdialysis samples were analysed for PPX using LC-MS/MS. Mobile phase “A” and “B” consisted of 0.1 % formic acid in LC-MS grade water and acetonitrile, respectively. The gradient was chosen as follows: 0.00 min: 100% A, 1.40 min 100% A, 1.41 min 0% A, 2.00 min 0% A, 2.10 min 100% A, 2.50 min 100 % A and delivered at 0.5 ml/min onto a reversed-phase column (Synergi Polar-RP 80 A, 150 x 2.0 mm i. d., 5 µm particles, Phenomenex, Inc., Aschaffenburg, Germany) at 20 °C. The column switching valve was set at 0.00 min to the waste, at 0.75 min to the mass spectrometer and at 2.00 min to the waste again.

Three transitions were chosen: 212-153 (DP 56 V, CE 21 V, CXP 10 V), 212-111 (DP 56 V, CE 37 V, CXP 8 V), 212-126 (DP 56 V, CE 39 V, CXP 8 V) and transition 212-153 was used for the quantification of PPX. As internal standard [D_7]-PPX was analysed using transition 219-153 (DP 86 V, CE 21 V, CXP 10 V).

Drugs and chemicals

Please see chapter II “General methodology” page 26.

Statistical analysis

Please see chapter II “General methodology” page 27. The time course of haloperidol-induced catalepsy test as well as reserpine-induced akinesia was analysed by a two-way ANOVA with treatment as independent and time as dependent factor followed by a Bonferroni post hoc test. Statistical analysis of the cumulative data of the haloperidol-induced catalepsy and the reserpine-induced akinesia test was carried out using one-way ANOVA with treatment as independent factor followed by a Bonferroni post hoc test. For comparison of basal DA and PPX levels an unpaired t-test was performed.

RESULTS

Haloperidol-induced catalepsy

Fig. 19 shows the effect of PPX on haloperidol-induced catalepsy. Statistical analysis yielded a significant interaction of time x treatment ($F(12;144) = 6.388$; $P < 0.001$) as well as significant effects on time ($F(6;144) = 4.786$; $P < 0.001$) and treatment ($F(2;144) = 15.33$; $P < 0.001$) (Fig. 19A). Time spent on the bar of the PPX-CR group was significantly decreased in comparison to the vehicle group during the whole experiment (0h, 2h, 8h, 10h: $P < 0.001$; 4h: $P < 0.05$; 6h, 12h: $P < 0.01$). The PPX-IR and vehicle group did not display a significant difference 2 h after haloperidol injection (time point 0; pre-test before PPX/vehicle injection) indicating that pre-treatment with PPX the day before did not show an effect on haloperidol-induced catalepsy the next morning. treatment with PPX the day before did not show an effect on haloperidol-induced catalepsy the next morning. Following injection with PPX, the time spent on the bar decreased significantly in the PPX-IR group at time points 2h and 4h ($P < 0.001$) as well as 6h ($P < 0.05$). Regarding the cumulative data (Fig. 19B), PPX-CR ($P < 0.001$) as well as PPX-IR ($P < 0.05$) showed an improvement of haloperidol-induced catalepsy, whilst PPX-CR revealed a significant higher effect in comparison to PPX-IR ($P < 0.05$).

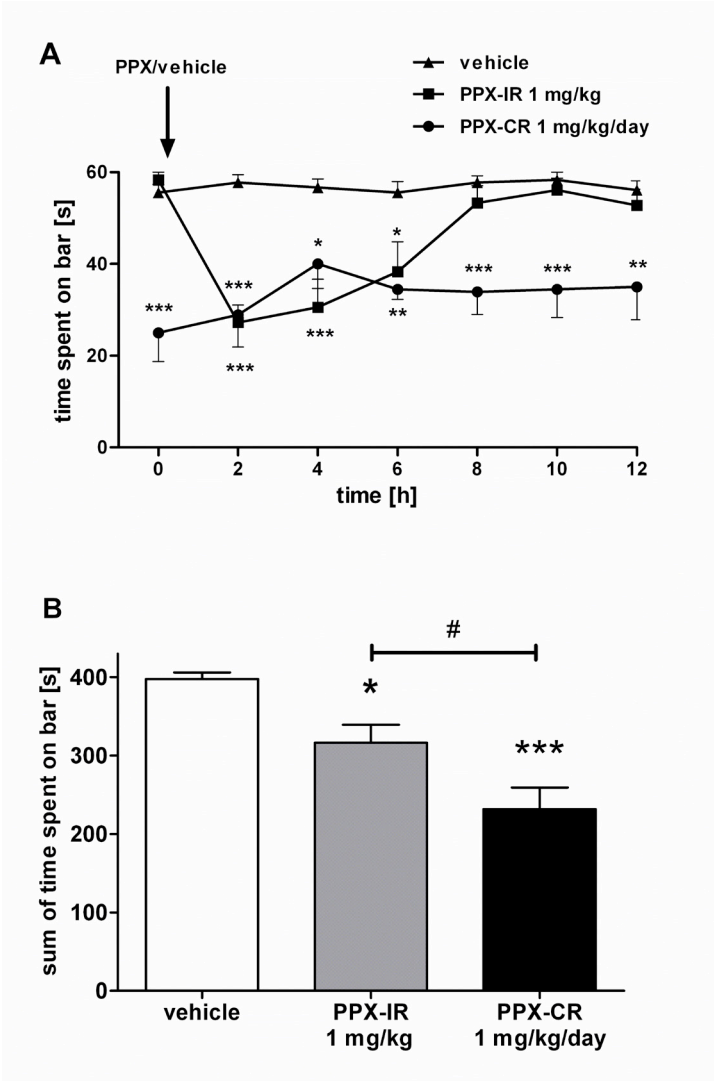


Fig. 19. Effects of PPX-IR (1 mg/kg, s.c., n = 9), PPX-CR (1 mg/kg/day, s.c., n = 9) and vehicle (n = 9, s.c.) on the time course (Fig. 19A) and cumulative data (Fig. 19B) of haloperidol-induced catalepsy. The PPX-IR group was treated with PPX 3 times on the day before the catalepsy experiment. Haloperidol (0.5 mg/kg, i.p.) was injected 2 hours prior to the first catalepsy measurement. Catalepsy was maintained for 12 hours by administration of haloperidol (0.1 mg/kg, i.p.). Data are expressed as mean ± SEM. The time course was analysed by a two-way ANOVA followed by a Bonferroni post hoc test (***P < 0.001, **P < 0.01, *P < 0.05 vs. vehicle). Cumulative data was analysed using one-way ANOVA followed by a Bonferroni post hoc test (***P < 0.001, *P < 0.05 vs. vehicle, #P < 0.05 PPX-IR vs. PPX-CR).

Reserpine-induced akinesia

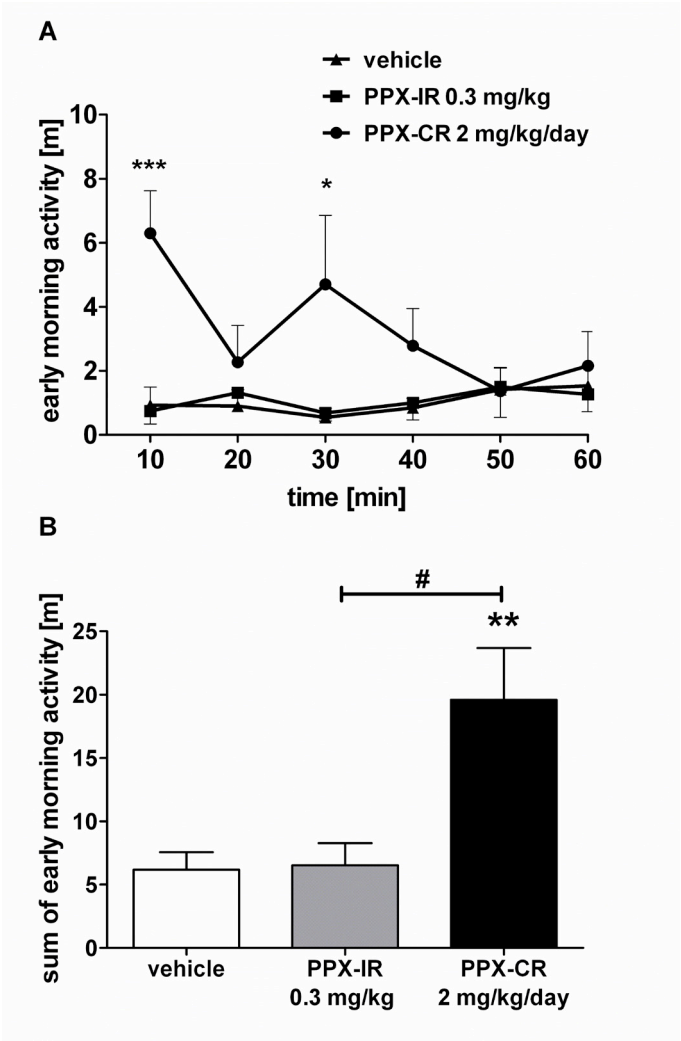


Fig. 20. Effects of PPX-IR (2 mg/kg, s.c., n = 7), PPX-CR (2 mg/kg/day, s.c., n = 6) and vehicle (n = 6, s.c.) on the time course (Fig. 20A) and cumulative data (Fig. 20B) of reserpine-induced akinesia. The PPX-IR group was treated with PPX 3 times on the day before the akinesia measurement. Reserpine (1 mg/kg, s.c.) was injected 17 hours prior to the experiment. Data are expressed as mean ± SEM. The time course was analysed by a two-way ANOVA followed by a Bonferroni post hoc test (***P < 0.001, *P < 0.05 vs. vehicle). The cumulative data was analysed using one-way ANOVA followed by a Bonferroni post hoc test (**P < 0.01 vs. vehicle, #P < 0.05 PPX-IR vs. PPX-CR).

The effects of PPX on reserpine-induced early morning akinesia are shown in Fig. 20. Statistical analysis revealed a significant treatment effect ($F(2;18) = 7.266$; $P < 0.01$). No significant differences were observed between the vehicle and the PPX-IR group indicating that pre-treatment with PPX the day before does not alter early morning akinesia. In contrast, akinesia was improved by treatment with PPX-CR at 10 min ($P < 0.001$) and 30 min ($P < 0.05$) (Fig. 20A) as well as considering the whole experiment over 60 min ($P < 0.01$) (Fig. 20B).

Measurement of extracellular DA levels

Effects of PPX on extracellular DA levels in the striatum are displayed in Fig. 21A. Pre-dose basal levels of DA in the PPX-IR were found to be 1.86 nM. In the PPX-CR group no pre-dose values could be measured because the microdialysis surgery and the implantation of the pump were carried out at the same time. At the time of DA measurement in the PPX-CR stable levels of approximately 0.07 nM were obtained which did not vary over time implicating steady state conditions. Statistical analysis

revealed significantly lower DA levels in the PPX-CR group (96.2 %) in comparison to the pre-dose values of the PPX-IR group ($P < 0.01$). The maximum effect in the PPX-IR group was observed 90 minutes after PPX treatment (44.4 % in comparison to basal DA levels).

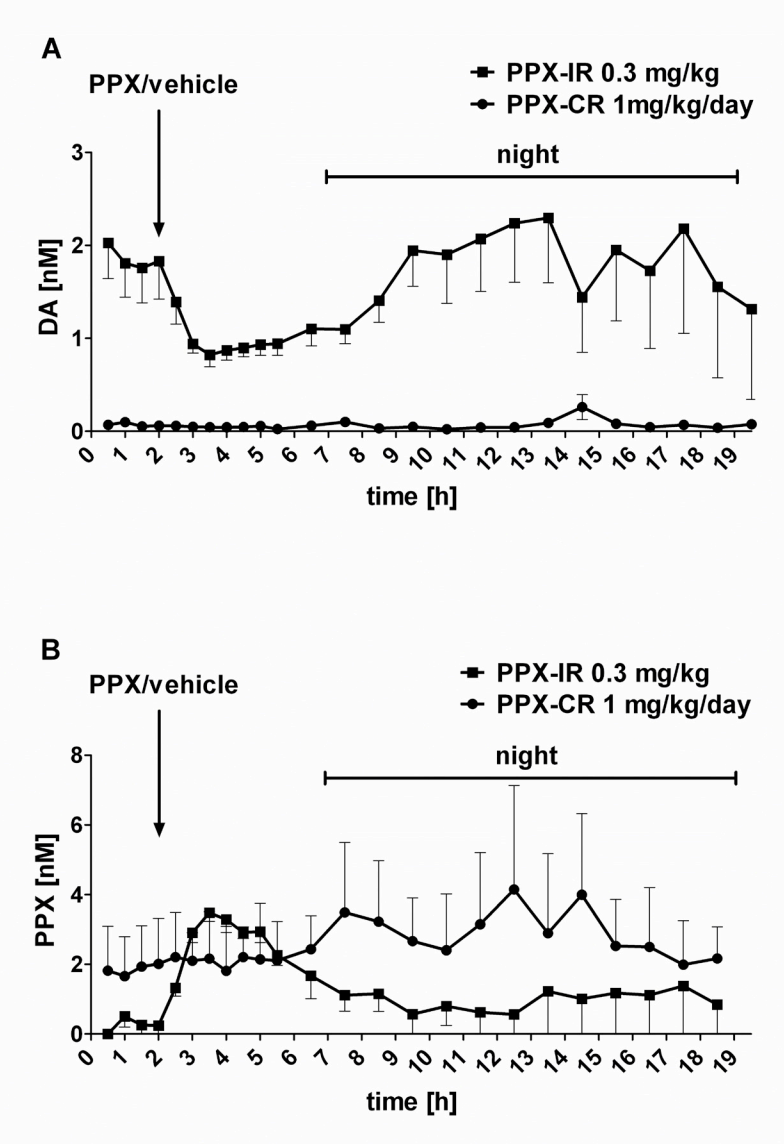


Fig. 21. In vivo microdialysis. Effects of PPX-IR (0.3 mg/kg, s.c., n = 4) and PPX-CR (1 mg/kg/day, s.c., n = 4) on extracellular levels of DA (Fig. 21A) and PPX (Fig. 21B) in the striatum. Data are expressed as mean ± SEM.

Measurement of micro-dialysate PPX levels

Extracellular PPX levels in the striatum are displayed in Fig. 21B. Pre-dose levels of PPX in the PPX-IR were found to be 0.25 nM. As mentioned before, no pre-dose levels in the PPX-CR were obtained and basal PPX levels of the PPX-CR were found to be 1.86 nM. Injection of PPX in the PPX-IR group led to an increase in PPX levels, which was maximum 90 min following PPX injection (3.48 nM).

DISCUSSION

The present neuropharmacological study demonstrated the effects of acute and continuous exposure of the DA D_3/D_2 agonist PPX in two symptomatic animal models of PD including in vivo microdialysis measurements of PPX and DA.

We found in both animal models of PD that the effects of PPX are dependent on the PPX exposure in the brain. In particular, the day following acute PPX pre-treatment the symptomatic effects of PPX were no longer present which resulted in early morning akinesia. In contrast, continuous PPX exposure using subcutaneously implanted Alzet® minipumps prevented early morning akinesia. Additionally, continuous PPX exposure produced significantly lower extracellular DA levels than the peak decrease obtained after acute PPX administration, although the PPX exposure was lower in the PPX-CR group.

Haloperidol is able to induce parkinsonian-like symptoms such as muscle rigidity (Lorenc-Koci *et al.* 1996) and catalepsy (De Ryck and Teitelbaum 1983; Fischer *et al.* 2002). Haloperidol-induced catalepsy is considered as an animal model of parkinsonian akinesia which reflects the exaggerated reflex reaction necessary to maintain postural stability, the obstruction to actively challenge stable static equilibrium and to initiate phasic locomotor movements (De Ryck *et al.* 1980). Haloperidol-induced akinesia is a result of the blockade of DA D_2 receptors in the corpus striatum (Ellenbroek *et al.* 1985). We found a pronounced effect of PPX-IR which was reversible, declined after 6 hours and was absent at 8 hours. Because the effect of a single haloperidol injection led only to significant catalepsy within 6 hours, we adapted the catalepsy model to maintain catalepsy over a longer period of time by multiple haloperidol injections using low haloperidol doses. This allowed us to study the effects of PPX-IR and PPX-CR over a long observation period of 12 hours. Only PPX-CR antagonised the haloperidol-induced catalepsy over the whole observation period which is in agreement with PPX exposure measurements (please see above). In terms of maximum efficacy the PPX-IR and PPX-CR groups did not differ. The duration of the anti-cataleptic effect was longer in the PPX-CR group. The anti-cataleptic effects are in line with a previous study on haloperidol-induced catalepsy in which a single subcutaneous injection of PPX (1 and 3 mg/kg) led to a 2.5-3 h lasting relieve of catalepsy (Maj *et al.* 1997). In contrast, higher doses of PPX (3 and 5 mg/kg) were necessary to antagonise the haloperidol-induced muscle rigidity (Lorenc-Koci and Wolfarth 1999).

In the second symptomatic animal model of PD, reserpine was used to investigate the effects of PPX-IR and PPX-CR on the motor behaviour symptom akinesia. In comparison to haloperidol which has a high affinity for DA D_2-like receptors (D_2, D_3, D_4: K_i value 1.2, 7, 2.3 nM, respectively) (Seeman and Van Tol 1994) reserpine acts

presynaptically by blocking the uptake of monoamines by the vesicular monoamine transporter 2. This inhibition unselectively affects the storage of monoamine neurotransmitters such as adrenaline, noradrenaline, DA, histamine and 5-HT in the CNS and also in the periphery. Although not specific to a single neurotransmitter pathway and without involvement of neurodegenerative events the reserpine model is still a valuable tool to investigate symptomatic effects of DA receptor agonists in PD (Maj *et al.* 1997) as well as to study non-dopaminergic mechanisms in PD (Kreitzer and Malenka 2007; Niswender *et al.* 2008). We optimised the typical reserpine model by reducing the dose of reserpine to 1 mg/kg.

As seen in the haloperidol-induced catalepsy model, PPX-CR antagonised the motor impairment. PPX-CR was effective over the whole observation period including the first measurement on early morning akinesia. Using a higher dose of reserpine (5 mg/kg) in combination with α-methyl-p-tyrosine (250 mg/kg, i.p.) to additionally block DA biosynthesis, Maj *et al.* (1997) showed that a single subcutaneous injection of PPX (0.3 and 1 mg/kg) increased locomotor activity. The effect of PPX was even higher than obtained in the vehicle+reserpine/α-MT group as well as in the vehicle+vehicle control group. Under our conditions we did not observe any hyperactivity neither in the PPX-IR nor in the PPX-CR group.

Unbound PPX brain tissue levels, CSF or microdialysate levels have not been published so far. Most of the pharmacokinetic data on PPX exposure relied on studies measuring PPX plasma levels. For example in healthy volunteers the plasma concentrations of PPX were proportional to dose under steady-state conditions (elimination half-life $t_{1/2}$ 8-12 hours, t_{max} 1-3 hours, c_{max} 0.375-4.5 ng/ml) (Kvernmo *et al.* 2006; Wright *et al.* 1997). Recently, we reported that PPX accumulated in the brain indicated by a brain plasma ratio of 6.7 obtained in mice after oral administration (Danzeisen *et al.* 2006), which is in accordance with the proposed active transport of PPX through the blood-brain barrier by an organic cation-sensitive transporter (Okura *et al.* 2007). In vivo microdialysis is the method of choice to analyse both the drug as well as the biomarker at the target side (i.e. the striatum in PD) in the same sample. We took advantage of the DA measurement using HPLC-ECD which is very sensitive and additionally established a method to analyse PPX in microdialysates using LC-MS/MS. The PPX exposure in the rat striatum was maximum at 90 minutes (3.48 nM) following injection in the PPX-IR group and declined over a period of 3 hours. Animals continuously treated with PPX showed

lower maximum PPX levels and revealed an almost constant striatal PPX exposure of 2.46 nM over the whole experiment.

In the present study PPX-IR and PPX-CR affected extracellular DA levels. The reduction of extracellular DA levels can be explained by stimulation of presynaptic DA receptors in dopaminergic nerve terminals. This effect is characteristic for DA receptor agonists including PPX and reflects both the involvement on regulation of DA synthesis and on Ca^{2+} dependent exocytotic DA release by the DA autoreceptor mediated feedback inhibition (Wolf and Roth 1987). PPX binds preferentially to DA D_3 receptors followed by DA D_2 receptors (Mierau *et al.* 1995; Piercey *et al.* 1996), which fits to the role of presynaptic DA D_3 receptors on DA release (Gainetdinov *et al.* 1996) and DA synthesis (Wolf and Roth 1990). Additionally, it was demonstrated that DA D_3 preferring compounds modulate the DA uptake in vitro and in vivo suggesting that the DA D_3 receptor activation increases DA uptake by modulating the DA transporter activity (Zapata and Shippenberg 2002).

Indeed, systemic PPX administration caused a long-lasting reduction of extracellular DA and DA metabolite levels in rat striatum (Carter and Muller 1991; Robertson *et al.* 1993). This effect was reversed by the D_2 DA receptor antagonist sulpiride but not by the D_1 DA receptor antagonist SCH 23390 (Carter and Muller 1991). Local PPX administration reduced the 6-OHDA-induced increase of extracellular DA in rat striatum (Ferger *et al.* 2000) and reduced the L-type Ca^{2+} channel activator (BAY K 8644)-induced rise in extracellular DA levels (Maruya *et al.* 2003). Cumulative evidence underlines that extracellular DA levels measured by in vivo microdialysis is a suitable biomarker to indicate DA D_2/D_3 receptor stimulation and therefore can be used to compare the effects of PPX-IR and PPX-CR. Although slightly higher peak PPX levels were obtained after PPX-IR, the extracellular DA levels were consistently lower in the PPX-CR group which speaks against desensitization and a tolerance effect on regulation of extracellular DA levels after CDS by PPX. (Chernoloz *et al.* 2009) performed an electrophysiological experiment in anesthetised rats which were subcutaneously implanted with osmotic minipumps delivering PPX at a dose of 1 mg/kg per day for 2 or 14 days. They demonstrated a decrease in the spontaneous firing of dopaminergic neurons by 40 % after 2 days of treatment, whereas after 14 days of PPX treatment the firing rate of DA neurons had recovered and implicated a desensitization of D_2/D_3 cell body autoreceptors after long-lasting continuous PPX treatment.

Continuous DA receptor stimulation using DA agonists delivered by miniosmotic pumps or by external infusion systems are useful in preclinical animal experiments but are impractical in most patients suffering from PD. In patients the most convenient route of administration is the oral one. Subcutaneous administration of DA receptor agonists as used in the present study can be alternatively used when a rapid onset of action is needed. A continuous subcutaneous infusion is suitable to stabilise motor functions (Nyholm 2006). A drawback of long-term subcutaneous infusion, however, could be adverse events such as skin reactions and nodules accompanied by variable drug absorption (Deleu *et al.* 2004). These disadvantages prompted the development to oral sustained or extended release formulations in several drug development programmes. In case of low bioavailability of a compound, transdermal delivery of dopaminergic drugs can be a practical method to achieve CDS and may be useful in patients with swallowing difficulties (Steiger 2008).

Particularly at night and in the early morning hours constant plasma levels of short acting DA agonists cannot be maintained because of drug clearance. In fact, the therapeutic efficacy of the symptomatic effects of DA agonists including PPX is closely related to sufficient drug exposure levels. As a matter of fact cardinal PD symptoms appear if dopaminergic receptor stimulation cannot be maintained. Another issue beyond the prolongation of the symptomatic effects of CDS by DA agonists is the reduced risk to develop motor complications such as dyskinesia as demonstrated in parkinsonian cynomolgus monkeys (Bibbiani *et al.* 2005). Furthermore, the reversal of motor deficits without dyskinesia induction in MPTP-treated common marmosets argues for the concept of CDS (Stockwell *et al.* 2008).

In conclusion, this study highlights the potential benefit of CDS using PPX-CR and the advantage over PPX-IR in two symptomatic PD models. Currently, a once-daily PPX extended release formulation (tablet) undergoes regulatory review. The outcome of clinical studies will show if the beneficial effects of CDS provided by PPX-CR translates into daily life of individuals affected with PD

This study was performed in collaboration with colleagues. My contribution was the measurement of DA levels using HPLC-ECD as well as preparation of the manuscript by preparing figures and writing the "materials and methods" as well as "results" part.

CHAPTER IX

Effects of L-DOPA on striatal monoamines in mice with L-DOPA-induced hyperactivity

ABSTRACT

L-DOPA remains the "gold standard" pharmacological treatment for PD, but has a tendency to produce disabling hyperactive movements called dyskinesias in a substantial number of these patients. As a precursor molecule, exogenous L-DOPA is converted to DA in the striatum of PD patients, but its utilization in other monoamine pathways, as related to its hyperactive side effects, is still not well understood. Using HPLC and a C57 mouse model of L-DOPA-induced dyskinesia that was developed in our laboratory, the present study examined the effects of exogenous L-DOPA with carbidopa (CD) on monoamines and their metabolites in the striatum versus the olfactory bulb as a control. Thus, mice previously exposed to MPTP and later made hyperactive with high dose exogenous L-DOPA (n = 10) were compared to normal acting mice who received L-DOPA without previous MPTP (n = 4) or no treatment at all (n = 4). In the olfactory bulb, exogenous L-DOPA uniformly caused increased DA levels and increased DA-, 5-HT- and NA turnover rates, but decreased 5-HT and NA levels, regardless of animal activity. These trends were also seen in the striatum, but, DA and 5-HT levels were less and DA- and 5-HT-turnover rates were higher, in dyskinetic animals. In addition, the expected increased NA-turnover rate was not seen in the striatum of dyskinetic animals. The results of the present study demonstrates that striatal monoamines in dyskinetic animals have a unique profile that must be considered when trying to explain the effects of antidyskinetic drugs that utilize monoamine receptors.

INTRODUCTION

As a precursor molecule, exogenous L-DOPA can theoretically be converted to DA by any cell containing the enzyme AADC. This enzyme is found not only in all neurons that produce catecholamines, including DA, and NA, but also in those that produce indolamines such as 5-HT (5-HT). In normal adult C57 mice given large amounts exogenous L-DOPA, brain DA levels increased while brain 5-HT levels decreased in a dose dependent manner (Everett and Borcherding 1970). These

experiments and others (Arai *et al.* 1994; Arai *et al.* 1995; Carta *et al.* 2007; Ng *et al.* 1970) suggested that AADC in 5-HT neurons, if overwhelmed by large amounts of exogenous L-DOPA, can produce DA and that this would be done at the expense of 5-HT production. In NA producing cells, on the other hand, it was suspected that exogenous L-DOPA would be converted to DA, but then this would be further converted by the enzyme dopamine beta hydroxylase (DBH) to increase NA production. Indeed, when large doses of exogenous L-DOPA were given to normal rats, levels of brain NA increased, but only transiently (Chalmers *et al.* 1971; Romero *et al.* 1972). In PD, it is well known that catecholamine neurons, such as those in the dopaminergic nigrostriatal pathway, and noradrenergic neurons of the locus coeruleus are severely degenerated (Alvord 1968; Forno 1966). Prior to the use of L-DOPA, striatal DA and NA in PD patients were found to be decreased by approximately 90% and 80%, respectively (Hornykiewicz 1975). As a result, it has long been suspected that the efficacy of L-DOPA as one of the best pharmacological treatments for PD, depends upon surviving striatal 5-HT pathways containing AADC to convert a significant amount of the exogenous L-DOPA into DA; however, prior to the use of L-DOPA, striatal 5-HT levels were also found to be decreased by about 56% (Hornykiewicz 1975). Due to the fact that striatal DA, NA and 5-HT levels seem to be affected both by the pathophysiological processes of PD and by exposure to exogenous L-DOPA, it has also been theorized that the side effect of debilitating, hyperactive involuntary movements referred to as L-DOPA-induced dyskinesia that have been reported in approximately 50% of PD patients taking L-DOPA therapy over 5 years (Ahlskog and Muenter 2001), are related to a combination of these monoamine changes (Brotchie 2005). However, the influence of exogenous L-DOPA on all striatal monoamines as it relates to the emergence of this hyperactive side effect of PD treatment has not yet been fully examined. In a C57 mouse model of PD, it has been shown that the neurotoxin MPTP depletes striatal DA by approximately 84% (Nishi *et al.* 1991; Sundstrom *et al.* 1987) and NA by 46% (Nishi *et al.* 1991), while leaving striatal 5-HT virtually intact (Heikkila *et al.* 1984; Sundstrom *et al.* 1987). Using this model, the present study examines the influence of high dose L-DOPA given with the peripheral AADC inhibitor CD, on monoamine production and metabolism in the striatum of hyperactive mice previously exposed to MPTP, compared to normal acting mice that either received high dose L-DOPA/CD without previous MPTP exposure or no treatment at all. Olfactory bulbs from these animals

served as controls, since they represent an easily accessible, non-motor brain area containing the same monoamines as those present in the striatum and were shown in C57 mice to result in similar MPTP-induced depletions of DA (Sundstrom *et al.* 1987). The results should determine if exogenous L-DOPA influences monoamine levels and metabolism of different brain areas in different ways and if there is a unique monoamine profile in the striatum of dyskinetic animals in particular.

MATERIALS AND METHODS

Methods for preparing the mouse model of L-DOPA-induced dyskinesia were performed in accordance with NIH guidelines and described previously (Nicholas 2007). Briefly, 10-12 month old male C57 BL/6 mice (Charles River) were divided into three groups (Fig 26), including 1) untreated normoactive controls (n = 4), 2) hyperactive animals previously exposed to MPTP (30 mg/kg in 5% ethanol) for 10 consecutive days and later given high dose L-DOPA after an additional 10-14 days of rest (n = 10), and 3) normoactive mice that also received high dose L-DOPA but were not previously exposed to MPTP (n = 4). Stable motor responses of surviving MPTP-treated and non-treated animals were measured using an external cage rack Photobeam Activity System (San Diego Instruments, San Diego, California). Behavioural responses of the animals used in this study have been previously reported (Nicholas 2007). Animals that received L-DOPA (Sigma, 200 mg/kg) were given intraperitoneal injections mixed with CD (DuPont, 25 mg/kg) in normal saline, twice daily and were sacrificed by decapitation 60 minutes after the fifth L-DOPA dose at the peak of hyperactivity in dyskinetic mice and at the same time as control animals receiving L-DOPA that were not hyperactive. The striatum and olfactory bulbs of these mice were quickly removed, frozen on dry ice and stored at –80ºC. Prior to HPLC analysis, all tissue samples were transferred separately to 15ml plastic tubes and weighed. Ice-cooled perchloric acid (0.4 M) was added to the striatum (500 µl), and olfactory bulbs (300 µl) and the tissue was homogenised for 10 s using an ultrasonicator and centrifuged for at least 20 min at 3200 x g at 4 °C. The supernatant was passed through a 0.2 µm filter (Minisart RC4, Sartorius AG, Germany) and frozen again at -80 °C. Using HPLC-ECD, the samples were analysed for DA and its metabolite DOPAC, 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) and NA and its metabolite 3-methoxy-4- hydroxyphenylglycol (MHPG),

under isocratic conditions. Chromatographic separation was performed using a reversed-phase column (Discovery C18, 150 x 4.6 mm i.d., 5 µm particles, with pre-column, Supelco, USA). The mobile phase included 1-octanesulfonic acid sodium salt (0.32 mM), Na_2EDTA x 2 H_20 (0.27 mM), NaCl (8.0 mM) and KH_2PO_4 (50.0 mM) After the pH was adjusted to 4.00 with H_3PO_4, the mobile phase was then passed through a 0.22-µm filter, mixed with acetonitrile (95:5, v/v) and delivered at a flow rate of 1.0 ml/min. Quantification of brain monoamine levels were calculated as mean ± standard deviation and compared among experimental groups using ANOVA and two-tailed t-tests.

RESULTS AND DISCUSSION

The results showed that, compared to untreated animals and regardless of animal activity and previous MPTP exposure, exogenous L-DOPA tended to significantly increase DA and deplete 5-HT and NA in the olfactory bulb (Fig. 22A) and striatum with a few important caveats (Fig. 22B). For example, the highest DA levels were found in normal acting animals that received L-DOPA but not MPTP, proving that high striatal DA alone was not responsible for hyperactivity. In dyskinetic mice, striatal DA was increased and 5-HT and NA decreased as compared to untreated controls, but only DA and 5-HT were statistically significantly decreased as compared to normal acting mice that received L-DOPA but not MPTP. These results suggest that exogenous L-DOPA tends to increase DA at the expense of 5-HT and NA, but these are even more pronounced in the striatum where MPTP has an additional detrimental effect. It also seems that in the striatum, the ability to decrease NA is more due to MPTP than to L-DOPA exposure.

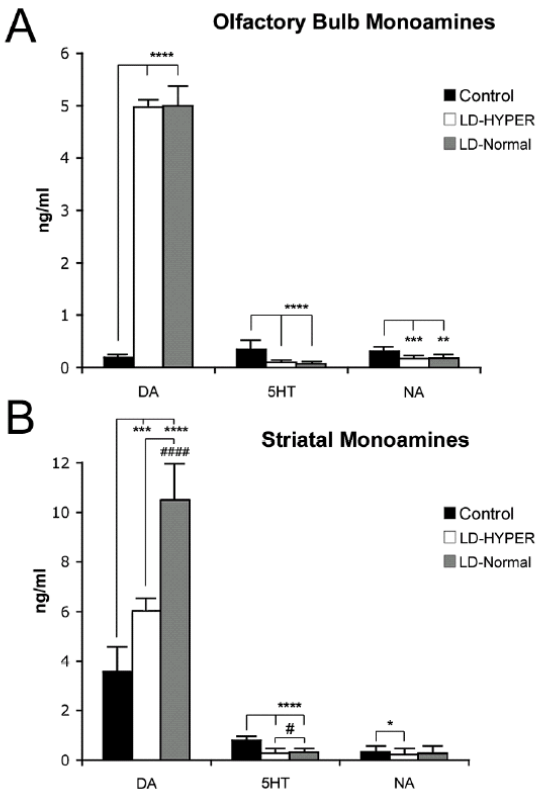


Fig. 22. Monoamines in the olfactory bulb (A) and striatum (B) of untreated normal acting control mice (Control), animals with L-DOPA-induced dyskinesia (L-DOPA-HYPER) and L-DOPA-treated normal acting control mice (L-DOPA-Normal). P values of L-DOPA-treated groups vs untreated group: **** < 0.0001, *** < 0.001, ** < 0.01, * < 0.05; P values between L-DOPA-treated groups: #### < 0.0001, # < 0.05.

Turnover rates were determined after examining monoamines and their metabolites (Table 6) and L-DOPA treatment was shown to universally increase DA-, 5-HT- and NA-turnover rates (Fig. 23), with a few important exceptions. In the olfactory bulb, monoamine turnover rates were not statistically significantly different between hyperactive mice and normoactive mice receiving L-DOPA (Fig. 23A). In contrast, DA- and 5-HT-turnover rates in the striatum of dyskinetic mice were highest, while striatal NA-turnover was not significantly different from normal acing control animals, whether they received L-DOPA or not (Fig. 23B). As a result, the expected increase in striatal NA-turnover was not seen in mice with L-DOPA-induced dyskinesia.

	Normoactive (n=4) no treatment	Hyperactive (n=10) MPTP/carbidopa/ L-DOPA	Normoactive (n=4) carbidopa/ L-DOPA
Olfactory bulb monoamine metabolites: mean ng/mg wet tissue weight ± SEM			
DOPAC	0.1455 ± 0.0072	11.5616 ± 0.5087***	12.8095 ± 0.8265***
HVA	0.2604 ± 0.0188	3.5230 ± 0.1604***	3.1426 ± 0.2245***
5-HIAA	0.3677 ± 0.0099	0.1946 ± 0.0068***	0.1711 ± 0.0171***
MHPG	0.5595 ± 0.0407	0.5275 ± 0.0492	0.3833 ± 0.0755
Striatal monoamine metabolites: mean ng/mg wet tissue weight ± SEM			
DOPAC	0.9818 ± 0.1150	20.6017 ± 1.1671***	19.8405 ± 0.8227***
HVA	0.6805 ± 0.0878	6.5243 ± 0.2871***,#	5.5688 ± 0.2680***
5-HIAA	0.6856 ± 0.0716	0.6230 ± 0.0336***,##	0.4943 ± 0.0193***
MHPG	0.2393 ± 0.0137	0.5862 ± 0.2937	0.2141 ± 0.0177

Table 6. Monoamine metabolites in olfactory bulb and striatum in LID vs. control mice. ***P < 0.0001 vs. non-treated controls; ##P < 0.001, #P < 0.01 hyperactive vs. normoactive carbidopa/L-DOPA treated groups.

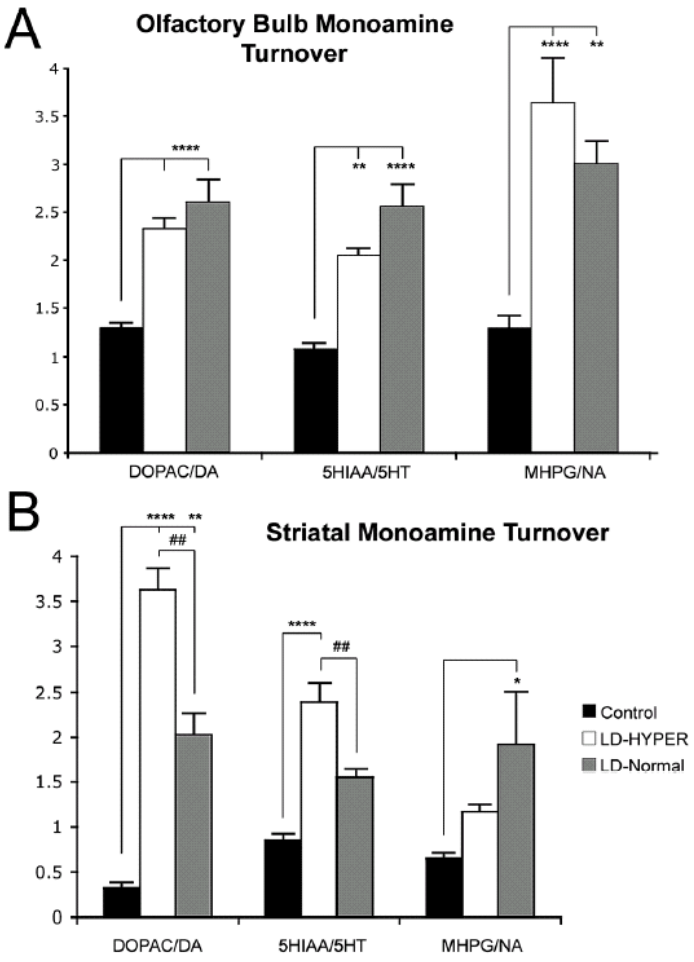


Fig. 23. Monoamine turnover rates in the olfactory bulb (A) and striatum (B) of untreated normal acting control mice (Control), animals with L-DOPA-induced dyskinesia (L-DOPA-HYPER) and L-DOPA-treated normal acting control mice (L-DOPA-Normal). DA turnover (DOPAC/DA); 5-HT turnover (5HIAA/5-HT); NA turnover (MHPG/NA); P values for L-DOPA-treated groups vs untreated group: **** < 0.0001, ** < 0.01, * < 0.05; P values between L-DOPA-treated groups: ## < 0.007.

These findings are consistent with the hypothesis that when the nigrostriatal system is damaged, as in the PD or one of its animal models, a relatively better intact 5-HT terminal networks converts much of the exogenous L-DOPA into DA and this is done at the expense of 5-HT production. However, these results also show that previous damage of DA pathways is not a prerequisite to causing decreases in 5-HT, as conversion of exogenous L-DOPA to DA at the expense of 5-HT takes place even in animals not exposed to MPTP. The results of the present study can be explained by the high likelihood that AADC in both DA and 5-HT neurons were saturated by excessive amounts of L-DOPA given in these experiments, regardless of previous damage to DA pathways. This is supported by studies in normal rats where dual colour fluorescence showed previously unseen DA immunoreactivity localized in serotonergic fibers of the striatum and cell bodies of the midbrain raphe nuclei only after rats were treated with exogenous L-DOPA (Arai *et al.* 1995). Using HPLC analysis, L-DOPA treatment in normal rats resulted in significant loss of 5-HT in many serotonergic and dopaminergic regions of the brain, especially the striatum, while striatal DA and DA turnover (Borah and Mohanakumar 2007) and enhancement of NA turnover in rat brain (Keller *et al.* 1974) were significantly higher. These findings are consistent with the present study. Furthermore, depression was

seen in L-DOPA-treated animals, as revealed in a forced swim test (Borah and Mohanakumar 2007), suggesting that L-DOPA-induced decreased 5-HT levels were partly responsible. In the 6- OHDA lesioned striatum of rats that received L-DOPA, 5-HT levels were decreased (Carta *et al.* 2007) and DA was detected within increased numbers of serotonergic varicose fibers, suggesting that striatal serotonergic hyperinnervation may compensate for the lost function of dopaminergic neurons in this model (Maeda *et al.* 2005). Sprouting of serotonergic afferents into the striatum was also seen after MPTP exposure in C57 mice (Rozas *et al.* 1998). In the present study, striatal 5-HT levels were the lowest in dyskinetic animals, suggesting an additional depleting effect of both MPTP and L-DOPA. It was previously shown in C57 mice that MPTP alone had no significant effect on striatal 5-HT levels (Heikkila *et al.* 1984; Sundstrom *et al.* 1987), but the MPTP dosing schedules used in these prior experiments differed from the present study. In contrast to 5-HT, NA production and breakdown seemed to differ slightly in different brain locations. Since DA is a substrate for NA, it could be theorized that exogenous L-DOPA should cause universal increases in both DA and NA. Thus, L-DOPA first is converted to DA in the cytosol, since the DA-synthesizing enzyme AADC is found in this location. DA would then be packaged in vesicles and should be converted to NA in that location, since the NA-synthesizing enzyme DBH is bound to the insides of vesicular membranes. However, one surprising finding in the present study was that exogenous L-DOPA treatment actually tended to universally decrease NA levels, especially in the olfactory bulb, and this effect was seen regardless of motor activity or previous MPTP exposure. These results suggest that in animals treated with L-DOPA, the cytosolic pool of DA in these noradrenergic terminals is so high, that perhaps the increased production of synaptic vesicles happens so rapidly that DBH incorporation into these vesicles is lacking. This is consistent with previous studies in normal rodents showing that exogenous L-DOPA increased primarily the cytoplasmic pool of DA and that significant increases in vesicular DA required previous catecholamine depletion (Buu 1989). However, in the striatum, NA production was not statistically different in normal acting animals, whether they previously received L-DOPA or not. Striatal NA levels were only lower in hyperactive animals, suggesting an MPTP-induced NA-depleting effect that was previously reported (Nishi *et al.* 1991). Although there is a trend that L-DOPA treatment results in increased NA turnover, the present study demonstrates that the MHPG/NA ratio also differs slightly, depending upon the site

examined. In the olfactory bulb, L-DOPA-induced NA turnover is universally increased, similar to both DA and 5-HT turnover rates. However, the expected increased turnover of NA in the striatum is not seen in mice with L-DOPA-induced dyskinesia, suggesting that in these mice the small amounts of NA that are being produced are not being recycled as quickly, perhaps resulting in abnormally sustained NA-receptor activation. This hypothesis would concur with the findings that highly selective alpha-2 noradrenergic blocking agents such as idazoxan and fipamezole have been successfully used to counteract dyskinesia in the MPTP monkey model of L-DOPA-induced dyskinesia (Grondin *et al.* 2000; Henry *et al.* 1999; Savola *et al.* 2003) and in human patients with these involuntary movements (Rascol *et al.* 2001) and that these receptors seem to be plentiful in the striatum (Nicholas *et al.* 1993; Rosin *et al.* 1996; Scheinin *et al.* 1994). Antidyskinetic effects utilizing serotonergic mechanisms have also been shown with various drugs in animal models and humans with L-DOPA-induced dyskinesia, although most of these agents also bind to other monoamine receptors as well (Nicholson and Brotchie 2002). However, using more specific 5-HT receptor binding drugs, recent evidence suggests that in 6-OHDA rats, L-DOPA but not DA depletion results in up-regulation of striatal 5-HT_{1B} receptors (Zhang *et al.* 2008) and an antidyskinetic effect can be obtained with 5-HT_{1A} (Carta *et al.* 2007) and 5-HT_{1B} agonists (Carta *et al.* 2007; Zhang *et al.* 2008), believed to be working at autoreceptors on serotonergic neuronal cell bodies/dendrites and terminals, respectively (Carta *et al.* 2007). The results of the present study suggest that exogenous L-DOPA can influence brain monoamine levels and metabolism regardless of animal activity; however, there is a unique monoamine profile in the striatum of dyskinetic animals in particular. Other motor brain areas in which monoamines are present should also similarly be examined in future studies, as well as other animal models of L-DOPA-induced dyskinesia to see if these trends are consistent. In any case, the present study suggests that in designing new drugs to treat PD patients with L-DOPA-induced dyskinesia, careful consideration should be made regarding choosing pharmacological profiles that would optimally affect these altered DA, 5-HT and NA brain pathways.

This study was performed in collaboration with Prof. Anthony Nicholas (University of Alabama at Birmingham, USA), which conducted the in vivo part. My contribution was the development of the HPLC method as well as the preparation and HPLC-analysis of the tissue samples.

CHAPTER X

Summary

At present, several animal models mimicking L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia are applied to investigate mechanisms underlying the induction and manifestation of dyskinesia as well as investigate potential drug targets for the alleviation of L-DOPA-induced dyskinesia. I used the 6-hydroxydopamine (6-OHDA) dyskinesia model in rats, since it is a very robust and reliable model which is characterised by a clear behavioural outcome. The aim of my thesis was to investigate neurochemical and behavioural mechanisms underlying the expression of dyskinetic movements and thus characterise the rat L-DOPA dyskinesia model in depth. I shed light on various pathogenic factors, which are important for the manifestation of L-DOPA-induced dyskinesia, as well as tested novel drug targets to obtain new strategies for the treatment of L-DOPA-induced dyskinesia.

One of the techniques I preferentially applied during my thesis is the technique of in vivo microdialysis. It is an invasive technique where neurotransmitter alterations as well as pharmacokinetic profiles of drugs in the brain extracellular fluid can be analysed. This bears the advantage to monitor neurotransmitter release and turnover not only under baseline conditions but also upon stimulation or inhibition with drugs since neurotransmitter or compound changes can be determined over time in the same animal resulting in fewer animal numbers per study. Additionally, behaviour can be observed in parallel and correlated to neurochemical changes. In vivo microdialysis can also be used reciprocally to administer compounds locally into various brain areas of interest, which is called reverse in vivo microdialysis. I frequently applied the technique of reverse in vivo microdialysis to assess different parameters, which may be important for the evocation of dyskinetic movements such as the choice of neurotransmitter as well as the factors lesion and brain area. Moreover, I used several behavioural tasks including the measurement of exploratory behaviour in an open-field system, the haloperidol-induced catalepsy test and the reserpine-induced akinesia test. In addition, monitoring of dyskinetic behaviour was one of the main behavioural tests I performed during my thesis. Furthermore, I used various chromatography techniques including high performance liquid chromatography (HPLC) combined with either electrochemical (ECD) or fluorescence

detection (FD) as well as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the measurement of neurotransmitters and pharmacokinetics of compounds.

The introduction comprises a review on the pathophysiology of L-DOPA-induced dyskinesia, in which the test strategy to assess the antidyskinetic effect in animal models as well as the translational aspect of preclinical and clinical studies is outlined.

In the first study (**chapter III: Intrastriatal inhibition of aromatic amino acid decarboxylase prevents L-DOPA-induced dyskinesia**), I demonstrated that the increase of intrastriatal dopamine (DA) levels was instrumental for L-DOPA- and DA-induced dyskinetic behaviour, whereas intrastriatal L-DOPA administration itself was not able to induce dyskinetic behaviour in the 6-OHDA rat model of dyskinesia. This result was obtained by striatal administration of benserazide via reverse in vivo microdialysis blocking the conversion of L-DOPA to DA. For the first time the striatal role of DA was clearly correlated with dyskinetic movements and a possible contribution of DA metabolites has been ruled out. In order to further investigate the downstream pathway of DA, I studied the effect of noradrenaline (NA) on the ability to provoke dyskinesia (**chapter IV: Comparison of intrastriatal administration of noradrenaline and L-DOPA on dyskinetic movements**). I clearly showed that NA is able to evoke dyskinesia in the same manner as L-DOPA. For the first time NA was discovered as dyskinesiogenic agent, which hypothesizes a novel form of dyskinesia, the “NA-induced” dyskinesia. Subsequently, I investigated the ability of different adrenoceptor antagonists to reduce dyskinetic movements induced by L-DOPA (**chapter V: The selective α_1 adrenoceptor antagonist HEAT reduces L-DOPA-induced dyskinesia in a rat model of Parkinson’s disease**). I demonstrated that the selective α_1 adrenoceptor antagonist HEAT, the α_2 adrenoceptor antagonist idazoxan as well as the non selective β_1/β_2 adrenoceptor antagonist propranolol alleviated L-DOPA-induced dyskinesia in a dose-related manner. For the first time, dyskinesia was reduced by administration of a α_1 adrenoceptor antagonist suggesting a novel treatment option for L-DOPA-induced dyskinesia. However, it remains unclear if α_1 adrenoceptor antagonists at antidyskinetic doses might interfere with the cardiovascular system since some α_1 adrenoceptor antagonists are generally applied for the treatment of hypertension. In more detail, I investigated the mechanisms of the α_2 adrenoceptor antagonist

idazoxan involved in the antidyskinetic effects. Particularly, I studied alterations of L-DOPA-induced extracellular DA and L-DOPA levels in the lesioned as well as intact striatum using in vivo microdialysis (**chapter VI: The α_2 adrenoceptor antagonist idazoxan alleviates L-DOPA-induced dyskinesia by reduction of striatal dopamine levels**). I found that idazoxan at antidyskinetic doses reduced extracellular L-DOPA levels in the lesioned as well as in the intact striatum. Moreover, idazoxan at therapeutic doses led to a decrease in extracellular DA levels in the 6-OHDA-lesioned striatum. In contrast, no reduction of L-DOPA or DA levels was found when idazoxan was given at doses which did not alleviate dyskinetic movements. Thus, a reduction of L-DOPA and DA levels may contribute to the antidyskinetic effect of idazoxan.

Another objective of my thesis was to focus on the role of the glutamatergic/GABAergic system in L-DOPA-induced dyskinesia. In the next study (**chapter VII: Striatal L-DOPA but not GABA and glutamate is the crucial trigger to evoke dyskinetic movements in 6-OHDA-lesioned rats**), I investigated the effects of GABA and glutamate on the ability to provoke dyskinesia in rats with already established dyskinetic history. Neither GABA nor glutamate did evoke dyskinesia after local administration in the striatum, GP as well as substantia nigra pars reticulata (SNr). In contrast, local L-DOPA administration in the lesioned striatum produced dyskinetic movements. I postulate a site specific action of L-DOPA since L-DOPA can provoke dyskinesia in the lesioned striatum but not after local administration into the GP or SNr.

Another approach to reduce dyskinetic movements is based on the concept of continuous dopaminergic stimulation (CDS), which takes advantage of the fact that continuous stimulation of dopaminergic receptors may be less prone to induce dyskinesia. CDS can be achieved by administration of longer-lasting DA agonists, which prevent dramatic fluctuations of plasma and brain levels, and L-DOPA or DA agonist formulations, which extendedly release the drug. Moreover, CDS may also target alleviation of early morning akinesia because of a sustained stimulation of dopaminergic receptors during the night. In collaboration with colleagues, I assessed the CDS approach using a continuous release formulation of the non-ergoline DA agonist pramipexole (PPX) by investigating symptomatic effects of PPX-CR (continuous release) in two animal models of Parkinson´s disease (PD) (**chapter VIII: Continuous dopaminergic stimulation by pramipexole is effective to treat early**

morning akinesia in animal models of Parkinson's disease: a pharmacokinetic-pharmacodynamic study using in vivo microdialysis). I found that PPX-CR provides a higher therapeutic benefit than PPX-IR (immediate release) since PPX-CR significantly reduced early morning akinesia as well as constantly provided an anticataleptic response in vivo.

Besides the 6-OHDA rat model of dyskinesia, various other rodent models of L-DOPA-induced dyskinesia such as the L-DOPA-induced hyperactivity model following reserpine and transgenic mouse models including the regulator of G-protein signaling 9-2 (RGS9-2) knock-out mice and aphakia mice are available (please see chapter I for more details). Another mouse model for L-DOPA-induced dyskinesia relies on the dopaminergic degeneration using systemic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treatment followed by chronic administration of L-DOPA. Mouse models are more appreciated in the drug discovery process because of lower absolute amount of compounds needed. Moreover, mouse models may be more valuable for the investigation of basic mechanisms since transgenic mice overexpressing or lacking proteins involved in the disease can be used. In collaboration with Prof. Anthony Nicholas (University of Alabama at Birmingham; USA), the new mouse model of L-DOPA-induced dyskinesia was characterised by post mortem neurochemistry studies focusing on the monoamine neurotransmitters DA, serotonin (5-HT) and NA including metabolites (**chapter IX: Effects of L-DOPA on striatal monoamines in mice with L-DOPA-induced hyperactivity**). I found that there is a unique monoamine profile in the striatum of animals conducive to develop dyskinesia.

Taken together, this thesis demonstrated that the 6-OHDA rat dyskinesia model is a valuable and reliable model for both investigating mechanisms underlying L-DOPA-induced dyskinesia as well as identifying and validating novel drug targets. It is a well characterised model from the behavioural as well as molecular and biochemical side. The results from the present thesis demonstrate that 1) the neurotransmitter DA is instrumental for the manifestation of dyskinesia; 2) the DA-denervated striatum is the crucial brain area for L-DOPA-induced dyskinesia and 3) the noradrenergic systemic plays a role in dyskinesia, whilst NA can induce dyskinesia and anti-adrenergic drugs can alleviate L-DOPA-induced dyskinesia (for summary please see fig. 24).

Numerous preclinical findings obtained in the 6-OHDA dyskinesia rat model have been successfully translated into the clinic (chapter I). However, testing potential drug

candidates in the model have to be critically evaluated regarding false positive/negative outcome since there are some confounding factors such as sedation, motor depressant effects, pharmacokinetic interactions as well as species-related differences. Considering these issues, the model provides a reliable and good opportunity to gain insights into the behavioural and molecular mechanisms underlying L-DOPA-induced dyskinesia. At present no antidyskinetic compounds are in the market. Only the NMDA antagonist amantadine is off-label used by PD patients, but with controversial success. Thus, there is an unmet medical need for the treatment of dyskinesia in PD and it is indispensable to focus on the development of new therapeutic strategies to limit the burden on PD patients, their families and carers. In conclusion, validated animal models, which reliably predict clinical efficacy, are essential for the drug discovery process.

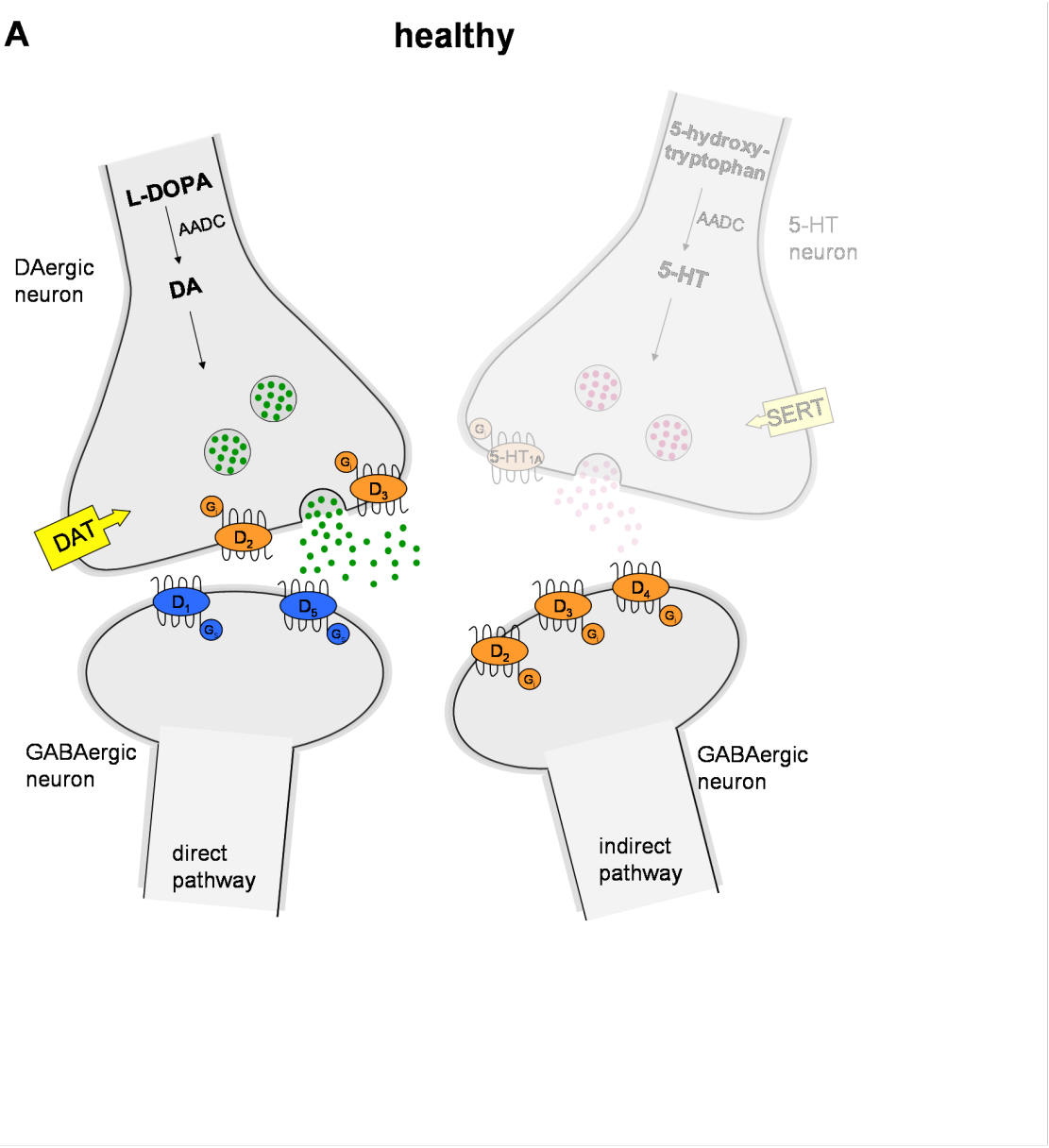
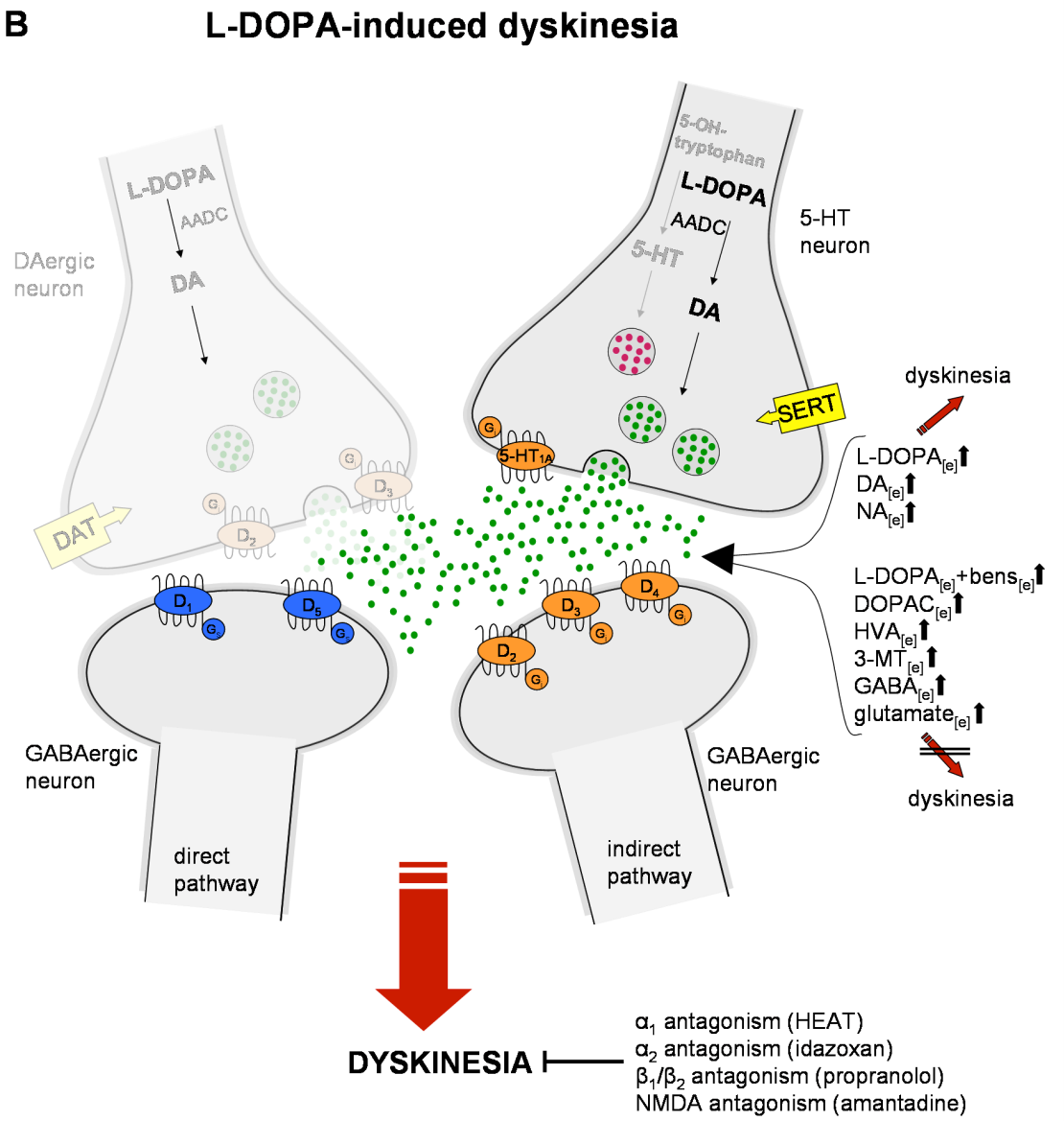


Fig. 24. Schematic drawing of presynaptic nigrostriatal neurons and postsynaptic GABAergic neurons in the healthy (A) and dyskinetic (B) brain.

(A) In the healthy brain, L-DOPA is decarboxylated via the enzyme AADC to DA in dopaminergic nigrostriatal neurons. DA is then stored in synaptic vesicles and released into the extracellular space in an activity-dependent manner. DA acts on dopaminergic receptors, which are located on GABAergic neurons of the direct and indirect pathway of the basal ganglia loops. The direct pathway comprises the DA D_1 receptor family consisting of DA D_1 and DA D_5 receptors. The DA D_2 receptor family (D_2, D_3 and D_4 receptors) are located on GABAergic neurons of the indirect pathway. Release of DA can be regulated by DA D_2 and D_3 autoreceptors, which provide a negative feedback control. Additionally, DA can be metabolised by different enzymes such as MAO and COMT (not shown), which further reduce the level of DA in the synaptic cleft.

(B) As PD progresses and L-DOPA-induced dyskinesia arises, dopaminergic neurons of the nigrostriatal pathway are more and more lost. Thus, fewer dopaminergic neurons remain to decarboxylate L-DOPA. Instead, L-DOPA is decarboxylated to DA in 5-HT neurons, which contain



the enzyme AADC as well. This results in an uncontrolled, excessive increase of extracellular DA in the DA denervated striatum, since the negative feedback control, normally provided by DA D_2 and D_3 autoreceptors, is lost. In addition, 5-HT terminals are hyperactivated caused by depletion of endogenous 5-HT because of DA accumulating in the storage vesicles. It is suggested that this imbalance between the capacity of the 5-HT neurons to release DA derived by L-DOPA and the lack of feedback control mechanisms is the driving force for the induction of dyskinesia. In the present thesis, it was shown that dyskinesia can be alleviated by α_1 antagonism, α_2 antagonism, β_1/β_2 antagonism as well as NMDA antagonism. Furthermore, it was demonstrated that local administration of L-DOPA, DA and NA delivered into the extracellular space of the striatum (abbreviation in the figure: [e]) can provoke dyskinesia. No dyskinesia was observed, when L-DOPA+benserazide was administered locally into the striatum, since the conversion of L-DOPA to DA was blocked. Furthermore, neither DA metabolites (DOPAC, HVA and 3-MT), GABA nor glutamate evoked dyskinetic behaviour.

APPENDIX

Rapid analysis of GABA and glutamate in microdialysis samples using high performance liquid chromatography and tandem mass spectrometry

ABSTRACT

A LC-MS/MS method has been established for the rapid and reliable determination of GABA and glutamate in brain microdialysates. The microdialysis samples were analysed using a HILIC column, which is able to retain the polar amino acid neurotransmitters. The mobile phase consisted of a binary gradient elution profile comprising 0.1 % formic acid in water and acetonitrile. GABA, glutamate as well as the respective internal standards $[D_6]$-GABA and $[D_5]$-glutamate were detected by a triple quadrupole mass spectrometer in the positive ESI mode within a running time of 3 minutes. The linearity ranged from 1 nM to 10 µM for GABA and 10 nM to 10 µM for glutamate. The LOQ was found to be 1 nM for GABA and 10 nM for glutamate (injection volume 10 µl). The present LC-MS/MS method was compared to the classical method for analysis of GABA and glutamate using HPLC and fluorescence detection. Eventually, the feasibility of the LC-MS/MS method was demonstrated using in vivo microdialysis in rats by monitoring changes of the extracellular concentrations of GABA and glutamate in the GP following stimulation with potassium.

INTRODUCTION

GABA and glutamate are the most prominent amino acid neurotransmitters in the CNS. The inhibitory neurotransmitter GABA is widely distributed throughout the brain and is found in 30-40% of all synapses (van der Zeyden *et al.* 2008; van der Zeyden *et al.* 2008). GABA arises through decarboxylation of glutamate via the enzyme glutamic acid decarboxylase and regulates many neuronal processes. The excitatory neurotransmitter glutamate is released by approximately 40% of the synapses in the CNS (Coyle and Puttfarcken 1993) and is involved in many aspects of normal brain functioning including memory, learning (Hertz 2006) and synaptic plasticity of the CNS. Dysfunction of both GABAergic and glutamatergic neurotransmission can result in a variety of neurological disorders such as Alzheimer's disease (Advokat and

Pellegrin 1992) and PD (Blandini *et al.* 1996; Chen and Yung 2004) as well as other CNS disorders (Chen and Yung 2004; Meldrum 1994).

In PD, pathophysiological alterations in the neurotransmitter system of the basal ganglia circuits play a key role. The basal ganglia comprise the direct pathway projecting from the striatum to the GPi and the SNr as well as the indirect pathway connecting the striatum with the GPi/SNr via synaptic projections to the GPe and STN (Alexander and Crutcher 1990). In PD, the striatal DA depletion leads to an overactivation of the striatopallidal synapse, which contains GABA as a neurotransmitter. Our motivation to investigate amino acid neurotransmitters using in vivo microdialysis is to study neurochemical alterations in experimental models of PD.

Changes of extracellular GABA and glutamate levels were studied extensively using in vivo microdialysis. In vivo microdialysis is a widely used technique to continuously monitor alterations of neurotransmitters in the extracellular fluid of the brain. The analytical standard technique to measure GABA and glutamate in microdialysates is HPLC coupled with fluorescence detection (HPLC-FD) (Ballini *et al.* 2008; Bianchi *et al.* 1999; Kehr 1998a; Kehr 1998b; Rea *et al.* 2005) or HPLC-ECD (Kehr 1998b; Macinnes and Duty 2008). Especially for the analysis of GABA in microdialysis samples, HPLC conditions are critical (Rea *et al.* 2005). It was observed that some unknown peaks of biological origin elute close to the GABA peak and that they sometimes cannot be separated from GABA unless keeping very stringent requirements as a very long running time of 60 minutes. In addition, some liquid chromatography/mass spectrometry (LC-MS) (Ma *et al.* 1999) and LC-MS/MS (Bourcier *et al.* 2006; Eckstein *et al.* 2008; Piraud *et al.* 2003; Song *et al.* 2005) methods have been developed for the analysis of GABA and glutamate in biological samples. However, the sensitivity of the latter methods would be insufficient to quantify GABA in microdialysates.

Accordingly, the objective of the present study was to develop a rapid and reliable method for the simultaneous quantification of GABA and glutamate in microdialysis samples using LC-MS/MS. The linearity, LOQ, reproducibility and accuracy of the present method will be presented. Additionally, the present LC-MS/MS method was compared to the widely used HPLC-FD analysis of GABA and glutamate. In order to demonstrate the feasibility of the LC-MS/MS method we determined alterations of

extracellular GABA and glutamate levels in the GP following stimulation with potassium using in vivo microdialysis in rats.

MATERIALS AND METHODS

Animals

Please see chapter II “General methodology” page 22.

In vivo microdialysis surgery in the GP

The in vivo microdialysis surgery in the GP was performed as described in chapter II “General methodology” page 23 with minor modifications of the type of guide cannula used as well as the coordinates. A intracerebral guide cannula was implanted aiming at the GP (MAB 4.9.IC, Microbiotech, Stockholm, Sweden) at the following coordinates relative to bregma: AP: - 1.2 mm, ML: + 3.0 mm, DV: - 5.2 mm (from skull).

In vivo microdialysis procedure in the GP

The in vivo microdialysis procedure in the GP was performed as described in chapter II “General methodology” page 24 with minor modifications of the type of probe used (MAB 4.9.2.Cu, 2 mm membrane length, Microbiotech, Stockholm, Sweden). n = 6 rats were used for the experiment. The mean of fractions 1 to 4 (0 – 80 min) were used for calculation of the basal levels which were regarded as 100 %. During fraction 5 (80 -100 min) aCSF containing a high amount of potassium (49.7 mM NaCl, 100 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$ and 1 mM Na_2HPO_4, pH 7.0-7.4) was perfused for 20 minutes into the GP. The sampling was then continued up to fraction 14 (100 – 280 min).

Standard solutions

As internal standards [D_6]-GABA and [D_5]-glutamate were used. The internal standard stock solutions as well as GABA and glutamate stock solutions were prepared separately by dissolving the compounds in LC-MS grade water at a concentration of 1 mM. All solutions were subsequently frozen at -80 °C. All further

standard and internal standard samples were obtained by diluting the stock solutions with aCSF and acetonitrile, respectively.

Sample preparation procedure

Ten µl of the microdialysis and standard sample, respectively, were diluted with 10 µl of internal standard solution (500 nM of [D_6]-GABA and [D_5]-glutamate in acetonitrile). Ten µl of this solution were injected into the LC-MS/MS.

Gradient elution profile

Mobile phase “A” and “B” consisted of 0.1 % formic acid in water (LC-MS grade) and acetonitrile (LC-MS grade), respectively. The gradient elution profile was chosen as follows: 0 min: 15 % A (1000 µl/min), 0.10 min: 15 % A (200 µl/min), 1.00 min: 80 % A (200 µl/min), 1.50 min: 80 % A (200 µl/min), 1.60 min: 15 % A (200 µl/min), 2.40 min: 15 % A (200 µl/min), 2.50 min: 15% A (1000 µl/min), 3.00 min: 15 % A (1000 µl/min).

LC-MS/MS system

Please see chapter II “General methodology” page 26. Chromatographic retention was obtained using a HILIC column (ZIC®-HILIC, 20 x 2.1 mm i.d., 3.5 µm, SeQuant AB, Umeå, Sweden) with a pre-microfilter (0.5 µm, Upchurch Scientific, Oak Harbor, WA, USA) at 20°C. The column switching valve was set at 0.00 minutes to the waste, at 0.70 minutes to the mass spectrometer and at 2.50 minutes to the waste again.

Drugs and chemicals

Please see chapter II “General methodology” page 26.

RESULTS AND DISCUSSION

Optimisation of mass spectrometric conditions

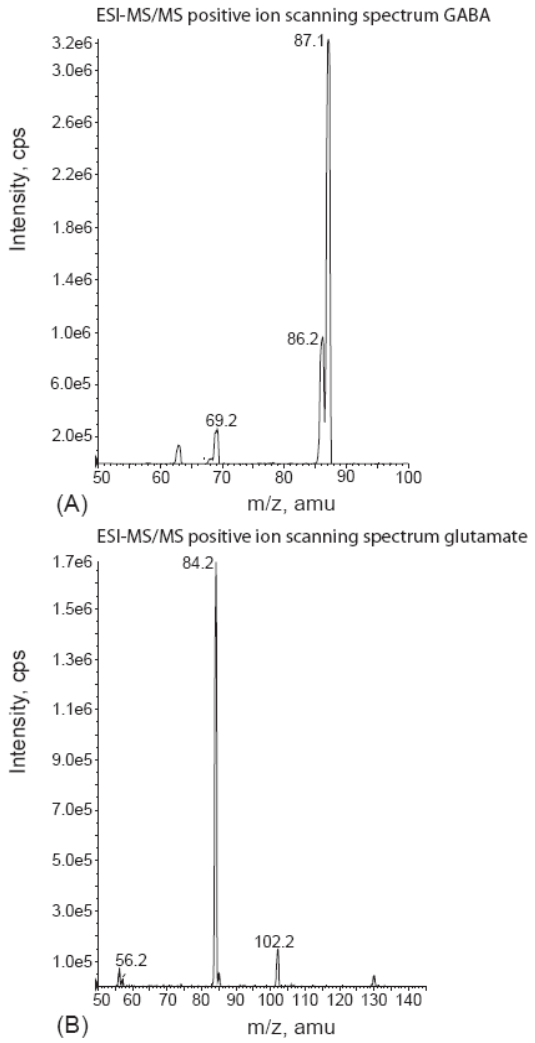


Fig. 25. ESI-MS/MS positive ion scanning spectra for (A) GABA and (B) glutamate.

Positive ESI generates mostly protonated ions ([M+H^+]) from molecules containing a functional group with the potential for ionisation. These ions are produced by applying a high voltage to a very fine spray of the analyte. GABA and glutamate are amino acids, which offer an ionisable amine function and can be protonated under the present conditions. The positive charged molecular ions *m/z* 104 and 148 for GABA and glutamate were generated, respectively. Using the multiple reaction monitoring (MRM) mode, several fragment ions were generated (Fig. 25). For the determination of GABA and glutamate three transitions were measured, whilst one transition was used for quantification (Table 7). For the internal standards [D_6]-GABA and [D_5]-glutamate one transition was selected. The compound specific parameters for each transition are shown in Table 7. Mass spectrometric conditions were optimised using the flow injection analysis (FIA) program provided by the "Quantitative Optimisation" function of the Analyst® software.

	Dwell time (ms)	DP (V)	CE (V)	CXP (V)
GABA				
104.1-87.1	**150**	**46**	**15**	**4**
104.1-86.1	150	46	15	6
104.1-69.2	150	46	23	12
glutamate				
148.1-84.2	**150**	**41**	**21**	**6**
148.1-102.0	150	41	17	8
148.1-56.2	150	41	41	2
[D6]-GABA				
110.1-93.0	150	26	15	6
[D5]-glutamate				
153.1-88.1	150	46	23	6

Table 7. Specific parameters of the API 4000™ triple quadrupole mass spectrometer for the measured transitions. The transition which is presented in bold was used for quantification.

Due to the high amount of salts in the microdialysis samples, the ionisation source can be clogged. Therefore, a switching valve was used to avoid delivering of salts into the mass spectrometer and thus increase sensitivity.

Stable deuterated internal standards for both amino acids were used. Hence, a compensation of potentially confounding matrix effects is obtained by using deuterated internal standards achieving a reliable quantification of the analytes. We chose the internal standards in a concentration range (500 nM), in which they do not interfere with the quantification of the analytes.

Optimisation of chromatographic conditions

GABA and glutamate are not retainable in a simple fashion on reversed phased columns due to their high polarity. Classically, the retention time can be increased by using ion pairing reagents or derivatisation procedures. The usage of ion pairing reagents in LC-MS/MS methods is limited due to the fact that most of the reagents are not volatile. Therefore, we chose a HILIC column, which is suitable for the retention and separation of very polar and hydrophilic compounds. The zwitterionic sulfobetain ZIC®-HILIC stationary phase is covalently attached to porous silica. Within this stationary phase, water is bound in a liquid layer and the polar analyte is distributed in this water enriched compartment. Factors which are responsible for the retention of the analytes are both hydrogen bonding and dipole-dipole interactions. Hence, at least 3% water should be included in the mobile phase in order to hydrate the stationary phase sufficiently. Accordingly, a higher concentration of organic solvent in the mobile phase increases the retention time of the hydrophilic analytes. Using the HILIC column it was possible to develop a method for the analysis of GABA and glutamate without a derivatisation step. Furthermore, the mobile phase consisted only of the solvents 0.1 % formic acid in water and acetonitrile, which are commonly used for LC-MS/MS.

During the method development, different flow rates were applied and it was found that especially the sensitivity of glutamate depends on the flow rate. The sensitivity was the higher, the lower the flow rate was. Therefore, we decided to use a low flow rate of 200 µl/min. However, we applied a higher flow rate of 1000 µl/min at the end of the run to equilibrate the system faster.

Linearity, LOQ, reproducibility and accuracy

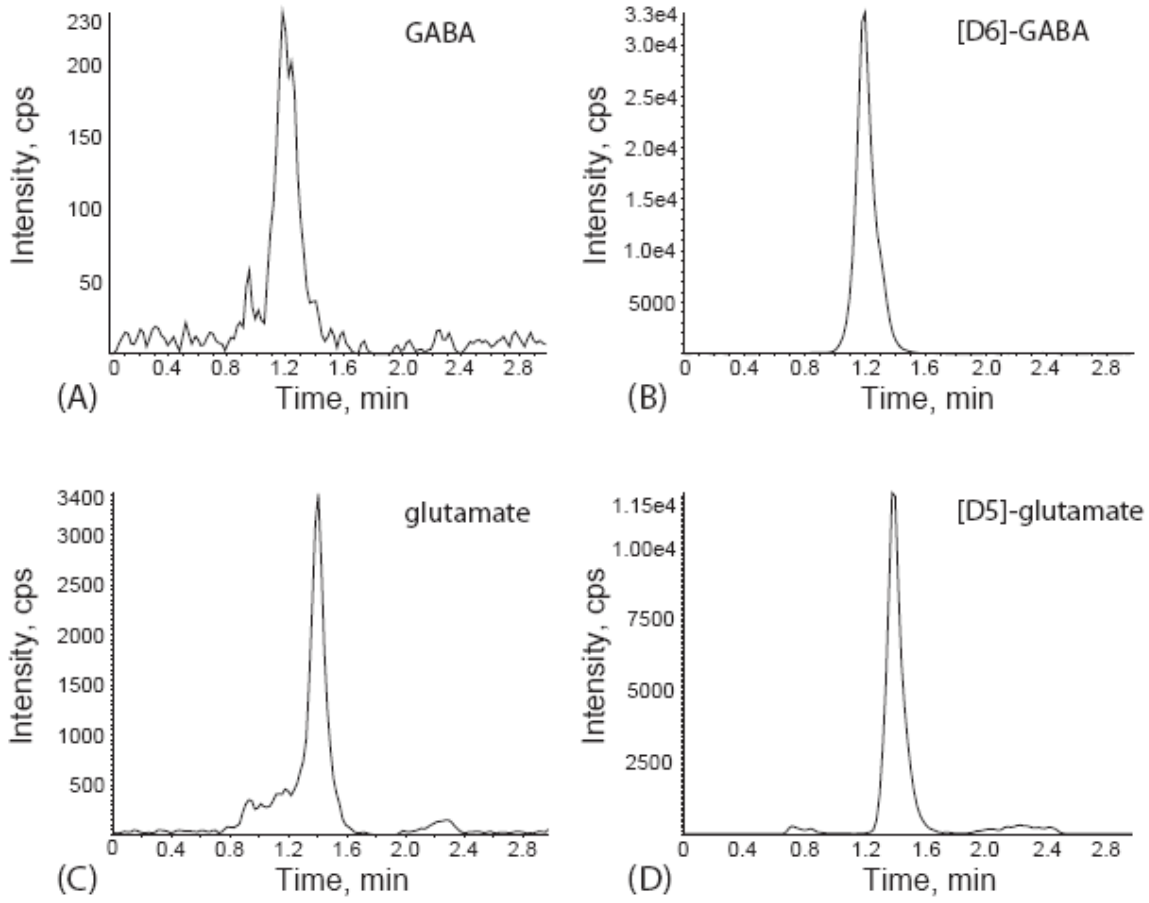


Fig. 26. Representative chromatograms of a standard sample: (A) GABA (1 nM); (B) [D_6]-GABA (500 nM); (C) glutamate (10 nM) and (D) [D_5]-glutamate (500 nM).

Linearity was given in the tested range of 1 nM to 10 µM for GABA and 10 nM to 10 µM for glutamate. The calibration curve was analysed with a weighting of $1/x^2$. Over the considered range of concentrations a correlation coefficient of 0.9981 for GABA and 0.9940 for glutamate was obtained. The LOQ for GABA and glutamate was 1 and 10 nM (injection volume 10 µl), respectively, with a signal-to-noise ratio of 10:1 (Fig. 26). The intra- and inter-batch reproducibility was measured using a GABA and glutamate concentration of 50 nM and 500 nM, respectively. The intra-batch reproducibility for 10 repeat injections was 2.7 % (R.SD) for GABA and 4.0 % (R.SD) for glutamate. The accuracy for GABA and glutamate was 112.2 ± 3.2 % (mean ± SD) and 102.7 ± 4.0 % (mean ± SD), respectively. The inter-batch reproducibility for 10 repeat injections in 4 batches was found to be 5.0 % (R.SD) and 5.3 % (R.SD) for GABA and glutamate, respectively.

In vivo GABA and glutamate monitoring of microdialysis samples

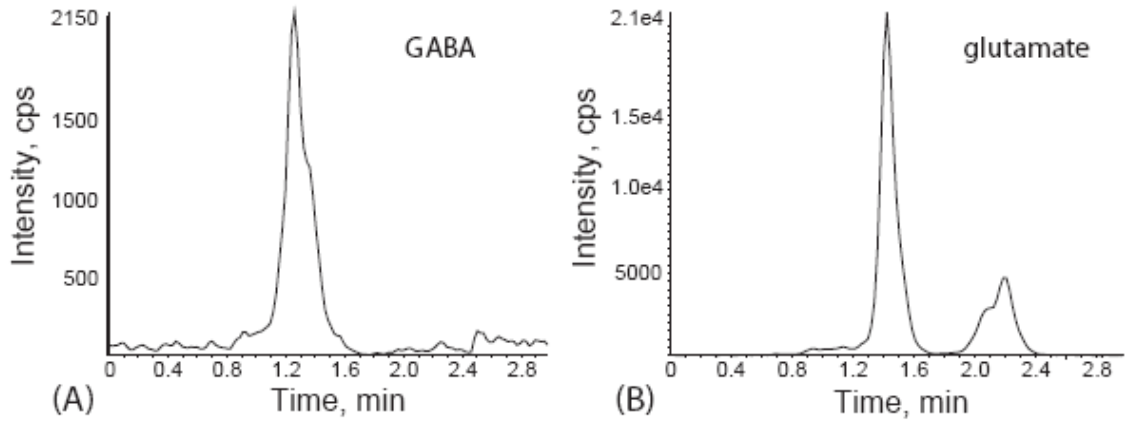


Fig. 27. Chromatograms of a basal microdialysis sample from the GP: (A) GABA (12.8 nM) and (B) glutamate (201.0 nM).

In order to demonstrate the feasibility of the present LC-MS/MS method, GABA and glutamate levels were monitored via the in vivo microdialysis technique in freely moving rats. Alterations of extracellular GABA and glutamate levels were measured in the GP following stimulation with potassium to depolarize neurons and facilitate neurotransmitter release. Stimulation with a high concentration of potassium is known to primarily release amino acid neurotransmitters from neuronal tissue but also from non neuronal sources such as glia (Timmerman and Westerink 1997). Fig. 27 shows representative chromatograms of the basal levels of GABA and glutamate in the GP. The basal levels were found to be 12.72 ± 1.31 nM (mean ± SEM) and 189.02 ± 14.76 nM (mean ± SEM) for GABA and glutamate, respectively. These results are consistent with previous studies, in which the basal levels of GABA and glutamate in the GP ranged from 12 to 48 nM and 100 to 650 nM, respectively (Mela *et al.* 2007; Windels *et al.* 2005; Ochi *et al.* 2004; Grimm and See 2000). Stimulation with potassium enriched aCSF resulted in a 14- and 8-fold increase in extracellular GABA and glutamate levels, respectively (Fig. 28).

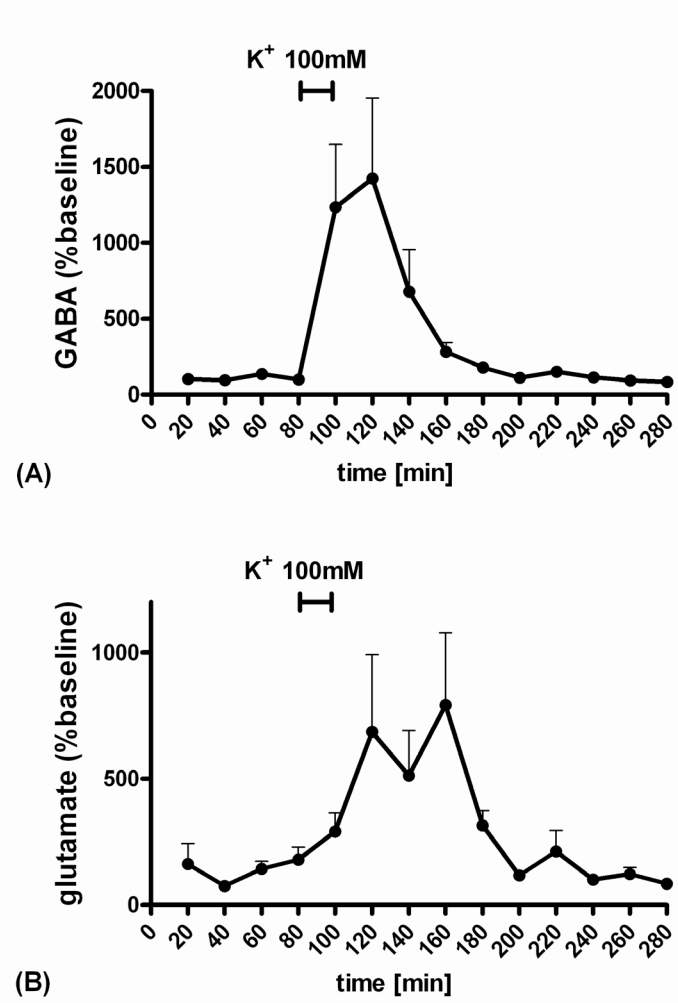


Fig. 28. Time course of potassium-evoked (A) GABA and (B) glutamate release in the GP (n = 6). From time point 80 – 100 min, the aCSF was switched to the potassium enriched aCSF to stimulate neurotransmitter release. Data are presented as mean ± SEM

To date, the most common method for the detection of GABA and glutamate in microdialysates is HPLC-FD (Ballini *et al.* 2008; Bianchi *et al.* 1999; Kehr 1998a; Kehr 1998b; Rea *et al.* 2005) or more rarely HPLC-ECD (Kehr 1998b; Macinnes and Duty 2008). Amino acids are polar molecules which offer low affinity for reversed phase columns. Its hydrophobic character and molecular size can be increased by a precolumn derivatisation mostly with the o-phthaldialdehyde/mercaptoethanol (OPA) reagent (Lindroth and Mopper 1979; Tossman *et al.* 1983). Subsequently, the amino acid/OPA derivative can be analysed by FD or ECD. To verify the present LC-MS/MS method, a HPLC-FD method was applied for the analysis of GABA and glutamate (Fig. 29).

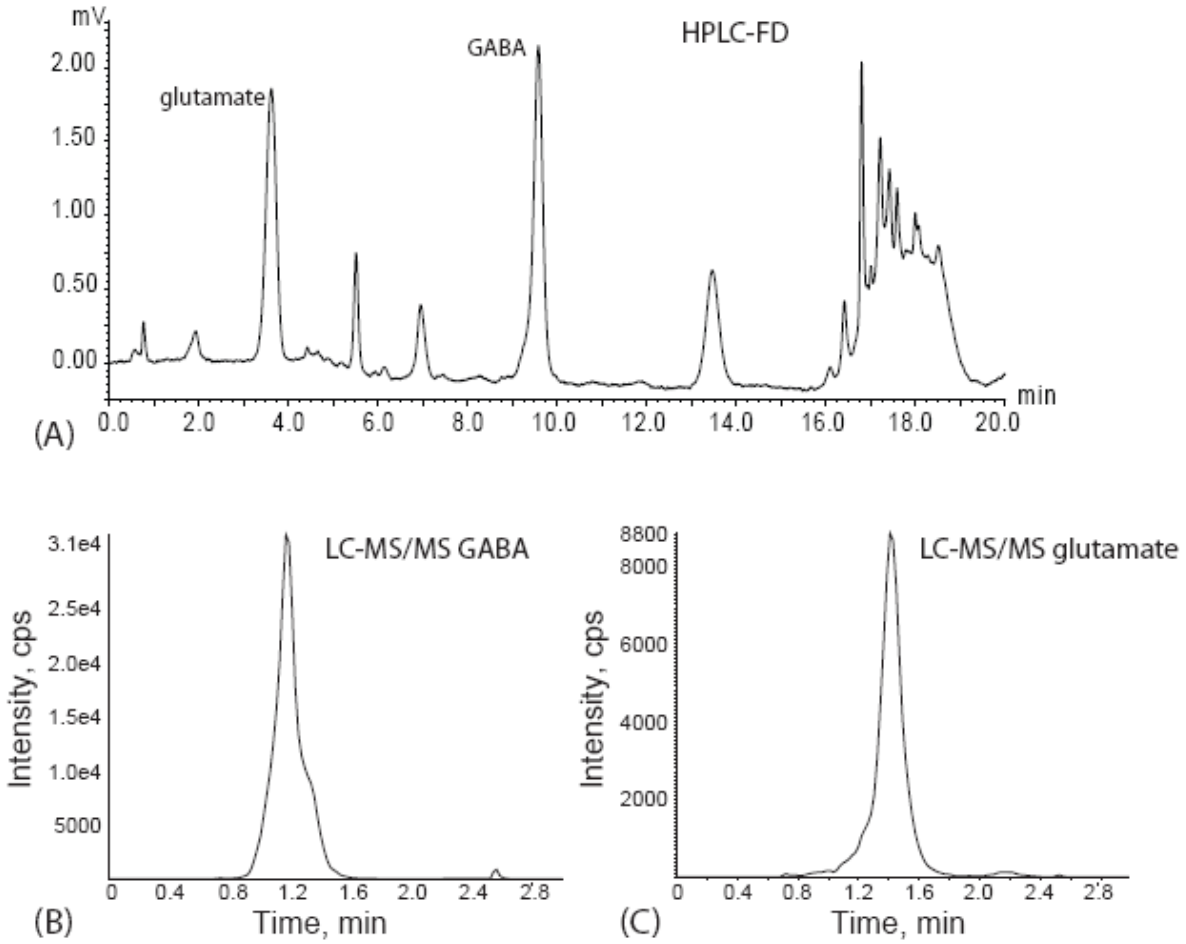


Fig. 29. Chromatograms of the same standard sample containing GABA (100 nM) and glutamate (100 nM) analysed with (A) HPLC-FD (mobile phase: binary gradient elution profile comprising 0.1 M sodium acetate buffer (pH 6.95) and methanol; flow rate : 1.0 ml/min; reversed-phase column: Nucleosil 120 C18, 60 x 4.0 mm i.d., 5 µm, (MZ Analysentechnik, Mainz, Germany); 30 °C), (B) LC-MS/MS (GABA) and (C) LC-MS/MS (glutamate).

A chromatographic separation of GABA and glutamate in standard samples was achieved within 20 minutes. The LOQ of the HPLC-FD method was found to be 5 nM for both GABA and glutamate (injection volume 10µl), which is in the same range as the LOQ of the present LC-MS/MS method. However, the HPLC-FD method requires a precolumn derivatisation and is more time-consuming due to the 7-fold longer running time. Moreover, GABA in microdialysis samples of the GP could not be reliably analysed with the HPLC-FD since unknown peaks of biological origin coelute with GABA in some of the samples. Other groups have also demonstrated previously that HPLC conditions for the analysis of GABA in microdialysates are critical (Rea *et al.* 2005). They described that discrepancies concerning basal extracellular levels of GABA in the same brain region as well as effects of pharmacological compounds

may be due to the difficult chromatographic separation of GABA using HPLC. However, under optimised conditions as keeping an exact pH value of the mobile phase (5.26 ± 0.01) and extending the retention time up to 60 minutes, GABA can be reliably separated from unknown peaks of biological origin and subsequently quantified. The present LC-MS/MS method does not require such prerequisites, it is rather a rapid method, which allows detection of the analytes in a short running time of 3 minutes. Moreover, pre-treatment of the samples is not required due to the usage of HILIC columns which allows separating the polar analytes from the matrix. In addition, the LC-MS/MS technique provides superior selectivity. Indeed, a triple quadrupole MS operating in the MRM mode offers an additional dimension of selectivity due to the monitoring of specific fragments. Thus, the analytes are identified not only by the retention time but also by the interplay of characteristic product ions indicated by their molecular weight.

An alternative method used for the analysis of GABA and glutamate out of microdialysates involves capillary electrophoresis with laser-induced fluorescence detection (Sauvinet *et al.* 2003). Neurotransmitters are tagged with the fluorogene agent naphthaline-2,3-dicarboxaldehyde and separated within 10 minutes by micellar electrokinetic chromatography followed by detection with laser-induced fluorescence. The limit of detection (LOD) was found to be 3 nM and 15 nM for GABA and glutamate, respectively.

A new technique was developed for the detection of glutamate by implantation of ceramic-based multisite microelectrodes achieving a LOD of 500 nM (Burmeister *et al.* 2002). Recently, an enzyme-based microelectrode array with fast response time and low detection limits for glutamate has been developed (Hascup *et al.* 2008). Additionally, a novel biosensor based on quartz crystal microbalance for the analysis of GABA was designed with an LOD of 42 µM (Wang and Muthuswamy 2008).

GABA and glutamate levels in biological samples have been measured previously using LC-MS (Ma *et al.* 1999) and LC-MS/MS (Bourcier *et al.* 2006; Eckstein *et al.* 2008; Piraud *et al.* 2003; Song *et al.* 2005). However, most of these methods require ion-pairing or ion-exchange techniques or usage of derivatisation reagents, which display a complex and time-consuming procedure. These prerequisites are necessary since the amino acids are very hydrophilic and are retained poorly on a reversed phase column. Ma *et al.* (1999) investigated GABA and glutamate in rat brain tissue using atmospheric pressure chemical ionization LC-MS. Preparation of

the sample was performed using a cation-exchange column and a subsequent evaporation step. The LOD for GABA and glutamate were 2.5 µg/ml (= 24 µM) and 5.0 µg/ml (= 34 µM), respectively. Song *et al.* (2005) developed a capillary LC-MS/MS method for quantification of GABA in human plasma and CSF. Prior to the separation, a time-consuming pre-treatment of the sample was performed by derivatisation with the reagent 7-fluoro-4-nitrobenzoxadiazole and a clean-up and concentration step on an extraction column. Using this method a LOD of 5 ng/ml (= 48 nM) was achieved. Recently, Eckstein *et al.* (2008) analysed GABA and glutamate levels in CSF using positive ESI LC-MS/MS. In this study, heptafluorobutyric acid as ion-pairing agent was used. For both GABA and glutamate a LOQ of 7.8 ng/ml (= 75 nM for GABA, 53 nM for glutamate) was achieved. Zhang *et al.* (2007) developed a capillary LC-MS/MS method for the determination of 6 neurotransmitters including GABA and glutamate in the extracellular brain fluid of monkeys obtained by the push-pull sampling method. Using a fused-silica capillary tubing (200 µm, i.d.) packed with polyhydroxyethyl aspartamide particles as HILIC stationary phase, a running time of 26 minutes including reconditioning was required. The LOD (signal-to-noise ratio 3:1) for GABA and glutamate was reported to be 4 nM and 20 nM, respectively, and the linearity ranged from 20 – 4000 nM for GABA and from 100 – 100000 nM for glutamate.

Obviously, the latter methods are applicable for brain tissue, plasma, CSF and push-pull samples, but insufficient to quantify basal GABA concentrations in microdialysis samples of the GP. In contrast, the LOQ (signal-to-noise ratio 10:1) in the LC-MS/MS method presented here was found to be 1 nM for GABA and 10 nM for glutamate requiring a volume of 10 µl of the microdialysate. Taking advantage of this method, a higher time resolution for the microdialysis experiment may be achieved due to the requirement of lower sample volumes and thus shorter sampling intervals are allowed.

CONCLUSION

A rapid and reliable LC-MS/MS method was developed for the simultaneous quantification of GABA and glutamate in brain microdialysates. The present method has several advantages compared to previously reported methods as it provides superior sensitivity, no sample pre-treatment and a very short running time of 3

minutes. Moreover, in comparison to the widely used HPLC-FD as well as the HPLC-ECD analysis the selectivity is higher in the present LC-MS/MS method. The assay achieves a LOQ of 1 nM for GABA and 10 nM for glutamate (injection volume 10 µl), which is sensitive to quantify extracellular levels of GABA and glutamate in microdialysis samples of the GP.

This study was performed together with Patrizia Voehringer. I developed the method and performed the in vivo studies. Patrizia Voehringer conducted reproducibility, accuracy and linearity measurements as well as helped with preparation of the manuscript.

LIST OF ABBREVIATIONS

3-MT	3-methoxytyramine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
6-OHDA	6-hydroxydopamine
AADC	aromatic amino acid decarboxylase
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
AUC	area under the curve
CD	carbidopa
CDS	continuous dopaminergic stimulation
CE	collision energy
COMT	catechol-O-methyltransferase
CR	continuous release
CSF	Cerebrospinal fluid
CXP	cell exit potential
DA	dopamine
DBH	dopamine beta hydroxylase
DBS	deep brain stimulation
DIMPEA	3,4-dimethoxyphenylethylamine
DOPAC	3,4-dihydroxyphenylacetic acid
DP	declustering potential
ECD	electrochemical detection
ESI	electrospray ionisation
FD	fluorescence detection
GABA	gamma aminobutyric acid
GP	globus pallidus
GPe	globus pallidus externus
GPi	globus pallidus internus
HEAT	(2-[[β-(-4-hydroxyphenyl)ethyl]aminomethyl]-1-tetralone)
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
HVA	4-hydroxy-3-methoxy-phenylacetic acid

IR	immediate release
LC-MS	liquid chromatography/mass spectrometry
LC-MS/MS	liquid chromatography/tandem mass spectrometry
L-DOPA	L-3,4-dihydroxyphenylalanine
LOD	limit of detection
LOQ	limit of quantitation
LPS	lipopolysaccharide
MAO	monoaminoxidase
MFB	medial forebrain bundle
MHPG	3-methoxy-4- hydroxyphenylglycol
MPEP	2-methyl-6-(phenylethynyl)-pyridine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRM	multiple reaction monitoring
MTEP	3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine
NA	noradrenaline
NMDA	N-methyl-D-aspartate
OPA	o-phthaldialdehyde/mercaptoethanol
PCA	principal component analysis
PD	Parkinson’s disease
PPX	pramipexole
rAAV	recombinant adeno-associated viruses
RGS9-2	regulator of G-protein signaling 9-2
SNc	substantia nigra pars compacta
STN	subthalamic nucleus
UPDRS	Unified Parkinson’s Disease Rating Scale

REFERENCES

1. Abercrombie E. D., Bonatz A. E. and Zigmond M. J. (1990) Effects of L-dopa on extracellular dopamine in striatum of normal and 6-hydroxydopamine-treated rats. *Brain Res.* **525,** 36-44.

2. Advokat C. and Pellegrin A. I. (1992) Excitatory amino acids and memory: evidence from research on Alzheimer's disease and behavioral pharmacology. *Neurosci. Biobehav. Rev.* **16,** 13-24.

3. Ahlskog J. E. and Muenter M. D. (2001) Frequency of levodopa-related dyskinesias and motor fluctuations as estimated from the cumulative literature. *Mov Disord.* **16,** 448-458.

4. Alachkar A., Brotchie J. and Jones O. T. (2006) alpha2-Adrenoceptor-mediated modulation of the release of GABA and noradrenaline in the rat substantia nigra pars reticulata. *Neurosci. Lett.* **395,** 138-142.

5. Alexander G. E. and Crutcher M. D. (1990) Functional architecture of basal ganglia circuits: neural substrates of parallel processing. *Trends Neurosci.* **13,** 266-271.

6. Alexander S. P., Mathie A. and Peters J. A. (2005) Guide to receptors and channels, 1st edition (2005 revision). *Br. J. Pharmacol.* **144 Suppl 1,** S1-128.

7. Alvord E. C. D. (1968) The pathology of parkinsonism, in *Pathology of the Nervous System,* (Minckler J., ed), pp. 1152-1161. New York.

8. Andersson M., Hilbertson A. and Cenci M. A. (1999) Striatal fosB expression is causally linked with l-DOPA-induced abnormal involuntary movements and the associated upregulation of striatal prodynorphin mRNA in a rat model of Parkinson's disease. *Neurobiol. Dis.* **6,** 461-474.

9. Arai R., Karasawa N., Geffard M. and Nagatsu I. (1995) L-DOPA is converted to dopamine in serotonergic fibers of the striatum of the rat: a double-labeling immunofluorescence study. *Neurosci. Lett.* **195,** 195-198.

10. Arai R., Karasawa N., Geffard M., Nagatsu T. and Nagatsu I. (1994) Immunohistochemical evidence that central serotonin neurons produce dopamine from exogenous L-DOPA in the rat, with reference to the involvement of aromatic L-amino acid decarboxylase. *Brain Res.* **667,** 295-299.

11. Auclair A., Drouin C., Cotecchia S., Glowinski J. and Tassin J. P. (2004) 5-HT2A and alpha1b-adrenergic receptors entirely mediate dopamine release,

locomotor response and behavioural sensitization to opiates and psychostimulants. *Eur. J. Neurosci.* **20,** 3073-3084.

12. Ballini C., Corte L. D., Pazzagli M., Colivicchi M. A., Pepeu G., Tipton K. F. and Giovannini M. G. (2008) Extracellular levels of brain aspartate, glutamate and GABA during an inhibitory avoidance response in the rat. *J. Neurochem.* **106,** 1035-1043.

13. Bara-Jimenez W., Bibbiani F., Morris M. J., Dimitrova T., Sherzai A., Mouradian M. M. and Chase T. N. (2005) Effects of serotonin 5-HT1A agonist in advanced Parkinson's disease. *Mov Disord.* **20,** 932-936.

14. Bara-Jimenez W., Dimitrova T. D., Sherzai A., Aksu M. and Chase T. N. (2006) Glutamate release inhibition ineffective in levodopa-induced motor complications. *Mov Disord.* **21,** 1380-1383.

15. Bara-Jimenez W., Sherzai A., Dimitrova T., Favit A., Bibbiani F., Gillespie M., Morris M. J., Mouradian M. M. and Chase T. N. (2003) Adenosine A(2A) receptor antagonist treatment of Parkinson's disease. *Neurology* **61,** 293-296.

16. Baron M. S. and Dalton W. B. (2003) Quetiapine as treatment for dopaminergic-induced dyskinesias in Parkinson's disease. *Mov Disord.* **18,** 1208-1209.

17. Bedard P. J., Di P. T., Falardeau P. and Boucher R. (1986) Chronic treatment with L-DOPA, but not bromocriptine induces dyskinesia in MPTP-parkinsonian monkeys. Correlation with [3H]spiperone binding. *Brain Res.* **379,** 294-299.

18. Belujon P., Bezard E., Taupignon A., Bioulac B. and Benazzouz A. (2007) Noradrenergic modulation of subthalamic nucleus activity: behavioral and electrophysiological evidence in intact and 6-hydroxydopamine-lesioned rats. *J. Neurosci.* **27,** 9595-9606.

19. Bennett J. P., Jr., Landow E. R., Dietrich S. and Schuh L. A. (1994) Suppression of dyskinesias in advanced Parkinson's disease: moderate daily clozapine doses provide long-term dyskinesia reduction. *Mov Disord.* **9,** 409-414.

20. Bennett J. P., Jr., Landow E. R. and Schuh L. A. (1993) Suppression of dyskinesias in advanced Parkinson's disease. II. Increasing daily clozapine doses suppress dyskinesias and improve parkinsonism symptoms. *Neurology* **43,** 1551-1555.

21. Bezard E., Brefel C., Tison F., Peyro-Saint-Paul H., Ladure P., Rascol O. and Gross C. E. (1999) Effect of the alpha 2 adrenoreceptor antagonist, idazoxan, on motor disabilities in MPTP-treated monkey. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **23,** 1237-1246.

22. Bezard E., Brotchie J. M. and Gross C. E. (2001) Pathophysiology of levodopa-induced dyskinesia: potential for new therapies. *Nat. Rev. Neurosci.* **2,** 577-588.

23. Bezard E., Ferry S., Mach U., Stark H., Leriche L., Boraud T., Gross C. and Sokoloff P. (2003) Attenuation of levodopa-induced dyskinesia by normalizing dopamine D3 receptor function. *Nat. Med.* **9,** 762-767.

24. Bianchi L., Della C. L. and Tipton K. F. (1999) Simultaneous determination of basal and evoked output levels of aspartate, glutamate, taurine and 4-aminobutyric acid during microdialysis and from superfused brain slices. *J. Chromatogr. B Biomed. Sci. Appl.* **723,** 47-59.

25. Bibbiani F., Costantini L. C., Patel R. and Chase T. N. (2005) Continuous dopaminergic stimulation reduces risk of motor complications in parkinsonian primates. *Exp. Neurol.* **192,** 73-78.

26. Bibbiani F., Oh J. D. and Chase T. N. (2001) Serotonin 5-HT1A agonist improves motor complications in rodent and primate parkinsonian models. *Neurology* **57,** 1829-1834.

27. Blanchet P. J., Konitsiotis S. and Chase T. N. (1998) Amantadine reduces levodopa-induced dyskinesias in parkinsonian monkeys. *Mov Disord.* **13,** 798-802.

28. Blandini F., Porter R. H. and Greenamyre J. T. (1996) Glutamate and Parkinson's disease. *Mol. Neurobiol.* **12,** 73-94.

29. Bonifati V., Fabrizio E., Cipriani R., Vanacore N. and Meco G. (1994) Buspirone in levodopa-induced dyskinesias. *Clin. Neuropharmacol.* **17,** 73-82.

30. Borah A. and Mohanakumar K. P. (2007) Long-term L-DOPA treatment causes indiscriminate increase in dopamine levels at the cost of serotonin synthesis in discrete brain regions of rats. *Cell Mol. Neurobiol.* **27,** 985-996.

31. Bourcier S., Benoist J. F., Clerc F., Rigal O., Taghi M. and Hoppilliard Y. (2006) Detection of 28 neurotransmitters and related compounds in biological fluids by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **20,** 1405-1421.

32. Braz C. A., Borges V. and Ferraz H. B. (2004) Effect of riluzole on dyskinesia and duration of the on state in Parkinson disease patients: a double-blind, placebo-controlled pilot study. *Clin. Neuropharmacol.* **27,** 25-29.

33. Brotchie J. M. (2005) Nondopaminergic mechanisms in levodopa-induced dyskinesia. *Mov Disord.* **20,** 919-931.

34. Brotchie J. M., Lee J. and Venderova K. (2005) Levodopa-induced dyskinesia in Parkinson's disease. *J. Neural Transm.* **112,** 359-391.

35. Buck K. and Ferger B. (2008) Intrastriatal inhibition of aromatic amino acid decarboxylase prevents l-DOPA-induced dyskinesia: a bilateral reverse in vivo microdialysis study in 6-hydroxydopamine lesioned rats. *Neurobiol. Dis.* **29,** 210-220.

36. Buck K. and Ferger B. (2009a) Comparison of intrastriatal administration of noradrenaline and l-DOPA on dyskinetic movements: A bilateral reverse in vivo microdialysis study in 6-hydroxydopamine-lesioned rats. *Neuroscience* **159,** 16-20.

37. Buck K. and Ferger B. (2009b) The selective α_1 adrenoceptor antagonist HEAT reduces L-DOPA-induced dyskinesia in a rat model of Parkinson's disease. *Synapse*.

38. Burmeister J. J., Pomerleau F., Palmer M., Day B. K., Huettl P. and Gerhardt G. A. (2002) Improved ceramic-based multisite microelectrode for rapid measurements of L-glutamate in the CNS. *J. Neurosci. Methods* **119,** 163-171.

39. Buu N. T. (1989) Vesicular accumulation of dopamine following L-DOPA administration. *Biochem. Pharmacol.* **38,** 1787-1792.

40. Bylund D. B., Eikenberg D. C., Hieble J. P., Langer S. Z., Lefkowitz R. J., Minneman K. P., Molinoff P. B., Ruffolo R. R., Jr. and Trendelenburg U. (1994) International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.* **46,** 121-136.

41. Carlsson T., Winkler C., Lundblad M., Cenci M. A., Bjorklund A. and Kirik D. (2006) Graft placement and uneven pattern of reinnervation in the striatum is important for development of graft-induced dyskinesia. *Neurobiol. Dis.* **21,** 657-668.

42. Carpentier A. F., Bonnet A. M., Vidailhet M. and Agid Y. (1996) Improvement of levodopa-induced dyskinesia by propranolol in Parkinson's disease. *Neurology* **46,** 1548-1551.

43. Carta A. R., Tronci E., Pinna A. and Morelli M. (2005) Different responsiveness of striatonigral and striatopallidal neurons to L-DOPA after a subchronic intermittent L-DOPA treatment. *Eur. J. Neurosci.* **21,** 1196-1204.

44. Carta M., Carlsson T., Kirik D. and Bjorklund A. (2007) Dopamine released from 5-HT terminals is the cause of L-DOPA-induced dyskinesia in parkinsonian rats. *Brain* **130,** 1819-1833.

45. Carta M., Carlsson T., Munoz A., Kirik D. and Bjorklund A. (2008) Involvement of the serotonin system in L-dopa-induced dyskinesias. *Parkinsonism. Relat Disord.* **14 Suppl 2,** S154-S158.

46. Carta M., Lindgren H. S., Lundblad M., Stancampiano R., Fadda F. and Cenci M. A. (2006) Role of striatal L-DOPA in the production of dyskinesia in 6-hydroxydopamine lesioned rats. *J. Neurochem.* **96,** 1718-1727.

47. Carter A. J. and Muller R. E. (1991) Pramipexole, a dopamine D2 autoreceptor agonist, decreases the extracellular concentration of dopamine in vivo. *Eur. J. Pharmacol.* **200,** 65-72.

48. Cenci M. A. (2007) Dopamine dysregulation of movement control in l-DOPA-induced dyskinesia. *Trends Neurosci.* **30,** 236-243.

49. Cenci M. A., Lee C. S. and Bjorklund A. (1998) L-DOPA-induced dyskinesia in the rat is associated with striatal overexpression of prodynorphin- and glutamic acid decarboxylase mRNA. *Eur. J. Neurosci.* **10,** 2694-2706.

50. Cenci M. A. and Lundblad M. (2006) Post- versus presynaptic plasticity in L-DOPA-induced dyskinesia. *J. Neurochem.* **99,** 381-392.

51. Cenci M. A. and Lundblad M. (2007) Ratings of L-DOPA-induced dyskinesia in the unilateral 6-OHDA lesion model of Parkinson's disease in rats and mice. *Curr. Protoc. Neurosci.* **Chapter 9,** Unit.

52. Chalmers J. P., Baldessarini R. J. and Wurtman R. J. (1971) Effects of L-dopa on norepinephrine metabolism in the brain. *Proc. Natl. Acad. Sci. U. S. A* **68,** 662-666.

53. Charlton C. G. and Crowell B., Jr. (2000) Effects of dopamine metabolites on locomotor activities and on the binding of dopamine: relevance to the side effects of L-dopa. *Life Sci.* **66,** 2159-2171.

54. Chen L. and Yung W. H. (2004) GABAergic neurotransmission in globus pallidus and its involvement in neurologic disorders. *Sheng Li Xue. Bao.* **56,** 427-435.

55. Chernoloz O., El M. M. and Blier P. (2009) Sustained administration of pramipexole modifies the spontaneous firing of dopamine, norepinephrine, and serotonin neurons in the rat brain. *Neuropsychopharmacology* **34,** 651-661.

56. Chopin P., Colpaert F. C. and Marien M. (1999) Effects of alpha-2 adrenoceptor agonists and antagonists on circling behavior in rats with unilateral 6-hydroxydopamine lesions of the nigrostriatal pathway. *J. Pharmacol. Exp. Ther.* **288,** 798-804.

57. Chung K. A., Carlson N. E. and Nutt J. G. (2005) Short-term paroxetine treatment does not alter the motor response to levodopa in PD. *Neurology* **64,** 1797-1798.

58. Clarke C. E., Sambrook M. A., Mitchell I. J. and Crossman A. R. (1987) Levodopa-induced dyskinesia and response fluctuations in primates rendered parkinsonian with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *J. Neurol. Sci.* **78,** 273-280.

59. Colosimo C. and Craus A. (2003) Noradrenergic drugs for levodopa-induced dyskinesia. *Clin. Neuropharmacol.* **26,** 299-305.

60. Cooper J. R., Bloom F. E. and Roth R. H. (2003) The biochemical Basis of Neuropharmacology, Oxford University Press, Inc., New York.

61. Cotzias G. C., Papavasiliou P. S. and Gellene R. (1969) Modification of Parkinsonism--chronic treatment with L-dopa. *N. Engl. J. Med.* **280,** 337-345.

62. Coyle J. T. and Puttfarcken P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262,** 689-695.

63. Danzeisen R., Schwalenstoecker B., Gillardon F., Buerger E., Krzykalla V., Klinder K., Schild L., Hengerer B., Ludolph A. C., Dorner-Ciossek C. and Kussmaul L. (2006) Targeted antioxidative and neuroprotective properties of the dopamine agonist pramipexole and its nondopaminergic enantiomer SND919CL2x [(+)2-amino-4,5,6,7-tetrahydro-6-Lpropylamino-benzathiazole dihydrochloride]. *J. Pharmacol. Exp. Ther.* **316,** 189-199.

64. De Ryck M., Schallert T. and Teitelbaum P. (1980) Morphine versus haloperidol catalepsy in the rat: a behavioral analysis of postural support mechanisms. *Brain Res.* **201,** 143-172.

65. De Ryck M. and Teitelbaum P. (1983) Morphine versus haloperidol catalepsy in the rat: an electromyographic analysis of postural support mechanisms. *Exp. Neurol.* **79,** 54-76.

66. Dekundy A., Lundblad M., Danysz W. and Cenci M. A. (2007) Modulation of L-DOPA-induced abnormal involuntary movements by clinically tested compounds: further validation of the rat dyskinesia model. *Behav. Brain Res.* **179,** 76-89.

67. Dekundy A., Pietraszek M., Schaefer D., Cenci M. A. and Danysz W. (2006) Effects of group I metabotropic glutamate receptors blockade in experimental models of Parkinson's disease. *Brain Res. Bull.* **69,** 318-326.

68. Del D. P., Pavese N., Gambaccini G., Bernardini S., Metman L. V., Chase T. N. and Bonuccelli U. (2001) Intravenous amantadine improves levadopa-

induced dyskinesias: an acute double-blind placebo-controlled study. *Mov Disord.* **16,** 515-520.

69. Deleu D., Hanssens Y. and Northway M. G. (2004) Subcutaneous apomorphine : an evidence-based review of its use in Parkinson's disease. *Drugs Aging* **21,** 687-709.

70. Ding Y., Restrepo J., Won L., Hwang D. Y., Kim K. S. and Kang U. J. (2007) Chronic 3,4-dihydroxyphenylalanine treatment induces dyskinesia in aphakia mice, a novel genetic model of Parkinson's disease. *Neurobiol. Dis.* **27,** 11-23.

71. Dorsey E. R., Constantinescu R., Thompson J. P., Biglan K. M., Holloway R. G., Kieburtz K., Marshall F. J., Ravina B. M., Schifitto G., Siderowf A. and Tanner C. M. (2007) Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. *Neurology* **68,** 384-386.

72. Dupre K. B., Eskow K. L., Barnum C. J. and Bishop C. (2008) Striatal 5-HT1A receptor stimulation reduces D1 receptor-induced dyskinesia and improves movement in the hemiparkinsonian rat. *Neuropharmacology* **55,** 1321-1328.

73. Durif F., Debilly B., Galitzky M., Morand D., Viallet F., Borg M., Thobois S., Broussolle E. and Rascol O. (2004) Clozapine improves dyskinesias in Parkinson disease: a double-blind, placebo-controlled study. *Neurology* **62,** 381-388.

74. Durif F., Vidailhet M., Assal F., Roche C., Bonnet A. M. and Agid Y. (1997) Low-dose clozapine improves dyskinesias in Parkinson's disease. *Neurology* **48,** 658-662.

75. Durif F., Vidailhet M., Bonnet A. M., Blin J. and Agid Y. (1995) Levodopa-induced dyskinesias are improved by fluoxetine. *Neurology* **45,** 1855-1858.

76. Eckstein J. A., Ammerman G. M., Reveles J. M. and Ackermann B. L. (2008) Analysis of glutamine, glutamate, pyroglutamate, and GABA in cerebrospinal fluid using ion pairing HPLC with positive electrospray LC/MS/MS. *J. Neurosci. Methods* **171,** 190-196.

77. Ehringer H. and Hornykiewicz O. (1960) [Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system.]. *Klin. Wochenschr.* **38,** 1236-1239.

78. Ellenbroek B., Schwarz M., Sontag K. H. and Cools A. (1985) The importance of the striato-nigro-collicular pathway in the expression of haloperidol-induced tonic electromyographic activity. *Neurosci. Lett.* **54,** 189-194.

79. Everett G. M. and Borcherding J. W. (1970) L-Dopa: Effect on Concentrations of Dopamine, Norepinephrine, and Serotonin in Brains of Mice. *Science* **168,** 849-850.

80. Fahn S. (2003) Description of Parkinson's disease as a clinical syndrome. *Ann. N. Y. Acad. Sci.* **991,** 1-14.

81. Fahn S. (2008) How do you treat motor complications in Parkinson's disease: Medicine, surgery, or both? *Ann. Neurol.* **64 Suppl 2,** S56-S64.

82. Ferger B., Teismann P. and Mierau J. (2000) The dopamine agonist pramipexole scavenges hydroxyl free radicals induced by striatal application of 6-hydroxydopamine in rats: an in vivo microdialysis study. *Brain Res.* **883,** 216-223.

83. Feuerstein C., Tanche M., Serre F., Gavend M., Pellat J. and Perret J. (1977) Does O-methyl-dopa play a role in levodopa-induced dyskinesias? *Acta Neurol. Scand.* **56,** 79-82.

84. Figueiras-Mendez R., Marin-Zarza F., Antonio M. J., Jimenez-Jimenez F. J., Orti-Pareja M., Magarinos C., Lopez-Pino M. A. and Martinez V. (1999) Subthalamic nucleus stimulation improves directly levodopa induced dyskinesias in Parkinson's disease. *J. Neurol. Neurosurg. Psychiatry* **66,** 549-550.

85. Fischer D. A., Ferger B. and Kuschinsky K. (2002) Discrimination of morphine- and haloperidol-induced muscular rigidity and akinesia/catalepsy in simple tests in rats. *Behav. Brain Res.* **134,** 317-321.

86. Follett K. A. (2004) Comparison of pallidal and subthalamic deep brain stimulation for the treatment of levodopa-induced dyskinesias. *Neurosurg. Focus.* **17,** E3.

87. Fornai F., di Poggio A. B., Pellegrini A., Ruggieri S. and Paparelli A. (2007) Noradrenaline in Parkinson's disease: from disease progression to current therapeutics. *Curr. Med. Chem.* **14,** 2330-2334.

88. Forno L. S. (1966) Pathology of parkinsonism. *J. Neurosurg.* **24 (Suppl. 2),** 266-271.

89. Fox S., Silverdale M., Kellett M., Davies R., Steiger M., Fletcher N., Crossman A. and Brotchie J. (2004) Non-subtype-selective opioid receptor antagonism in treatment of levodopa-induced motor complications in Parkinson's disease. *Mov Disord.* **19,** 554-560.

90. Fox S. H., Henry B., Hill M., Crossman A. and Brotchie J. (2002) Stimulation of cannabinoid receptors reduces levodopa-induced dyskinesia in the MPTP-lesioned nonhuman primate model of Parkinson's disease. *Mov Disord.* **17,** 1180-1187.

91. Fox S. H., Henry B., Hill M. P., Peggs D., Crossman A. R. and Brotchie J. M. (2001) Neural mechanisms underlying peak-dose dyskinesia induced by

levodopa and apomorphine are distinct: evidence from the effects of the alpha(2) adrenoceptor antagonist idazoxan. *Mov Disord.* **16,** 642-650.

92. Fozard J. R., Mir A. K. and Middlemiss D. N. (1987) Cardiovascular response to 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) in the rat: site of action and pharmacological analysis. *J. Cardiovasc. Pharmacol.* **9,** 328-347.

93. Gabriel K. R. and Odoroff C. L. (1990) Biplots in biomedical research. *Stat. Med.* **9,** 469-485.

94. Gainetdinov R. R., Sotnikova T. D., Grekhova T. V. and Rayevsky K. S. (1996) In vivo evidence for preferential role of dopamine D3 receptor in the presynaptic regulation of dopamine release but not synthesis. *Eur. J. Pharmacol.* **308,** 261-269.

95. Goetz C. G., Damier P., Hicking C., Laska E., Muller T., Olanow C. W., Rascol O. and Russ H. (2007) Sarizotan as a treatment for dyskinesias in Parkinson's disease: a double-blind placebo-controlled trial. *Mov Disord.* **22,** 179-186.

96. Goetz C. G., Laska E., Hicking C., Damier P., Muller T., Nutt J., Warren O. C., Rascol O. and Russ H. (2008) Placebo influences on dyskinesia in Parkinson's disease. *Mov Disord.* **23,** 700-707.

97. Gomez-Mancilla B. and Bedard P. J. (1993) Effect of nondopaminergic drugs on L-dopa-induced dyskinesias in MPTP-treated monkeys. *Clin. Neuropharmacol.* **16,** 418-427.

98. Gregoire L., Samadi P., Graham J., Bedard P. J., Bartoszyk G. D. and Di P. T. (2009) Low doses of sarizotan reduce dyskinesias and maintain antiparkinsonian efficacy of l-Dopa in parkinsonian monkeys. *Parkinsonism. Relat Disord.*

99. Grimm J. W. and See R. E. (2000) Chronic haloperidol-induced alterations in pallidal GABA and striatal D(1)-mediated dopamine turnover as measured by dual probe microdialysis in rats. *Neuroscience* **100,** 507-514.

100. Grondin R., Bedard P. J., Hadj T. A., Gregoire L., Mori A. and Kase H. (1999a) Antiparkinsonian effect of a new selective adenosine A2A receptor antagonist in MPTP-treated monkeys. *Neurology* **52,** 1673-1677.

101. Grondin R., Doan V. D., Gregoire L. and Bedard P. J. (1999b) D1 receptor blockade improves L-dopa-induced dyskinesia but worsens parkinsonism in MPTP monkeys. *Neurology* **52,** 771-776.

102. Grondin R., Hadj T. A., Doan V. D., Ladure P. and Bedard P. J. (2000) Noradrenoceptor antagonism with idazoxan improves L-dopa-induced dyskinesias in MPTP monkeys. *Naunyn Schmiedebergs Arch. Pharmacol.* **361,** 181-186.

103. Guigoni C., Dovero S., Aubert I., Li Q., Bioulac B. H., Bloch B., Gurevich E. V., Gross C. E. and Bezard E. (2005) Levodopa-induced dyskinesia in MPTP-treated macaques is not dependent on the extent and pattern of nigrostrial lesioning. *Eur. J. Neurosci.* **22,** 283-287.

104. Hascup K. N., Hascup E. R., Pomerleau F., Huettl P. and Gerhardt G. A. (2008) Second-by-second measures of L-glutamate in the prefrontal cortex and striatum of freely moving mice. *J. Pharmacol. Exp. Ther.* **324,** 725-731.

105. Hauber W., Neuscheler P., Nagel J. and Muller C. E. (2001) Catalepsy induced by a blockade of dopamine D1 or D2 receptors was reversed by a concomitant blockade of adenosine A(2A) receptors in the caudate-putamen of rats. *Eur. J. Neurosci.* **14,** 1287-1293.

106. Hauser R. A., Shulman L. M., Trugman J. M., Roberts J. W., Mori A., Ballerini R. and Sussman N. M. (2008) Study of istradefylline in patients with Parkinson's disease on levodopa with motor fluctuations. *Mov Disord.* **23,** 2177-2185.

107. Heikkila R. E., Hess A. and Duvoisin R. C. (1984) Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. *Science* **224,** 1451-1453.

108. Henry B., Fox S. H., Crossman A. R. and Brotchie J. M. (2001) Mu- and delta-opioid receptor antagonists reduce levodopa-induced dyskinesia in the MPTP-lesioned primate model of Parkinson's disease. *Exp. Neurol.* **171,** 139-146.

109. Henry B., Fox S. H., Peggs D., Crossman A. R. and Brotchie J. M. (1999) The alpha2-adrenergic receptor antagonist idazoxan reduces dyskinesia and enhances anti-parkinsonian actions of L-dopa in the MPTP-lesioned primate model of Parkinson's disease. *Mov Disord.* **14,** 744-753.

110. Hertz L. (2006) Glutamate, a neurotransmitter--and so much more. A synopsis of Wierzba III. *Neurochem. Int.* **48,** 416-425.

111. Hill M. P. and Brotchie J. M. (1999) The adrenergic receptor agonist, clonidine, potentiates the anti-parkinsonian action of the selective kappa-opioid receptor agonist, enadoline, in the monoamine-depleted rat. *Br. J. Pharmacol.* **128,** 1577-1585.

112. Hill M. P., Ravenscroft P., Bezard E., Crossman A. R., Brotchie J. M., Michel A., Grimee R. and Klitgaard H. (2004) Levetiracetam potentiates the antidyskinetic action of amantadine in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned primate model of Parkinson's disease. *J. Pharmacol. Exp. Ther.* **310,** 386-394.

113. Holmberg M., Scheinin M., Kurose H. and Miettinen R. (1999) Adrenergic alpha2C-receptors reside in rat striatal GABAergic projection neurons:

comparison of radioligand binding and immunohistochemistry. *Neuroscience* **93,** 1323-1333.

114. Hornykiewicz O. (1975) Brain monoamines and Parkinsonism, in *Aminergic hypothesis of behavior: Reality or Cliché?,* (Bernard B. K., ed), pp. 13-21. Department of Health Education and Welfare, Washington, D.C.

115. Hornykiewicz O. (2002) L-DOPA: from a biologically inactive amino acid to a successful therapeutic agent. *Amino. Acids* **23,** 65-70.

116. Hume S. P., Opacka-Juffry J., Myers R., Ahier R. G., Ashworth S., Brooks D. J. and Lammertsma A. A. (1995) Effect of L-dopa and 6-hydroxydopamine lesioning on [11C]raclopride binding in rat striatum, quantified using PET. *Synapse* **21,** 45-53.

117. Invernizzi R. W., Garavaglia C. and Samanin R. (2003) The alpha 2-adrenoceptor antagonist idazoxan reverses catalepsy induced by haloperidol in rats independent of striatal dopamine release: role of serotonergic mechanisms. *Neuropsychopharmacology* **28,** 872-879.

118. Iravani M. M., Jackson M. J., Kuoppamaki M., Smith L. A. and Jenner P. (2003) 3,4-methylenedioxymethamphetamine (ecstasy) inhibits dyskinesia expression and normalizes motor activity in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated primates. *J. Neurosci.* **23,** 9107-9115.

119. Iravani M. M., Sadeghian M., Leung C. C., Tel B. C., Rose S., Schapira A. H. and Jenner P. (2008) Continuous subcutaneous infusion of pramipexole protects against lipopolysaccharide-induced dopaminergic cell death without affecting the inflammatory response. *Exp. Neurol.* **212,** 522-531.

120. Iravani M. M., Tayarani-Binazir K., Chu W. B., Jackson M. J. and Jenner P. (2006) In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated primates, the selective 5-hydroxytryptamine 1a agonist (R)-(+)-8-OHDPAT inhibits levodopa-induced dyskinesia but only with\ increased motor disability. *J. Pharmacol. Exp. Ther.* **319,** 1225-1234.

121. Jenner P. (2003) The MPTP-treated primate as a model of motor complications in PD: primate model of motor complications. *Neurology* **61,** S4-11.

122. Jenner P. (2008a) Molecular mechanisms of L-DOPA-induced dyskinesia. *Nat. Rev. Neurosci.* **9,** 665-677.

123. Jenner P. (2008b) Preventing and controlling dyskinesia in Parkinson's disease--a view of current knowledge and future opportunities. *Mov Disord.* **23 Suppl 3,** S585-S598.

124. Jenner P., Mori A., Hauser R., Morelli M., Fredholm B. B. and Chen J. F. (2009) Adenosine, adenosine A 2A antagonists, and Parkinson's disease. *Parkinsonism. Relat Disord.* **15,** 406-413.

125. Johnston T. H., Lee J., Gomez-Ramirez J., Fox S. H. and Brotchie J. M. (2005) A simple rodent assay for the in vivo identification of agents with potential to reduce levodopa-induced dyskinesia in Parkinson's disease. *Exp. Neurol.* **191,** 243-250.

126. Jonkers N., Sarre S., Ebinger G. and Michotte Y. (2002) MK801 suppresses the L-DOPA-induced increase of glutamate in striatum of hemi-Parkinson rats. *Brain Res.* **926,** 149-155.

127. Kanda T., Jackson M. J., Smith L. A., Pearce R. K., Nakamura J., Kase H., Kuwana Y. and Jenner P. (1998) Adenosine A2A antagonist: a novel antiparkinsonian agent that does not provoke dyskinesia in parkinsonian monkeys. *Ann. Neurol.* **43,** 507-513.

128. Kanda T., Jackson M. J., Smith L. A., Pearce R. K., Nakamura J., Kase H., Kuwana Y. and Jenner P. (2000) Combined use of the adenosine A(2A) antagonist KW-6002 with L-DOPA or with selective D1 or D2 dopamine agonists increases antiparkinsonian activity but not dyskinesia in MPTP-treated monkeys. *Exp. Neurol.* **162,** 321-327.

129. Kannari K., Yamato H., Shen H., Tomiyama M., Suda T. and Matsunaga M. (2001) Activation of 5-HT(1A) but not 5-HT(1B) receptors attenuates an increase in extracellular dopamine derived from exogenously administered L-DOPA in the striatum with nigrostriatal denervation. *J. Neurochem.* **76,** 1346-1353.

130. Katzenschlager R., Manson A. J., Evans A., Watt H. and Lees A. J. (2004) Low dose quetiapine for drug induced dyskinesias in Parkinson's disease: a double blind cross over study. *J. Neurol. Neurosurg. Psychiatry* **75,** 295-297.

131. Kehr J. (1998a) Determination of gamma-aminobutyric acid in microdialysis samples by microbore column liquid chromatography and fluorescence detection. *J. Chromatogr. B Biomed. Sci. Appl.* **708,** 49-54.

132. Kehr J. (1998b) Determination of glutamate and aspartate in microdialysis samples by reversed-phase column liquid chromatography with fluorescence and electrochemical detection. *J. Chromatogr. B Biomed. Sci. Appl.* **708,** 27-38.

133. Keller H. H., Bartholini G. and Pletscher A. (1974) Enhancement of noradrenaline turnover in rat brain by L-dopa. *J. Pharm. Pharmacol.* **26,** 649-651.

134. Kleedorfer B., Lees A. J. and Stern G. M. (1991) Buspirone in the treatment of levodopa induced dyskinesias. *J. Neurol. Neurosurg. Psychiatry* **54,** 376-377.

135. Kleiner-Fisman G., Herzog J., Fisman D. N., Tamma F., Lyons K. E., Pahwa R., Lang A. E. and Deuschl G. (2006) Subthalamic nucleus deep brain stimulation: summary and meta-analysis of outcomes. *Mov Disord.* **21 Suppl 14,** S290-S304.

136. Kleven M. S., Assie M. B., Cosi C., Barret-Grevoz C. and Newman-Tancredi A. (2005) Anticataleptic properties of alpha2 adrenergic antagonists in the crossed leg position and bar tests: differential mediation by 5-HT1A receptor activation. *Psychopharmacology (Berl)* **177,** 373-380.

137. Klintenberg R., Svenningsson P., Gunne L. and Andren P. E. (2002) Naloxone reduces levodopa-induced dyskinesias and apomorphine-induced rotations in primate models of parkinsonism. *J. Neural Transm.* **109,** 1295-1307.

138. Koga K., Kurokawa M., Ochi M., Nakamura J. and Kuwana Y. (2000) Adenosine A(2A) receptor antagonists KF17837 and KW-6002 potentiate rotation induced by dopaminergic drugs in hemi-Parkinsonian rats. *Eur. J. Pharmacol.* **408,** 249-255.

139. Konitsiotis S., Blanchet P. J., Verhagen L., Lamers E. and Chase T. N. (2000) AMPA receptor blockade improves levodopa-induced dyskinesia in MPTP monkeys. *Neurology* **54,** 1589-1595.

140. Konradi C., Westin J. E., Carta M., Eaton M. E., Kuter K., Dekundy A., Lundblad M. and Cenci M. A. (2004) Transcriptome analysis in a rat model of L-DOPA-induced dyskinesia. *Neurobiol. Dis.* **17,** 219-236.

141. Kovoor A., Seyffarth P., Ebert J., Barghshoon S., Chen C. K., Schwarz S., Axelrod J. D., Cheyette B. N., Simon M. I., Lester H. A. and Schwarz J. (2005) D2 dopamine receptors colocalize regulator of G-protein signaling 9-2 (RGS9-2) via the RGS9 DEP domain, and RGS9 knock-out mice develop dyskinesias associated with dopamine pathways. *J. Neurosci.* **25,** 2157-2165.

142. Kreitzer A. C. and Malenka R. C. (2007) Endocannabinoid-mediated rescue of striatal LTD and motor deficits in Parkinson's disease models. *Nature* **445,** 643-647.

143. Kubrusly R. C., Ventura A. L., de Melo Reis R. A., Serra G. C., Yamasaki E. N., Gardino P. F., de Mello M. C. and de Mello F. G. (2007) Norepinephrine acts as D1-dopaminergic agonist in the embryonic avian retina: late expression of beta1-adrenergic receptor shifts norepinephrine specificity in the adult tissue. *Neurochem. Int.* **50,** 211-218.

144. Kumar N., Van Gerpen J. A., Bower J. H. and Ahlskog J. E. (2005) Levodopa-dyskinesia incidence by age of Parkinson's disease onset. *Mov Disord.* **20,** 342-344.

145. Kvernmo T., Hartter S. and Burger E. (2006) A review of the receptor-binding and pharmacokinetic properties of dopamine agonists. *Clin. Ther.* **28,** 1065-1078.

146. Lanau F., Zenner M. T., Civelli O. and Hartman D. S. (1997) Epinephrine and norepinephrine act as potent agonists at the recombinant human dopamine D4 receptor. *J. Neurochem.* **68,** 804-812.

147. Levandis G., Bazzini E., Armentero M. T., Nappi G. and Blandini F. (2008) Systemic administration of an mGluR5 antagonist, but not unilateral subthalamic lesion, counteracts l-DOPA-induced dyskinesias in a rodent model of Parkinson's disease. *Neurobiol. Dis.* **29,** 161-168.

148. Lewitt P. A., Guttman M., Tetrud J. W., Tuite P. J., Mori A., Chaikin P. and Sussman N. M. (2008) Adenosine A2A receptor antagonist istradefylline (KW-6002) reduces "off" time in Parkinson's disease: a double-blind, randomized, multicenter clinical trial (6002-US-005). *Ann. Neurol.* **63,** 295-302.

149. Lin J. H. (2008) CSF as a surrogate for assessing CNS exposure: an industrial perspective. *Curr. Drug Metab* **9,** 46-59.

150. Linazasoro G., Van B. N., Ugedo L. and Ruiz Ortega J. A. (2008) Pharmacological treatment of Parkinson's disease: life beyond dopamine D2/D3 receptors? *J. Neural Transm.* **115,** 431-441.

151. Lindroth P. and Mopper K. (1979) High performance liquid chromatograph determination of subpicomol amounts of amino acids by precolumn fluorescence derivatization with o-phthaldialdehyde. *Anal. Chem.* **51,** 1667-1674.

152. Lorenc-Koci E. and Wolfarth S. (1999) Efficacy of pramipexole, a new dopamine receptor agonist, to relieve the parkinsonian-like muscle rigidity in rats. *Eur. J. Pharmacol.* **385,** 39-46.

153. Lorenc-Koci E., Wolfarth S. and Ossowska K. (1996) Haloperidol-increased muscle tone in rats as a model of parkinsonian rigidity. *Exp. Brain Res.* **109,** 268-276.

154. Luginger E., Wenning G. K., Bosch S. and Poewe W. (2000) Beneficial effects of amantadine on L-dopa-induced dyskinesias in Parkinson's disease. *Mov Disord.* **15,** 873-878.

155. Lundblad M., Andersson M., Winkler C., Kirik D., Wierup N. and Cenci M. A. (2002) Pharmacological validation of behavioural measures of akinesia and

dyskinesia in a rat model of Parkinson's disease. *Eur. J. Neurosci.* **15,** 120-132.

156. Lundblad M., Picconi B., Lindgren H. and Cenci M. A. (2004) A model of L-DOPA-induced dyskinesia in 6-hydroxydopamine lesioned mice: relation to motor and cellular parameters of nigrostriatal function. *Neurobiol. Dis.* **16,** 110-123.

157. Lundblad M., Usiello A., Carta M., Hakansson K., Fisone G. and Cenci M. A. (2005) Pharmacological validation of a mouse model of l-DOPA-induced dyskinesia. *Exp. Neurol.* **194,** 66-75.

158. Lundblad M., Vaudano E. and Cenci M. A. (2003) Cellular and behavioural effects of the adenosine A2a receptor antagonist KW-6002 in a rat model of l-DOPA-induced dyskinesia. *J. Neurochem.* **84,** 1398-1410.

159. Ma D., Zhang J., Sugahara K., Ageta T., Nakayama K. and Kodama H. (1999) Simultaneous determination of gamma-aminobutyric acid and glutamic acid in the brain of 3-mercaptopropionic acid-treated rats using liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr. B Biomed. Sci. Appl.* **726,** 285-290.

160. MacDonald E. and Scheinin M. (1995) Distribution and pharmacology of alpha 2-adrenoceptors in the central nervous system. *J. Physiol Pharmacol.* **46,** 241-258.

161. Macinnes N. and Duty S. (2008) Group III metabotropic glutamate receptors act as hetero-receptors modulating evoked GABA release in the globus pallidus in vivo. *Eur. J. Pharmacol.* **580,** 95-99.

162. Maeda T., Nagata K., Yoshida Y. and Kannari K. (2005) Serotonergic hyperinnervation into the dopaminergic denervated striatum compensates for dopamine conversion from exogenously administered l-DOPA. *Brain Res.* **1046,** 230-233.

163. Maj J., Rogoz Z., Skuza G. and Kolodziejczyk K. (1997) The behavioural effects of pramipexole, a novel dopamine receptor agonist. *Eur. J. Pharmacol.* **324,** 31-37.

164. Manson A. J., Iakovidou E. and Lees A. J. (2000) Idazoxan is ineffective for levodopa-induced dyskinesias in Parkinson's disease. *Mov Disord.* **15,** 336-337.

165. Manson A. J., Katzenschlager R., Hobart J. and Lees A. J. (2001) High dose naltrexone for dyskinesias induced by levodopa. *J. Neurol. Neurosurg. Psychiatry* **70,** 554-556.

166. Marin C., Aguilar E., Rodriguez-Oroz M. C., Bartoszyk G. D. and Obeso J. A. (2009) Local administration of sarizotan into the subthalamic nucleus attenuates levodopa-induced dyskinesias in 6-OHDA-lesioned rats. *Psychopharmacology (Berl)* **204,** 241-250.

167. Maruya H., Watanabe Y., Okita M., Lawlor G. F., Utsumi H. and Niitsuma T. (2003) Inhibitory effects of D2 agonists by striatal injection on excessive release of dopamine and hyperactivity induced by Bay K 8644 in rats. *Neuroscience* **118,** 1091-1098.

168. Mathe J. M., Nomikos G. G., Hildebrand B. E., Hertel P. and Svensson T. H. (1996) Prazosin inhibits MK-801-induced hyperlocomotion and dopamine release in the nucleus accumbens. *Eur. J. Pharmacol.* **309,** 1-11.

169. McCune S. K., Voigt M. M. and Hill J. M. (1993) Expression of multiple alpha adrenergic receptor subtype messenger RNAs in the adult rat brain. *Neuroscience* **57,** 143-151.

170. Meissner W., Ravenscroft P., Reese R., Harnack D., Morgenstern R., Kupsch A., Klitgaard H., Bioulac B., Gross C. E., Bezard E. and Boraud T. (2006) Increased slow oscillatory activity in substantia nigra pars reticulata triggers abnormal involuntary movements in the 6-OHDA-lesioned rat in the presence of excessive extracellular striatal dopamine. *Neurobiol. Dis.* **22,** 586-598.

171. Mela F., Marti M., Dekundy A., Danysz W., Morari M. and Cenci M. A. (2007) Antagonism of metabotropic glutamate receptor type 5 attenuates l-DOPA-induced dyskinesia and its molecular and neurochemical correlates in a rat model of Parkinson's disease. *J. Neurochem.* **101,** 483-497.

172. Meldrum B. S. (1994) The role of glutamate in epilepsy and other CNS disorders. *Neurology* **44,** S14-S23.

173. Merello M., Nouzeilles M. I., Cammarota A. and Leiguarda R. (1999) Effect of memantine (NMDA antagonist) on Parkinson's disease: a double-blind crossover randomized study. *Clin. Neuropharmacol.* **22,** 273-276.

174. Merims D., Ziv I., Djaldetti R. and Melamed E. (1999) Riluzole for levodopa-induced dyskinesias in advanced Parkinson's disease. *Lancet* **353,** 1764-1765.

175. Mesnage V., Houeto J. L., Bonnet A. M., Clavier I., Arnulf I., Cattelin F., Le F. G., Damier P., Welter M. L. and Agid Y. (2004) Neurokinin B, neurotensin, and cannabinoid receptor antagonists and Parkinson disease. *Clin. Neuropharmacol.* **27,** 108-110.

176. Mierau J. and Schingnitz G. (1992) Biochemical and pharmacological studies on pramipexole, a potent and selective dopamine D2 receptor agonist. *Eur. J. Pharmacol.* **215,** 161-170.

177. Mierau J., Schneider F. J., Ensinger H. A., Chio C. L., Lajiness M. E. and Huff R. M. (1995) Pramipexole binding and activation of cloned and expressed dopamine D2, D3 and D4 receptors. *Eur. J. Pharmacol.* **290,** 29-36.

178. Miller D. W. and Abercrombie E. D. (1999) Role of high-affinity dopamine uptake and impulse activity in the appearance of extracellular dopamine in striatum after administration of exogenous L-DOPA: studies in intact and 6-hydroxydopamine-treated rats. *J. Neurochem.* **72,** 1516-1522.

179. Miralles A., Olmos G., Sastre M., Barturen F., Martin I. and Garcia-Sevilla J. A. (1993) Discrimination and pharmacological characterization of I2-imidazoline sites with [3H]idazoxan and alpha-2 adrenoceptors with [3H]RX821002 (2-methoxy idazoxan) in the human and rat brains. *J. Pharmacol. Exp. Ther.* **264,** 1187-1197.

180. Misu Y., Goshima Y. and Miyamae T. (2002) Is DOPA a neurotransmitter? *Trends Pharmacol. Sci.* **23,** 262-268.

181. Misu Y., Kitahama K. and Goshima Y. (2003) L-3,4-Dihydroxyphenylalanine as a neurotransmitter candidate in the central nervous system. *Pharmacol. Ther.* **97,** 117-137.

182. Monville C., Torres E. M. and Dunnett S. B. (2005) Validation of the l-dopa-induced dyskinesia in the 6-OHDA model and evaluation of the effects of selective dopamine receptor agonists and antagonists. *Brain Res. Bull.* **68,** 16-23.

183. Morgese M. G., Cassano T., Gaetani S., Macheda T., Laconca L., Dipasquale P., Ferraro L., Antonelli T., Cuomo V. and Giuffrida A. (2009) Neurochemical changes in the striatum of dyskinetic rats after administration of the cannabinoid agonist WIN55,212-2. *Neurochem. Int.* **54,** 56-64.

184. Muller T. and Russ H. (2006) Levodopa, motor fluctuations and dyskinesia in Parkinson's disease. *Expert. Opin. Pharmacother.* **7,** 1715-1730.

185. Nadjar A., Gerfen C. R. and Bezard E. (2009) Priming for l-dopa-induced dyskinesia in Parkinson's disease: a feature inherent to the treatment or the disease? *Prog. Neurobiol.* **87,** 1-9.

186. Nagatsu T. and Sawada M. (2007) Biochemistry of postmortem brains in Parkinson's disease: historical overview and future prospects. *J. Neural Transm. Suppl* 113-120.

187. Newman-Tancredi A., udinot-Bouchez V., Gobert A. and Millan M. J. (1997) Noradrenaline and adrenaline are high affinity agonists at dopamine D4 receptors. *Eur. J. Pharmacol.* **319,** 379-383.

188. Ng K. Y., Chase T. N., Colburn R. W. and Kopin I. J. (1970) L-Dopa-induced release of cerebral monoamines. *Science* **170,** 76-77.

189. Nicholas A. P. (2007) Levodopa-induced hyperactivity in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Mov Disord.* **22,** 99-104.

190. Nicholas A. P., Hokfelt T. and Pieribone V. A. (1996) The distribution and significance of CNS adrenoceptors examined with in situ hybridization. *Trends Pharmacol. Sci.* **17,** 245-255.

191. Nicholas A. P., Pieribone V. and Hokfelt T. (1993) Distributions of mRNAs for alpha-2 adrenergic receptor subtypes in rat brain: an in situ hybridization study. *J. Comp Neurol.* **328,** 575-594.

192. Nicholson S. L. and Brotchie J. M. (2002) 5-hydroxytryptamine (5-HT, serotonin) and Parkinson's disease - opportunities for novel therapeutics to reduce the problems of levodopa therapy. *Eur. J. Neurol.* **9 Suppl 3,** 1-6.

193. Nishi K., Kondo T. and Narabayashi H. (1991) Destruction of norepinephrine terminals in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice reduces locomotor activity induced by L-dopa. *Neurosci. Lett.* **123,** 244-247.

194. Nishikawa T., Tanaka M., Tsuda A., Koga I. and Uchida Y. (1984) Clonidine therapy for tardive dyskinesia and related syndromes. *Clin. Neuropharmacol.* **7,** 239-245.

195. Niswender C. M., Johnson K. A., Weaver C. D., Jones C. K., Xiang Z., Luo Q., Rodriguez A. L., Marlo J. E., de P. T., Thompson A. D., Days E. L., Nalywajko T., Austin C. A., Williams M. B., Ayala J. E., Williams R., Lindsley C. W. and Conn P. J. (2008) Discovery, characterization, and antiparkinsonian effect of novel positive allosteric modulators of metabotropic glutamate receptor 4. *Mol. Pharmacol.* **74,** 1345-1358.

196. Nutt J. G. (1990) Levodopa-induced dyskinesia: review, observations, and speculations. *Neurology* **40,** 340-345.

197. Nutt J. G., Obeso J. A. and Stocchi F. (2000) Continuous dopamine-receptor stimulation in advanced Parkinson's disease. *Trends Neurosci.* **23,** S109-S115.

198. Nyholm D. (2006) Pharmacokinetic optimisation in the treatment of Parkinson's disease : an update. *Clin. Pharmacokinet.* **45,** 109-136.

199. Obeso J. A., Olanow C. W. and Nutt J. G. (2000a) Levodopa motor complications in Parkinson's disease. *Trends Neurosci.* **23,** S2-S7.

200. Obeso J. A., Rodriguez-Oroz M. C., Chana P., Lera G., Rodriguez M. and Olanow C. W. (2000b) The evolution and origin of motor complications in Parkinson's disease. *Neurology* **55,** S13-S20.

201. Ochi M., Shiozaki S. and Kase H. (2004) Adenosine A(2A) receptor-mediated modulation of GABA and glutamate release in the output regions of the basal ganglia in a rodent model of Parkinson's disease. *Neuroscience* **127,** 223-231.

202. Oh J. D., Bibbiani F. and Chase T. N. (2002) Quetiapine attenuates levodopa-induced motor complications in rodent and primate parkinsonian models. *Exp. Neurol.* **177,** 557-564.

203. Okura T., Ito R., Ishiguro N., Tamai I. and Deguchi Y. (2007) Blood-brain barrier transport of pramipexole, a dopamine D2 agonist. *Life Sci.* **80,** 1564-1571.

204. Olanow C. W., Damier P., Goetz C. G., Mueller T., Nutt J., Rascol O., Serbanescu A., Deckers F. and Russ H. (2004) Multicenter, open-label, trial of sarizotan in Parkinson disease patients with levodopa-induced dyskinesias (the SPLENDID Study). *Clin. Neuropharmacol.* **27,** 58-62.

205. Olanow C. W., Obeso J. A. and Stocchi F. (2006) Drug insight: Continuous dopaminergic stimulation in the treatment of Parkinson's disease. *Nat. Clin. Pract. Neurol.* **2,** 382-392.

206. Onali P., Olianas M. C. and Gessa G. L. (1985) Characterization of dopamine receptors mediating inhibition of adenylate cyclase activity in rat striatum. *Mol. Pharmacol.* **28,** 138-145.

207. Oueslati A., Sgambato-Faure V., Melon C., Kachidian P., Gubellini P., Amri M., Kerkerian-Le G. L. and Salin P. (2007) High-frequency stimulation of the subthalamic nucleus potentiates L-DOPA-induced neurochemical changes in the striatum in a rat model of Parkinson's disease. *J. Neurosci.* **27,** 2377-2386.

208. Paci C., Thomas A. and Onofrj M. (2001) Amantadine for dyskinesia in patients affected by severe Parkinson's disease. *Neurol. Sci.* **22,** 75-76.

209. Pan W. H., Yang S. Y. and Lin S. K. (2004) Neurochemical interaction between dopaminergic and noradrenergic neurons in the medial prefrontal cortex. *Synapse* **53,** 44-52.

210. Paquette M. A., Brudney E. G., Putterman D. B., Meshul C. K., Johnson S. W. and Berger S. P. (2008) Sigma ligands, but not N-methyl-D-aspartate antagonists, reduce levodopa-induced dyskinesias. *Neuroreport* **19,** 111-115.

211. Paquette M. A., Foley K., Brudney E. G., Meshul C. K., Johnson S. W. and Berger S. P. (2009) The sigma-1 antagonist BMY-14802 inhibits L-DOPA-

induced abnormal involuntary movements by a WAY-100635-sensitive mechanism. *Psychopharmacology (Berl)*.

212. Parkinson Study Group (2001) Evaluation of dyskinesias in a pilot, randomized, placebo-controlled trial of remacemide in advanced Parkinson disease. *Arch. Neurol.* **58,** 1660-1668.

213. Paul P., Lahaye C., Delagrange P., Nicolas J. P., Canet E. and Boutin J. A. (1999) Characterization of 2-[125I]iodomelatonin binding sites in Syrian hamster peripheral organs. *J. Pharmacol. Exp. Ther.* **290,** 334-340.

214. Paxinos G. and Watson C. (1998) The rat brain in stereotaxic coordinates. *Academic Press, San Diego*.

215. Pearce R. K., Jackson M., Smith L., Jenner P. and Marsden C. D. (1995) Chronic L-DOPA administration induces dyskinesias in the 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine-treated common marmoset (Callithrix Jacchus). *Mov Disord.* **10,** 731-740.

216. Pechevis M., Clarke C. E., Vieregge P., Khoshnood B., schaseaux-Voinet C., Berdeaux G. and Ziegler M. (2005) Effects of dyskinesias in Parkinson's disease on quality of life and health-related costs: a prospective European study. *Eur. J. Neurol.* **12,** 956-963.

217. Piercey M. F. (1998) Pharmacology of pramipexole, a dopamine D3-preferring agonist useful in treating Parkinson's disease. *Clin. Neuropharmacol.* **21,** 141-151.

218. Piercey M. F., Hoffmann W. E., Smith M. W. and Hyslop D. K. (1996) Inhibition of dopamine neuron firing by pramipexole, a dopamine D3 receptor-preferring agonist: comparison to other dopamine receptor agonists. *Eur. J. Pharmacol.* **312,** 35-44.

219. Piraud M., Vianey-Saban C., Petritis K., Elfakir C., Steghens J. P., Morla A. and Bouchu D. (2003) ESI-MS/MS analysis of underivatised amino acids: a new tool for the diagnosis of inherited disorders of amino acid metabolism. Fragmentation study of 79 molecules of biological interest in positive and negative ionisation mode. *Rapid Commun. Mass Spectrom.* **17,** 1297-1311.

220. Rajput A. H., Rajput A., Lang A. E., Kumar R., Uitti R. J. and Galvez-Jimenez N. (1998) New use for an old drug: amantadine benefits levodopa-induced dyskinesia. *Mov Disord.* **13,** 851.

221. Rascol O. (2000) Medical treatment of levodopa-induced dyskinesias. *Ann. Neurol.* **47,** S179-S188.

222. Rascol O., Arnulf I., Peyro-Saint P. H., Brefel-Courbon C., Vidailhet M., Thalamas C., Bonnet A. M., Descombes S., Bejjani B., Fabre N., Montastruc

J. L. and Agid Y. (2001) Idazoxan, an alpha-2 antagonist, and L-DOPA-induced dyskinesias in patients with Parkinson's disease. *Mov Disord.* **16,** 708-713.

223. Rascol O., Brooks D. J., Korczyn A. D., De Deyn P. P., Clarke C. E. and Lang A. E. (2000) A five-year study of the incidence of dyskinesia in patients with early Parkinson's disease who were treated with ropinirole or levodopa. 056 Study Group. *N. Engl. J. Med.* **342,** 1484-1491.

224. Rascol O. and Fabre N. (2001) Dyskinesia: L-dopa-induced and tardive dyskinesia. *Clin. Neuropharmacol.* **24,** 313-323.

225. Rascol O., Fabre N., Blin O., Poulik J., Sabatini U., Senard J. M., Ane M., Montastruc J. L. and Rascol A. (1994) Naltrexone, an opiate antagonist, fails to modify motor symptoms in patients with Parkinson's disease. *Mov Disord.* **9,** 437-440.

226. Rea K., Cremers T. I. and Westerink B. H. (2005) HPLC conditions are critical for the detection of GABA by microdialysis. *J. Neurochem.* **94,** 672-679.

227. Riederer P. and Wuketich S. (1976) Time course of nigrostriatal degeneration in parkinson's disease. A detailed study of influential factors in human brain amine analysis. *J. Neural Transm.* **38,** 277-301.

228. Robelet S., Melon C., Guillet B., Salin P. and Kerkerian-Le G. L. (2004) Chronic L-DOPA treatment increases extracellular glutamate levels and GLT1 expression in the basal ganglia in a rat model of Parkinson's disease. *Eur. J. Neurosci.* **20,** 1255-1266.

229. Robertson G. S., Tham C. S., Wilson C., Jakubovic A. and Fibiger H. C. (1993) In vivo comparisons of the effects of quinpirole and the putative presynaptic dopaminergic agonists B-HT 920 and SND 919 on striatal dopamine and acetylcholine release. *J. Pharmacol. Exp. Ther.* **264,** 1344-1351.

230. Rodriguez M., Morales I., Gonzalez-Mora J. L., Gomez I., Sabate M., Dopico J. G., Rodriguez-Oroz M. C. and Obeso J. A. (2007) Different levodopa actions on the extracellular dopamine pools in the rat striatum. *Synapse* **61,** 61-71.

231. Romero J. A., Chalmers J. P., Cottman K., Lytle L. D. and Wurtman R. J. (1972) Regional effects of L-dihydroxyphenylalanine (L-dopa) on norepinephrine metabolism in rat brain. *J. Pharmacol. Exp. Ther.* **180,** 277-285.

232. Rommelfanger K. S. and Weinshenker D. (2007) Norepinephrine: The redheaded stepchild of Parkinson's disease. *Biochem. Pharmacol.* **74,** 177-190.

233. Rosin D. L., Talley E. M., Lee A., Stornetta R. L., Gaylinn B. D., Guyenet P. G. and Lynch K. R. (1996) Distribution of alpha 2C-adrenergic receptor-like immunoreactivity in the rat central nervous system. *J. Comp Neurol.* **372,** 135-165.

234. Rozas G., Liste I., Guerra M. J. and Labandeira-Garcia J. L. (1998) Sprouting of the serotonergic afferents into striatum after selective lesion of the dopaminergic system by MPTP in adult mice. *Neurosci. Lett.* **245,** 151-154.

235. Rylander D., Recchia A., Mela F., Dekundy A., Danysz W. and Cenci M. A. (2009) Pharmacological modulation of glutamate transmission in a rat model of L-DOPA-induced dyskinesia: effects on motor behavior and striatal nuclear signaling. *J. Pharmacol. Exp. Ther.* **330,** 227-235.

236. Samadi P., Gregoire L. and Bedard P. J. (2003) Opioid antagonists increase the dyskinetic response to dopaminergic agents in parkinsonian monkeys: interaction between dopamine and opioid systems. *Neuropharmacology* **45,** 954-963.

237. Samadi P., Gregoire L., Hadj T. A., Di P. T., Rouillard C. and Bedard P. J. (2005) Naltrexone in the short-term decreases antiparkinsonian response to l-Dopa and in the long-term increases dyskinesias in drug-naive parkinsonian monkeys. *Neuropharmacology* **49,** 165-173.

238. Samadi P., Gregoire L., Morissette M., Calon F., Hadj T. A., Dridi M., Belanger N., Meltzer L. T., Bedard P. J. and Di P. T. (2008) mGluR5 metabotropic glutamate receptors and dyskinesias in MPTP monkeys. *Neurobiol. Aging* **29,** 1040-1051.

239. Sanberg P. R., Bunsey M. D., Giordano M. and Norman A. B. (1988) The catalepsy test: its ups and downs. *Behav. Neurosci.* **102,** 748-759.

240. Sastre-Coll A., Esteban S. and Garcia-Sevilla J. A. (1999) Effects of imidazoline receptor ligands on monoamine synthesis in the rat brain in vivo. *Naunyn Schmiedebergs Arch. Pharmacol.* **360,** 50-62.

241. Sauvinet V., Parrot S., Benturquia N., Bravo-Moraton E., Renaud B. and Denoroy L. (2003) In vivo simultaneous monitoring of gamma-aminobutyric acid, glutamate, and L-aspartate using brain microdialysis and capillary electrophoresis with laser-induced fluorescence detection: Analytical developments and in vitro/in vivo validations. *Electrophoresis* **24,** 3187-3196.

242. Savola J. M., Hill M., Engstrom M., Merivuori H., Wurster S., McGuire S. G., Fox S. H., Crossman A. R. and Brotchie J. M. (2003) Fipamezole (JP-1730) is a potent alpha2 adrenergic receptor antagonist that reduces levodopa-induced dyskinesia in the MPTP-lesioned primate model of Parkinson's disease. *Mov Disord.* **18,** 872-883.

243. Schapira A. H. (2005) Present and future drug treatment for Parkinson's disease. *J. Neurol. Neurosurg. Psychiatry* **76,** 1472-1478.

244. Schapira A. H., Bezard E., Brotchie J., Calon F., Collingridge G. L., Ferger B., Hengerer B., Hirsch E., Jenner P., Le N. N., Obeso J. A., Schwarzschild M. A., Spampinato U. and Davidai G. (2006) Novel pharmacological targets for the treatment of Parkinson's disease. *Nat. Rev. Drug Discov.* **5,** 845-854.

245. Scheinin M., Lomasney J. W., Hayden-Hixson D. M., Schambra U. B., Caron M. G., Lefkowitz R. J. and Fremeau R. T., Jr. (1994) Distribution of alpha 2-adrenergic receptor subtype gene expression in rat brain. *Brain Res. Mol. Brain Res.* **21,** 133-149.

246. Schmidt W. J., Bubser M. and Hauber W. (1992) Behavioural pharmacology of glutamate in the basal ganglia. *J. Neural Transm. Suppl* **38,** 65-89.

247. Seeman P. and Van Tol H. H. (1994) Dopamine receptor pharmacology. *Trends Pharmacol. Sci.* **15,** 264-270.

248. Shiozaki S., Ichikawa S., Nakamura J., Kitamura S., Yamada K. and Kuwana Y. (1999) Actions of adenosine A2A receptor antagonist KW-6002 on drug-induced catalepsy and hypokinesia caused by reserpine or MPTP. *Psychopharmacology (Berl)* **147,** 90-95.

249. Sieradzan K. A., Fox S. H., Hill M., Dick J. P., Crossman A. R. and Brotchie J. M. (2001) Cannabinoids reduce levodopa-induced dyskinesia in Parkinson's disease: a pilot study. *Neurology* **57,** 2108-2111.

250. Snow B. J., Macdonald L., Mcauley D. and Wallis W. (2000) The effect of amantadine on levodopa-induced dyskinesias in Parkinson's disease: a double-blind, placebo-controlled study. *Clin. Neuropharmacol.* **23,** 82-85.

251. Sommermeyer H., Frielingsdorf J. and Knorr A. (1995) Effects of prazosin on the dopaminergic neurotransmission in rat brain. *Eur. J. Pharmacol.* **276,** 267-270.

252. Song Y., Shenwu M., Dhossche D. M. and Liu Y. M. (2005) A capillary liquid chromatographic/tandem mass spectrometric method for the quantification of gamma-aminobutyric acid in human plasma and cerebrospinal fluid. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **814,** 295-302.

253. Srinivasan J. and Schmidt W. J. (2004) The effect of the alpha2-adrenoreceptor antagonist idazoxan against 6-hydroxydopamine-induced Parkinsonism in rats: multiple facets of action? *Naunyn Schmiedebergs Arch. Pharmacol.* **369,** 629-638.

254. Stacy M., Silver D., Mendis T., Sutton J., Mori A., Chaikin P. and Sussman N. M. (2008) A 12-week, placebo-controlled study (6002-US-006) of istradefylline in Parkinson disease. *Neurology* **70,** 2233-2240.

255. Steiger M. (2008) Constant dopaminergic stimulation by transdermal delivery of dopaminergic drugs: a new treatment paradigm in Parkinson's disease. *Eur. J. Neurol.* **15,** 6-15.

256. Stocchi F., Tagliati M. and Olanow C. W. (2008) Treatment of levodopa-induced motor complications. *Mov Disord.* **23 Suppl 3,** S599-S612.

257. Stockwell K. A., Virley D. J., Perren M., Iravani M. M., Jackson M. J., Rose S. and Jenner P. (2008) Continuous delivery of ropinirole reverses motor deficits without dyskinesia induction in MPTP-treated common marmosets. *Exp. Neurol.* **211,** 172-179.

258. Sundstrom E., Stromberg I., Tsutsumi T., Olson L. and Jonsson G. (1987) Studies on the effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on central catecholamine neurons in C57BL/6 mice. Comparison with three other strains of mice. *Brain Res.* **405,** 26-38.

259. Tanaka H., Kannari K., Maeda T., Tomiyama M., Suda T. and Matsunaga M. (1999) Role of serotonergic neurons in L-DOPA-derived extracellular dopamine in the striatum of 6-OHDA-lesioned rats. *Neuroreport* **10,** 631-634.

260. Tarazi F. I., Kula N. S. and Baldessarini R. J. (1997) Regional distribution of dopamine D4 receptors in rat forebrain. *Neuroreport* **8,** 3423-3426.

261. Timmerman W. and Westerink B. H. (1997) Brain microdialysis of GABA and glutamate: what does it signify? *Synapse* **27,** 242-261.

262. Tossman U., Eriksson S., Delin A., Hagenfeldt L., Law D. and Ungerstedt U. (1983) Brain amino acids measured by intracerebral dialysis in portacaval shunted rats. *J. Neurochem.* **41,** 1046-1051.

263. Ueda H., Sato K., Okumura F. and Misu Y. (1995) L-DOPA inhibits spontaneous acetylcholine release from the striatum of experimental Parkinson's model rats. *Brain Res.* **698,** 213-216.

264. Valastro B., Dekundy A., Danysz W. and Quack G. (2009) Oral creatine supplementation attenuates L-DOPA-induced dyskinesia in 6-hydroxydopamine-lesioned rats. *Behav. Brain Res.* **197,** 90-96.

265. van der Stelt M., Fox S. H., Hill M., Crossman A. R., Petrosino S., Di M., V and Brotchie J. M. (2005) A role for endocannabinoids in the generation of parkinsonism and levodopa-induced dyskinesia in MPTP-lesioned non-human primate models of Parkinson's disease. *FASEB J.* **19,** 1140-1142.

266. van der Zeyden M., Oldenziel W. H., Rea K., Cremers T. I. and Westerink B. H. (2008) Microdialysis of GABA and glutamate: analysis, interpretation and comparison with microsensors. *Pharmacol. Biochem. Behav.* **90,** 135-147.

267. Verhagen Metman L., Del D. P., LePoole K., Konitsiotis S., Fang J. and Chase T. N. (1999) Amantadine for Levodopa-Induced Dyskinesias: A 1-Year Follow-up Study. *Arch. Neurol.* **56,** 1383-1386.

268. Verhagen M. L., Blanchet P. J., van den M. P., Del D. P., Natte R. and Chase T. N. (1998a) A trial of dextromethorphan in parkinsonian patients with motor response complications. *Mov Disord.* **13,** 414-417.

269. Verhagen M. L., Del D. P., Blanchet P. J., van den M. P. and Chase T. N. (1998b) Blockade of glutamatergic transmission as treatment for dyskinesias and motor fluctuations in Parkinson's disease. *Amino. Acids* **14,** 75-82.

270. Verhagen M. L., Del D. P., Natte R., van den M. P. and Chase T. N. (1998c) Dextromethorphan improves levodopa-induced dyskinesias in Parkinson's disease. *Neurology* **51,** 203-206.

271. Visanji N. P., Fox S. H., Johnston T. H., Millan M. J. and Brotchie J. M. (2009) Alpha1-adrenoceptors mediate dihydroxyphenylalanine-induced activity in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned macaques. *J. Pharmacol. Exp. Ther.* **328,** 276-283.

272. Visanji N. P., Gomez-Ramirez J., Johnston T. H., Pires D., Voon V., Brotchie J. M. and Fox S. H. (2006) Pharmacological characterization of psychosis-like behavior in the MPTP-lesioned nonhuman primate model of Parkinson's disease. *Mov Disord.* **21,** 1879-1891.

273. Wachtel S. R. and Abercrombie E. D. (1994) L-3,4-dihydroxyphenylalanine-induced dopamine release in the striatum of intact and 6-hydroxydopamine-treated rats: differential effects of monoamine oxidase A and B inhibitors. *J. Neurochem.* **63,** 108-117.

274. Wang T. and Muthuswamy J. (2008) Immunosensor for detection of inhibitory neurotransmitter gamma-aminobutyric acid using quartz crystal microbalance. *Anal. Chem.* **80,** 8576-8582.

275. Westin J. E., Andersson M., Lundblad M. and Cenci M. A. (2001) Persistent changes in striatal gene expression induced by long-term L-DOPA treatment in a rat model of Parkinson's disease. *Eur. J. Neurosci.* **14,** 1171-1176.

276. Westin J. E., Vercammen L., Strome E. M., Konradi C. and Cenci M. A. (2007) Spatiotemporal pattern of striatal ERK1/2 phosphorylation in a rat model of L-DOPA-induced dyskinesia and the role of dopamine D1 receptors. *Biol. Psychiatry* **62,** 800-810.

277. Windels F., Carcenac C., Poupard A. and Savasta M. (2005) Pallidal origin of GABA release within the substantia nigra pars reticulata during high-frequency stimulation of the subthalamic nucleus. *J. Neurosci.* **25,** 5079-5086.

278. Winkler C., Kirik D., Bjorklund A. and Cenci M. A. (2002) L-DOPA-induced dyskinesia in the intrastriatal 6-hydroxydopamine model of parkinson's disease: relation to motor and cellular parameters of nigrostriatal function. *Neurobiol. Dis.* **10,** 165-186.

279. Wolf M. E. and Roth R. H. (1987) Dopamine neurons projecting to the medial prefrontal cortex possess release-modulating autoreceptors. *Neuropharmacology* **26,** 1053-1059.

280. Wolf M. E. and Roth R. H. (1990) Autoreceptor regulation of dopamine synthesis. *Ann. N. Y. Acad. Sci.* **604,** 323-343.

281. Wright C. E., Sisson T. L., Ichhpurani A. K. and Peters G. R. (1997) Steady-state pharmacokinetic properties of pramipexole in healthy volunteers. *J. Clin. Pharmacol.* **37,** 520-525.

282. Zapata A. and Shippenberg T. S. (2002) D(3) receptor ligands modulate extracellular dopamine clearance in the nucleus accumbens. *J. Neurochem.* **81,** 1035-1042.

283. Zhang W. and Ordway G. A. (2003) The alpha2C-adrenoceptor modulates GABA release in mouse striatum. *Brain Res. Mol. Brain Res.* **112,** 24-32.

284. Zhang X., Andren P. E., Greengard P. and Svenningsson P. (2008) Evidence for a role of the 5-HT1B receptor and its adaptor protein, p11, in L-DOPA treatment of an animal model of Parkinsonism. *Proc. Natl. Acad. Sci. U. S. A* **105,** 2163-2168.

285. Zhang X., Rauch A., Lee H., Xiao H., Rainer G. and Logothetis N. K. (2007) Capillary hydrophilic interaction chromatography/mass spectrometry for simultaneous determination of multiple neurotransmitters in primate cerebral cortex. *Rapid Commun. Mass Spectrom.* **21,** 3621-3628.

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LIST OF PUBLICATIONS

Peer-reviewed publications

K. Buck and B. Ferger. Intrastriatal inhibition of aromatic amino acid decarboxylase prevents L-DOPA-induced dyskinesia: a bilateral reverse in vivo microdialysis study in 6-hydroxydopamine lesioned rats. *Neurobiol Dis,* 2008; 29(2):210-220.

A. P. Nicholas, K. Buck, B. Ferger. Effects of levodopa on striatal monoamines in mice with levodopa-induced hyperactivity. *Neurosci Lett*, 2008; 443(3):204-208.

K. Buck and B. Ferger. Comparison of intrastriatal administration of noradrenaline and l-DOPA on dyskinetic movements: A bilateral reverse in vivo microdialysis study in 6-hydroxydopamine-lesioned rats. *Neuroscience,* 2009; 159(1):16-20.

K. Buck, P. Voehringer, B. Ferger. Rapid analysis of GABA and glutamate in microdialysis samples using high performance liquid chromatography and tandem mass spectrometry. *Journal of Neuroscience Methods*, 2009; 182:78-84.

K. Buck and B. Ferger. The selective α_1 adrenoceptor antagonist HEAT reduces L-DOPA-induced dyskinesia in a rat model of Parkinson's disease. *Synapse*, 2010; 64(2):117-126.

K. Buck, P. Voehringer, B. Ferger. The α_2 adrenoceptor antagonist idazoxan alleviates L-DOPA-induced dyskinesia by reduction of striatal dopamine levels: an in vivo microdialysis study in 6-hydroxydopamine lesioned rats. *J Neurochem*, 2010; 112(2):444-452.

B. Ferger, K. Buck, M. Shimasaki, E. Koros, P. Voehringer, E. Buerger. Continuous dopaminergic stimulation by pramipexole is effective to treat early morning akinesia in animal models of Parkinson's disease: a pharmacokinetic-pharmacodynamic study using in vivo microdialysis. *Synapse.* In Press.

K. Buck, P. Voehringer, B. Ferger. Site-specific action of L-DOPA in the striatum but not globus pallidus and substantia nigra pars reticulata evokes dyskinetic movements in chronic L-DOPA-treated 6-OHDA-lesioned rats. *Neurosci.* In Press.

Presentations at national and international conferences

K. Buck and B. Ferger. Local dopamine and L-DOPA induce functional behavioural alterations in the rat dyskinesia model: a bilateral reverse in vivo microdialysis study. Program No. 675.10. 2006 Neuroscience Meeting Planner. Atlanta, GA: Society for Neuroscience, 2006. Online (poster).

K. Buck and B. Ferger. Intrastriatal inhibition of aromatic amino acid decarboxylase prevents L-DOPA-induced dyskinesia: a bilateral reverse in vivo microdialysis study in rats. 7th Göttingen Meeting of the German Neuroscience Society 2007, Göttingen, Germany (poster).

K. Buck and B. Ferger. Intrastriatal administration of noradrenaline produces dyskinetic movements: a bilateral reverse in vivo microdialysis study in 6-hydroxydopamine lesioned rats. Program No. 796.6. 2007 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience, 2007. Online (poster).

A. P. Nicholas, K. Buck, D. Parsons, B. Ferger. Brain monoamines in levodopa-induced hyperactivity. Program No. 710.16. 2007 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience, 2007. Online (poster).

K. Buck and B. Ferger. Role of noradrenergic neurotransmission in the rat model of L-DOPA-induced dyskinesia. FENS Abstr. vol 4, 218.12, 2008, Geneva, Switzerland (poster).

S. Schuette, K. Buck, B. Ferger. The role of 5-HT_{1A} receptors on the anti-parkinsonian activity of MDMA in the reserpine akinesia model and in the haloperidol-induced catalepsy model in mice. FENS Abstr. vol 4, 218.57, 2008, Geneva, Switzerland (poster).

E. Koros, K. Buck, B. Ferger. Effects of the selective mGlu5 receptor antagonist MTEP on dopaminergic and glutamatergic neurotransmission in the medial prefrontal cortex of the rat. Monitoring Molecules in Neuroscience. 12th International Conference on In Vivo Methods. 2008, Vancouver, Canada (poster).

K. Buck and B. Ferger. The rat dyskinesia model: neurochemical and behavioural characterisation. Conference of Junior Neuroscientists 2008, Ellwangen, Germany (oral presentation).

K. Buck, M. Shimasaki, J. Steinhauer, E. Buerger, B. Ferger. Continuous dopaminergic stimulation using pramipexole is effective to treat early morning akinesia in animal models of Parkinson's disease. 9th International Conference AD/PD 2009, Prague, Czech Republic (poster).

K. Buck, P. Voehringer, B. Ferger. Striatal L-DOPA but not GABA and glutamate is the crucial trigger to switch on dyskinetic movements in 6-OHDA-lesioned rats. Program No. 629.8. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online (poster).

B. Ferger, K. Buck, M. Shimasaki, E. Koros, P. Voehringer, E. Buerger. Continuous dopaminergic stimulation by the dopamine agonist pramipexole is effective to treat early morning akinesia in animal models of Parkinson's disease: a pharmacokinetic-pharmacodynamic study using in vivo microdialysis. Program No. 629.9. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online (poster).

P. Voehringer, K. Buck, B. Ferger. CSF exposure of amantadine is the crucial parameter to predict therapeutic efficacy in L-DOPA-induced dyskinesia: a study supporting translational medicine. Program No. 629.10. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online (poster).