Investigation of motor and olfactory dysfunction in genetic and non-genetic animal models of Parkinson’s disease: implications for translational research in medicine

Dissertation
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Patrizia Vöhringer
Biberach an der Riß

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Acting Dean: Prof. Dr. Thomas Wirth
First Reviewer: Prof. Dr. Boris Ferger
Second Reviewer: PD Dr. Anke Witting
Day of Graduation: 15.04.2016
For my family
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<td>3-MT</td>
<td>3-methoxytyramine</td>
</tr>
<tr>
<td>5-HT</td>
<td>serotonin (5-hydroxytryptamine)</td>
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<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
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<tr>
<td>α-syn</td>
<td>α-synuclein</td>
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<tr>
<td>AAALAC</td>
<td>Association for Assessment and Accreditation of Laboratory Animal Care International</td>
</tr>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>AAV5</td>
<td>adeno-associated virus 5</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
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<tr>
<td>AMPT</td>
<td>α-methyl-p-tyrosine</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>AON</td>
<td>anterior olfactory nucleus</td>
</tr>
<tr>
<td>AP</td>
<td>anteroposterior</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>BO</td>
<td>bulbus olfactorius</td>
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<tr>
<td>C</td>
<td>carboxy</td>
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<tr>
<td>CBA</td>
<td>chicken beta actin</td>
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<td>CDS</td>
<td>continuous dopaminergic stimulation</td>
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<td>CE-MS</td>
<td>capillary electrophoresis mass spectrometry</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyltransferase</td>
</tr>
<tr>
<td>cps</td>
<td>counts per second</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>DA</td>
<td>dopamine</td>
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<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DV</td>
<td>dorsoventral</td>
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<tr>
<td>ECD</td>
<td>electrochemical detection</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionisation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>EIF4G1</td>
<td>eukaryotic translation initiation factor 4 gamma 1</td>
</tr>
<tr>
<td>FD</td>
<td>fluorescence detection</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FIA</td>
<td>flow injection analysis</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GC-MS</td>
<td>gas chromatography mass spectrometry</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GlyT</td>
<td>glycine transporter</td>
</tr>
<tr>
<td>GP</td>
<td>globus pallidus</td>
</tr>
<tr>
<td>GPe</td>
<td>globus pallidus externus</td>
</tr>
<tr>
<td>GPI</td>
<td>globus pallidus internus</td>
</tr>
<tr>
<td>h</td>
<td>human</td>
</tr>
<tr>
<td>HILIC</td>
<td>hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HVA</td>
<td>homovanillic acid</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>k&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LOD</td>
<td>limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>limit of quantitation</td>
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<tr>
<td>LRRK2</td>
<td>leucine-rich repeat kinase 2</td>
</tr>
<tr>
<td>MAO-B</td>
<td>monoamine oxidase B</td>
</tr>
<tr>
<td>MCE</td>
<td>mercaptoethanol</td>
</tr>
<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
</tr>
<tr>
<td>ML</td>
<td>mediolateral</td>
</tr>
<tr>
<td>MPP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1-methyl-4-phenylpyridinium ion</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
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<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>N</td>
<td>amino</td>
</tr>
<tr>
<td>NAC</td>
<td>non-amyloid component</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>OPA</td>
<td>o-phthaldialdehyde</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCP</td>
<td>phencyclidine</td>
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<td>PD</td>
<td>Parkinson's disease</td>
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<tr>
<td>PD</td>
<td>pharmacodynamic</td>
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<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
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<tr>
<td>PK</td>
<td>pharmacokinetic</td>
</tr>
<tr>
<td>p.o.</td>
<td>peroral</td>
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<tr>
<td>PPX</td>
<td>pramipexole</td>
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<td>PPX-CR</td>
<td>pramipexole continuous release</td>
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<td>PPX-IR</td>
<td>pramipexole immediate release</td>
</tr>
<tr>
<td>RM</td>
<td>repeated measures</td>
</tr>
<tr>
<td>RSD</td>
<td>relative standard deviation</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SNpc</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNpr</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>SPECT</td>
<td>single-photon emission computed tomography</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>UPSIT</td>
<td>University of Pennsylvania Smell Identification Test</td>
</tr>
<tr>
<td>vg</td>
<td>vector genomes</td>
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<tr>
<td>VMAT-2</td>
<td>vesicular monoamine transporter 2</td>
</tr>
<tr>
<td>VPS35</td>
<td>vacuolar protein sorting 35</td>
</tr>
<tr>
<td>WPRE</td>
<td>woodchuck hepatitis virus post-transcriptional regulatory element</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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Published results

Results gained in my thesis have previously been published in the following publications:


1. Introduction

1.1 Parkinson’s disease

1.1.1 Clinical features and pathology

Parkinson’s disease (PD) is one of the most common neurodegenerative disorders affecting more than 1 % of the population over the age of 65, with a prevalence rate steeply rising with age (Alves et al., 2008; de Lau and Breteler, 2006; Pringsheim et al., 2014; Wirdefeldt et al., 2011). The disorder is associated with a progressive course, and patients are faced with severe disability and escalating deterioration of quality of life. The factor age seems to be the most important risk factor for developing PD making it a serious health burden to an aging population with increased life expectancy, which emphasises the scientific interest in this devastating disease (Kalia and Lang, 2015).

PD is neuropathologically characterised by the progressive loss of dopaminergic neurons within the basal ganglia network, particularly those projecting from the substantia nigra pars compacta (SNpc) to the striatum. The resulting depletion of striatal dopamine (DA) leads to the clinical manifestation of motor symptoms including bradykinesia, rigidity, resting tremor as well as postural instability (Fahn, 2003; Meissner et al., 2011).

In addition to the motor phenotype, there are a variety of non-motor symptoms such as impaired olfaction, sleep disturbances, constipation, mood disorders as well as autonomic dysfunction which manifest during the course of PD (Chaudhuri et al., 2011; Chaudhuri and Naidu, 2008; Schapira and Tolosa, 2010; Visanji and Marras, 2015). Many of these antedate the onset of motor disturbances by years and characterise a premotor preclinical phase of PD (Hawkes et al., 2010; Kalia and Lang, 2015; Pont-Sunyer et al., 2015). A time course of PD symptoms is depicted in Figure 1.
Diagnosis of PD occurs with the appearance of motor symptoms. This clinical phase of PD can be preceded by a premotor preclinical phase which is characterised by the presence of non-motor symptoms. PD (Parkinson’s disease). See Kalia and Lang (2015).

Another pathological hallmark of PD is the presence of Lewy bodies and Lewy neurites in surviving neurons (Spillantini et al., 1997). These intracytoplasmic protein aggregates are predominantly composed of fibrillar forms of α-synuclein (α-syn) and can be found in several affected brain regions of patients suffering from the disease. Given the widespread distribution of Lewy bodies throughout the brain, PD is defined as Lewy body disease and categorised as α-synucleinopathy (Ferrer et al., 2012; Goedert et al., 2013). Of note, protein aggregation is supposed to be a key contributor to the progressive neurodegeneration observed in PD (McNaught and Olanow, 2006). In fact, there is increasing evidence demonstrating cell-to-cell transmission and spreading of pathological α-syn aggregates in a prion-like manner as a common mechanism for the progressiveness of PD (Herva and Spillantini, 2015; Olanow and Brundin, 2013; Recasens and Dehay, 2014).

Figure 1: Time course of PD symptoms. Diagnosis of PD occurs with the appearance of motor symptoms. This clinical phase of PD can be preceded by a premotor preclinical phase which is characterised by the presence of non-motor symptoms. PD (Parkinson’s disease). See Kalia and Lang (2015).
1.1.2 Etiology, pathological mechanisms and genetics of PD

The precise etiology and pathogenesis of PD remain largely elusive. In the majority of cases, PD is regarded as a sporadic disease of a yet unknown reason. Several theories concerning the pathophysiological pathways of the neuronal cell death are discussed, including altered protein handling, aggregation and spreading of misfolded α-syn, mitochondrial dysfunction, oxidative stress as well as neuroinflammation (Dexter and Jenner, 2013; Hirsch et al., 2013; Schapira and Jenner, 2011). However, epidemiological studies reveal that about 10 % of PD patients exhibit a familial history, suggesting an inherited disorder. Mutations in different genes such as α-syn, leucine-rich repeat kinase 2 (LRRK2), vacuolar protein sorting 35 (VPS35), eukaryotic translation initiation factor 4 gamma 1 (EIF4G1), parkin, PTEN-induced putative kinase 1 (PINK1) and DJ-1 have conclusively been demonstrated to cause familial parkinsonism (Bonifati, 2014; Houlden and Singleton, 2012; Puschmann, 2013; Singleton et al., 2013; Spatola and Wider, 2014). Since both forms of the disease share common clinical and neuropathological properties, defining the molecular mechanisms underlying familial PD will be relevant to sporadic PD as well.

1.1.3 Treatment strategies for PD

Currently, most treatment strategies for PD are symptomatic approaches and rely on restoration of the dopaminergic neurotransmission within the basal ganglia network. The pharmacological pipeline for the treatment of PD offers various dopaminergic drugs such as DA receptor agonists or L-3,4-dihydroxyphenylalanine (L-DOPA). Additionally, compounds affecting the enzymatic metabolism of DA such as monoamine oxidase B (MAO-B) or catechol-O-methyltransferase (COMT) inhibitors are administered in combination with L-DOPA improving its efficacy as well as increasing the availability of DA (LeWitt, 2008; Schapira and Olanow, 2008; Seeberger and Hauser, 2007).

A widely used DA receptor agonist of the non-ergot subtype is pramipexole (PPX). It offers high affinity for the DA D₃ receptor followed by the DA D₂ receptor, thereby producing beneficial effects on motor function, activities of daily living and quality of life (Ferger et al., 2010; Mierau et al., 1995; Piercey, 1998; Schapira and Olanow, 2008). PPX has been approved by the Food and Drug Administration
Introduction

(FDA) and other regulatory authorities for the treatment of early and advanced stages of PD in 1997 (Ferger et al., 2010; Lieberman et al., 1997; Shannon et al., 1997). Since this time, PPX has become one of the most commonly prescribed DA receptor agonists among PD patients and is in use as monotherapy as well as in combination with L-DOPA (Ferger et al., 2010; Fox et al., 2011).

None of the multiple drugs available for the treatment of PD have surpassed the therapeutic benefit provided by L-DOPA (Schapira and Olanow, 2008). Indeed, since its introduction more than 50 years ago, oral L-DOPA continues to be the most effective and widely used pharmacotherapy for PD, which initially improves the cardinal DA-related motor symptoms of the disease (Hornykiewicz, 2010; LeWitt, 2008; Olanow and Schapira, 2013; Schapira and Olanow, 2008; Sprenger and Poewe, 2013). However, a major limitation of the long-term use of L-DOPA is the development of disabling motor complications such as dyskinesia (Buck and Ferger, 2010), which is characterised by abnormal involuntary movements mainly comprising chorea and dystonia (Brotchie et al., 2005; Sharma et al., 2015). Dyskinesia emerges progressively over time, and about 40 % of PD patients suffer from dyskinesia within four to six years of L-DOPA treatment (Ahlskog and Mueenter, 2001; Mazzucchi et al., 2015).

Amantadine

Amantadine, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, has been discovered to be a potential treatment for PD motor symptoms in 1969 (Schwab et al., 1969). At present, amantadine has emerged as the most effective compound to alleviate L-DOPA-induced dyskinesia in patients suffering from PD (Fahn, 2008; Pilleri and Antonini, 2015; Schaeffer et al., 2014).

It has been previously shown that the development of L-DOPA-induced motor complications in PD is associated with an overactivation of excitatory glutamatergic pathways within the basal ganglia network (Brotchie, 2005; Chase et al., 2000; Verhagen Metman et al., 1998a; Verhagen Metman et al., 1998b). Thus, amantadine has long been considered to alleviate dyskinesia by blocking glutamatergic NMDA receptors. However, amantadine is also thought to block serotonin (5-HT) uptake and induce 5-HT overflow, which may consequently improve dyskinesia via stimulation of 5-HT1A autoreceptors (Baptista et al., 1997; Bishop et al., 2009; Paquette et al., 2012).
Amantadine is not equally effective in all PD patients and the drug is not always tolerated well due to the development of central side effects including hallucinations and confusion in some patients (Crosby et al., 2003). Additionally, the duration of the possible antidyskinetic effect is controversially ranging from a transient effect of several months (Paci et al., 2001; Stocchi et al., 2008; Thomas et al., 2004) to a long-lasting improvement of at least one year (Verhagen Metman et al., 1999) and even more than four years (Wolf et al., 2010).

**Continuous dopaminergic stimulation**

The concept of continuous dopaminergic stimulation (CDS) has received considerable attention as a therapeutic treatment approach for PD. Evidence suggests that motor complications in PD are related to therapy with short-acting dopaminergic drugs which induce non-physiological intermittent stimulation of striatal DA receptors that are normally tonically stimulated (Chase et al., 1989; Jenner, 2008c; Senek and Nyholm, 2014). This pulsatile stimulation leads to molecular and pathophysiological changes in the basal ganglia pathways and the development of motor complications such as dyskinesia (Senek and Nyholm, 2014). The strategy of CDS postulates that it is desirable to achieve continuous and more physiological dopaminergic stimulation, resulting in higher therapeutic benefit due to a lower propensity to develop motor complications (Jenner, 2008c; Olanow et al., 2006). Pharmacologically, CDS is attempted by strategies including the development of DA receptor agonists or L-DOPA formulations with longer plasma half-lives as well as continuous drug delivery, eventually preventing unwanted effects related to fluctuations in brain and plasma drug levels (Chaudhuri et al., 2013; Ferger et al., 2010; Jenner, 2013). 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 (Ferger et al., 2010; Kvernmo et al., 2006).

Despite the therapeutic progress achieved within the past decades, PD continues to be a progressive disease leading to severe disability. Beyond the symptomatic alleviation, there is still no therapy available which can halt or slow the progression of PD, and the development of neuroprotective and disease-modifying treatment
strategies remains an urgent, unmet clinical need (Kalia and Lang, 2015; Meissner et al., 2011; Valera and Masliah, 2015).

1.1.4 Biomarkers of PD

In 2001, the Biomarkers Definitions Working Group has defined ‘biomarkers’ as characteristics that can be objectively measured and evaluated as indicators of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001). This definition comprises clinically quantifiable parameters, biochemical assays or neuroimaging markers. In this regard, PD research is dealing with the investigation of potential biomarkers that can be used to aid in diagnosis, identify at-risk individuals, monitor disease progression and evaluate responses to therapeutic interventions (Saracchi et al., 2014; Schapira, 2013; Sharma et al., 2013). An example for a common clinically used neuroimaging biomarker is DA transporter single-photon emission computed tomography (SPECT) (Arena and Stoessl, 2016; Suwijn et al., 2015). This technique is effective in detecting nigrostriatal cell loss in parkinsonian patients and facilitates diagnostic accuracy.

The present work will focus on the investigation and analysis of the amino acid biomarkers γ-aminobutyric acid (GABA), glutamate and glycine as well as premotor biomarkers of the preclinical phase of PD.

GABA and glutamate

GABA and glutamate are the most prominent amino acid neurotransmitters in the central nervous system (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). 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 and learning as well as synaptic plasticity (Hertz, 2006). 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 systems of the basal ganglia circuits play a key role. The basal ganglia comprise the direct pathway projecting from the striatum to the globus pallidus internus (GPI) and the substantia nigra pars reticulata (SNPr) as well as the indirect pathway connecting the striatum with the GPI/SNPr via synaptic projections to the globus pallidus externus (GPE) and subthalamic nucleus (STN) (Alexander and Crutcher, 1990; Jenner, 2008b; Sharma et al., 2015). The major inputs into the basal ganglia are nigrostriatal dopaminergic as well as corticostriatal glutamatergic projections. The outputs from the basal ganglia are largely GABAergic (Jenner, 2008b). There is increasing evidence that changes related to GABA and glutamate occur during the appearance of L-DOPA-induced dyskinesia. Indeed, enhanced signalling of glutamatergic corticostriatal projections as well as overactivation of the GABAergic striatopallidal synapse seem to be involved (Brotchie et al., 2005; Jenner, 2008b). Using the technique of in vivo microdialysis, it is possible to monitor GABA and glutamate in biomarker studies, which is important for the investigation of neurochemical alterations in experimental models of PD and L-DOPA-induced dyskinesia.

**Glycine**

Glycine is the simplest amino acid and acts as an inhibitory neurotransmitter in the spinal cord, brainstem and several other areas of the CNS (Bowery and Smart, 2006; Legendre, 2001; Rampon et al., 1996). In addition to its inhibitory effect at ionotropic glycine receptors, glycine activates excitatory NMDA receptors, where it acts as a co-agonist to glutamate (Danysz and Parsons, 1998; Johnson and Ascher, 1987). Two different forms of glycine transporters (GlyT) can be found in glial and neuronal plasma membranes, GlyT1 and GlyT2 (Aragón and López-Corcuera, 2003; Gomeza et al., 2003; Harsing, Jr. and Matyus, 2013; Zafra et al., 1997). The inhibition of GlyT1 increases extracellular glycine levels by blocking its neuronal or glial reuptake resulting in enhanced NMDA receptor activity. Thus, GlyT1 inhibitors may prove useful in the treatment of diseases associated with NMDA receptor hypofunction such as schizophrenia (Chue, 2013; Coyle et al., 2012; Hashimoto, 2010; Javitt, 2012). Moreover, GlyT1 inhibitors are reported to
promote striatal dopaminergic axon sprouting via NMDA receptors expressed by dopaminergic neurons, which provides a clinical treatment option for disorders such as PD (Schmitz et al., 2013). These findings underpin the role of glycine to be a promising biomarker for the development of antipsychotic as well as antiparkinsonian drugs (Pich et al., 2012; Voehringer et al., 2013). In order to study neurochemical alterations in experimental models of PD or schizophrenia as well as biomarker studies, glycine levels can be measured in cerebrospinal fluid (CSF) samples and monitored via in vivo microdialysis (Alberati et al., 2012; Boulay et al., 2008; Nagy et al., 2010; Perry et al., 2008; Pinard et al., 2010).

**Premotor biomarkers**

Converging evidence suggests that the neurodegenerative process in PD begins years or even decades before the appearance of motor disturbances (Marek and Jennings, 2009; Meissner, 2012). During this premotor period, compensatory mechanisms of the brain are able to buffer the loss of nigrostriatal dopaminergic neurons and mask the onset of motor symptoms which become clinically evident for diagnosis when about 75 % of the nigrostriatal cells are lost (Lloyd, 1977; Visanji and Marras, 2015). Additionally, this premotor phase is characterised by the presence of several non-motor features such as olfactory dysfunction representing early symptomatic manifestations of the disease. Figure 2 shows a simplified time course of PD linking neuronal loss with the onset of motor signs and clinical diagnosis.

Identifying biomarkers in the premotor stage of PD is of major importance for early diagnosis and modulation of disease progression, since dopaminergic neurons are relatively spared and potential neuroprotective or disease-modifying therapies are expected to have their greatest impact.
**Figure 2: Time course of PD representing the premotor and clinical phase of the disease.**

The premotor phase of PD is characterised by decreasing function of nigrostriatal dopaminergic neurons. Clinical diagnosis of PD can be made when about 75 % of nigrostriatal cells are lost and motor symptoms become evident. Biomarkers of the premotor phase would identify PD before significant neuronal damage has occurred and potential neuroprotective or disease-modifying therapies could halt or slow disease progression. PD (Parkinson's disease). See Marek and Jennings (2009).

### 1.2 Non-motor symptoms in PD

As mentioned above, non-motor signs are becoming increasingly recognised as symptoms of PD (Modugno et al., 2013). Some of them, mainly olfactory deficits, sleep disturbances, mood disorders and constipation, are already present in early disease stages before the onset of motor disturbances and largely contribute to the reduced quality of life and disability associated with the disease (Pont-Sunyer et al., 2015; Visanji and Marras, 2015).
1.2.1 Olfactory dysfunction

Olfactory dysfunction is among the earliest manifestations in PD (Doty, 2012a; Haehner et al., 2007; Ruan et al., 2012) and comprises impairments in odour detection, identification and discrimination (Doty et al., 1988; Doty, 2012b; Tissingh et al., 2001). Total anosmia is rare, and many patients suffering from PD are not aware of the dysfunction until their smelling behaviour is formally tested. There are several methods used to assess the integrity of the olfactory system such as the University of Pennsylvania Smell Identification Test (UPSIT) or the Sniffin' Sticks test (Doty et al., 1984; Hummel et al., 1997). These procedures are frequently applied in clinical settings for an extended diagnosis of PD.

The prevalence of olfactory dysfunction in PD is high (Doty et al., 1988; Haehner et al., 2009), and the deficit seems to be unrelated to the duration or severity of the disease (Doty et al., 1988; Modugno et al., 2013). Medications used to alleviate the motor symptoms of PD such as L-DOPA or DA receptor agonists provide no benefit regarding smell loss, and the development of efficient treatment strategies targeting the whole spectrum of non-motor symptoms represents a major therapeutic challenge in PD (Modugno et al., 2013).

It is known that hyposmia can predate clinical PD by four years at least (Ross et al., 2008). Moreover, several studies demonstrate that olfactory deficits are associated with a greater probability of developing PD (Ponsen et al., 2004; Ross et al., 2008). Therefore, olfactory dysfunction is considered to have a promising role as a premotor biomarker for PD, allowing the identification of patients with an increased risk for future disease (Haas et al., 2012; Kranick and Duda, 2008; Morley and Duda, 2010; Postuma et al., 2012; Xiao et al., 2014).

1.2.2 Pathophysiology

The causative mechanisms accounting for the non-motor symptoms in PD remain complex, since pathological changes occur in widespread regions of the central and peripheral nervous system involving disturbances in dopaminergic, serotonergic, noradrenergic as well as cholinergic neurotransmitter systems (Goldman and Postuma, 2014; Sung and Nicholas, 2013). Many of the non-motor features are related to broad distribution of α-syn and Lewy bodies outside the substantia nigra (SN) (Jellinger, 2011). In 2003, Braak and colleagues proposed a
staging scheme for the progression of α-syn pathology in PD, which is reported to start in the lower brainstem and olfactory structures and progress in a caudal-rostral manner (Braak et al., 2003) (Figure 3). In later disease stages, α-synucleinopathy also involves the nigrostriatal motor complex resulting in the manifestation of movement disturbances. The Braak staging scheme illustrates that the bulbus olfactorius (BO) and the associated anterior olfactory nucleus (AON) are among the first brain regions exhibiting neuropathological changes in the form of Lewy bodies and α-syn aggregates (Braak stage 1) (Braak et al., 2003; Daniel and Hawkes, 1992; Pearce et al., 1995). This may explain smell loss as an early sign of PD, occurring preclinically before the classical motor symptoms (Hoyles and Sharma, 2013).

Figure 3: Staging of α-synuclein pathology according to the Braak model. PD-related α-synuclein pathology advances in predictable stages. Lewy bodies first appear in the bulbus olfactorius, the associated anterior olfactory nucleus and the dorsal motor nucleus of the vagal nerve (stage 1). Lewy pathology then spreads into further brainstem nuclei (stage 2) and the midbrain and basal forebrain (stages 3 and 4). In later disease stages (stages 5 to 6), pathology reaches the cerebral cortex. First clinical symptoms occur during stage 3 or early stage 4 when α-synuclein pathology involves significant regions of the substantia nigra and related brain areas. PD (Parkinson's disease). Reprinted from Thal et al. (2004) and Braak et al. (2003), Neurobiology of Aging, Vol. 24, Pages No. 197-211, Copyright (2015), with permission from Elsevier.
1.3 Animal models of PD

In order to better understand the molecular mechanisms underlying the pathophysiology of PD as well as to develop novel therapeutic strategies, scientists focus on producing accurate animal models which parallel the human disorder remarkably well. For the past several decades, animal models of PD have come in a variety of forms. Typically, they can be divided into those using pharmacological or neurotoxic agents, or those utilising the in vivo expression of PD-related genetic mutations (Blesa and Przedborski, 2014; Le et al., 2014).

1.3.1 Pharmacological models

Two common functional models of PD rely on administration of haloperidol or reserpine and are important to predict the efficacy of novel symptomatic therapies (Duty and Jenner, 2011). Both models are considered as pharmacological, symptomatic animal models of PD, producing specific features of the disease without reflecting nigrostriatal neurodegeneration (Ribeiro et al., 2013).

Haloperidol is known to induce extrapyramidal PD symptoms such as catalepsy and muscular rigidity (Alvarez-Fischer et al., 2002; Gerlach and Riederer, 1996; Lorenc-Koci et al., 1996). Cataleptic behaviour can be defined as a state in which an animal cannot correct an abnormal or unusual posture back to its normal position (Sanberg et al., 1988). Haloperidol is a classical antipsychotic neuroleptic drug most frequently prescribed for the management of schizophrenia (Joy et al., 2006; Settle, Jr. and Ayd, Jr., 1983). It blocks the nigrostriatal DA transmission at postsynaptic DA D\textsubscript{2} receptors (Ellenbroek et al., 1985; Sanberg, 1980), thereby causing adverse events such as parkinsonism, which is often utilised as an animal model for screening antiparkinsonian drugs (Ferger et al., 2010; Maj et al., 1997).

Treatment with reserpine leads to PD-resembling motor behaviour symptoms such as akinesia, tremor and rigidity (Betarbet et al., 2002; Colpaert, 1987; Lorenc-Koci et al., 1995). Unlike haloperidol, reserpine acts presynaptically by blocking the uptake of monoamines by the vesicular monoamine transporter 2 (VMAT-2) (Duty and Jenner, 2011). This inhibition unselectively affects the storage of monoamine neurotransmitters such as noradrenaline, DA and serotonin in the brain as well as
in the periphery (Carlsson et al., 1957; Dolphin et al., 1976). Although not specific to a single neurotransmitter pathway and without involvement of pathological neurodegenerative changes within the substantia nigra, the reserpine model still remains a tool of high convenience for the screening of potential symptomatic PD medications including L-DOPA and DA receptor agonists (Betarbet et al., 2002; Ferger et al., 2010; Gossel et al., 1995; Leao et al., 2015; Maj et al., 1997).

1.3.2 Neurotoxic models

Neurotoxic models of PD aim at reproducing the pathological and behavioural changes of the human disease and are based on the irreversible degeneration of nigrostriatal dopaminergic neurons as well as DA deficiency in the striatum. 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone and paraquat are the most widely used toxic compounds modelling PD in rodents and nonhuman primates, particularly by inducing cytotoxicity through mitochondrial inhibition and oxidative stress mechanisms (Ribeiro et al., 2013). However, these models induce acute effects which differ significantly from the slowly progressive pathology observed in human PD.

The 6-OHDA model is frequently used to replicate a PD-like damage of the nigrostriatal dopaminergic pathway in rats. Since 6-OHDA is not able to cross the blood-brain barrier, it has to be directly injected into the SN, medial forebrain bundle (MFB) or striatum via stereotaxic surgery. 6-OHDA has high affinity to the DA transporter which carries the toxin inside the dopaminergic neurons. There, 6-OHDA causes massive oxidative stress and mitochondrial dysfunction (Blandini et al., 2008; Ungerstedt, 1968). The 6-OHDA-lesioned rat provides a well-established model for PD, mimicking nigral dopaminergic cell loss, DA depletion as well as neurobehavioural motor deficits, but no Lewy bodies (Blesa et al., 2012). Additionally, administration of L-DOPA to 6-OHDA-lesioned rats causes debilitating abnormal involuntary movements which resemble dyskinesia as seen in humans (Cenci et al., 1998). Therefore, the 6-OHDA rat model proves useful for the investigation of the mechanisms responsible for dyskinesia following chronic treatment with L-DOPA (Buck and Ferger, 2010; Morin et al., 2014).
Administration of MPTP to mice and nonhuman primates represents a further specific and reproducible model for PD, replicating most of the key features of the disease including parkinsonian motor symptoms. Due to its high lipophilicity, MPTP readily crosses the blood-brain barrier upon systemic administration (Markey et al., 1984). Inside the brain, MPTP is converted into its active toxic metabolite 1-methyl-4-phenylpyridinium ion (MPP+) by the enzyme MAO-B. MPP+ is taken up by dopaminergic neurons via the DA transporter and causes damage to the nigrostriatal dopaminergic pathway by inhibiting the complex I component of the mitochondrial respiratory chain, resulting in oxidative stress and cell death (Blesa et al., 2012; Langston, 1987).

Epidemiological studies suggest an association between exposure to environmental toxins such as the pesticides rotenone and paraquat and the incidence of PD (Cicchetti et al., 2009). Therefore, several animal models of the disease are based on administration of these toxic compounds. Rotenone easily enters brain dopaminergic neurons independent of the DA transporter. Once inside the cell, rotenone provokes degenerative effects as a result of mitochondrial toxicity and oxidative stress events (Betarbet et al., 2000; Cicchetti et al., 2009). Chronic systemic exposure to rotenone in rats mimics specific features of PD including motor deficits as well as α-syn-positive cytoplasmic inclusions (Blesa and Przedborski, 2014). However, this rotenone model is reported to simulate atypical parkinsonism rather than the idiopathic form of the disease (Höglinger et al., 2006).

Paraquat, whose chemical structure is similar to MPP+, is reported to induce selective degeneration of nigral dopaminergic neurons (Cicchetti et al., 2009; McCormack et al., 2002). Carrier-mediated transport via the neutral amino acid transporter facilitates the entry into the brain, where paraquat elicits mitochondrial toxicity as well as oxidative damage.
1.3.3 Genetic models

During the last two decades, the involvement of genes in familial forms of PD has attracted increasing attention. This led to the generation of novel animal models based on genetic manipulations, which allow a detailed analysis of pathophysiological mechanisms occurring in PD and provide an outstanding potential for the development of new treatment strategies.

The first genetic mutation causing a familial type of PD has been found in the α-syn gene (Polymeropoulos et al., 1997) (Figure 4). To date, several missense mutations of α-syn (A30P, A53T, E46K, H50Q, G51D and A53E) are known to be linked to an autosomal dominant form of the disease (Krüger et al., 1998; Lesage et al., 2013; Pasanen et al., 2014; Polymeropoulos et al., 1997; Proukakis et al., 2013; Zarranz et al., 2004). Additionally, both duplications and triplications of the wild-type α-syn gene have been found to elicit familial PD, indicating that overexpression of the native protein itself can trigger a neurodegenerative process (Chartier-Harlin et al., 2004; Singleton et al., 2003). Since aggregation of α-syn is believed to play an essential role in the pathogenesis of PD, many animal models are based on overexpression of this protein in wild-type or mutant forms (Bezard et al., 2013; Löw and Aebischer, 2012).

![Figure 4: Schematic representation of the human α-synuclein gene. α-synuclein is a small protein of 140 amino acids and can be divided into three distinct regions: the N-terminal domain (residues 1-60), the central region termed NAC (residues 61-95) and the C-terminal domain (residues 96-140). The six missense mutations in the α-synuclein gene that are associated with dominantly inherited forms of PD are located in the N-terminal region. N (amino), C (carboxy), NAC (non-amyloid component), PD (Parkinson's disease). See Gallegos et al. (2015).](image)
Several α-syn transgenic mice have been developed so far (Magen and Chesselet, 2010). However, whilst many of these accurately replicate the α-syn neuropathology, in some cases accompanied by behavioural abnormalities, significant nigrostriatal degeneration has often not been observed (Duty and Jenner, 2011). Transgenic models carrying mutations in the LRRK2 gene, as well as models based on knockout of genes encoding parkin, PINK1 or DJ-1, equally fail to show considerable pathology in nigrostriatal structures in the majority of cases (Blesa and Przedborski, 2014).

Overall, transgenic mouse models have contributed enormously to the understanding of the mechanisms underlying PD, since they are able to recapitulate specific pathological features of the illness including Lewy body formation. However, the absence of appreciable dopaminergic neurodegeneration and no consistent phenotypes remain a major limitation (Dawson et al., 2010).

Another successful tool to model PD in rodents and nonhuman primates is viral vector-mediated overexpression of α-syn (Löw and Aebischer, 2012). Viral vectors provide a unique instrument to express disease-causing proteins in specific brain regions with high efficiency. In PD research, this technology has been successfully applied for many years to mimic some of the core symptoms of the disease such as nigral dopaminergic cell loss, motor impairment as well as α-syn cytoplasmic inclusions (Bezard et al., 2013; Kirik et al., 2002; Van der Perren et al., 2015).

Figure 5 represents a schematic overview of the molecular mechanisms involved in the development of animal models of PD by the use of pharmacological and neurotoxic agents as well as genetic manipulations.
Figure 5: Molecular mechanisms of pharmacological agents, neurotoxins and genetic manipulations used to develop animal models of PD. The drawing shows the cite of action of pharmacological and neurotoxic agents as well as genetic manipulations at a dopaminergic neuron with its cell body in the substantia nigra and its terminal in the striatum. (1) Haloperidol blocks the nigrostriatal DA transmission by inhibiting postsynaptic DA D$_2$ receptors. (2) Reserpine blocks the uptake of DA via the vesicular monoamine transporter 2, which affects the storage of DA and results in striatal DA deficiency. (3) 6-OHDA is taken up by the neuron via the DA transporter. There, it causes massive oxidative stress and mitochondrial dysfunction. (4) MPTP is converted to MPP$^+$ by MAO-B. MPP$^+$ is taken up by the neuron via the DA transporter and accumulates in mitochondria, leading to complex I inhibition and the generation of free radicals. (5) Rotenone is a direct inhibitor of complex I in mitochondria, which also leads to free radical generation. (6) The toxic mechanism of paraquat involves mitochondrial toxicity as well as oxidative damage. (7) The expression of α-synuclein can be increased in transgenic animal models or via viral vector-mediated overexpression, leading to protein aggregation and neuronal degeneration. DA (dopamine), 6-OHDA (6-hydroxydopamine), MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), MPP$^+$ (1-methyl-4-phenylpyridinium ion), MAO-B (monoamine oxidase B), PD (Parkinson’s disease). Modified from Betarbet et al. (2002), BioEssays, Vol. 24, Pages No. 308-318, Copyright (2015), with permission from John Wiley and Sons.
1.3.4 Validation of animal models

As already mentioned, animal models of PD serve as valuable tools for experimental scientists in their ambition to elucidate the underlying pathophysiological mechanisms as well as in their attempts to design new and more efficient treatment strategies for the disease (Blesa and Przedborski, 2014; Le et al., 2014). However, animal models prove to be useful just to the extent to which they properly simulate the pathological, histological, biochemical as well as functional characteristics of the disorder (Duty and Jenner, 2011; Jenner, 2008a). An ideal animal model of PD would preferably show both the behavioural as well as pathophysiological features of the disease, including motor and non-motor disturbances, progressive degeneration of dopaminergic and non-dopaminergic neurons as well as Lewy body-like inclusions. It should meet the following crucial requirements: 1) face validity which reflects the analogy in symptoms and pathological features between the animal model and human patients, 2) construct validity which shares etiology and underlying pathophysiological mechanisms and 3) predictive validity which suggests that treatment strategies that are effective in the model will also be successful in human patients (Morsink and Dukers, 2009; van der Staay et al., 2009). An example of characteristics for the validation of an ideal animal model of PD is illustrated in Figure 6.

![Diagram of validation criteria](image)

**Figure 6:** Example of characteristics for the validation of an ideal animal model of PD. DA (dopamine), PD (Parkinson's disease). See Chesselet and Richter (2011).
The results obtained in preclinical animal models are translated into the situation in humans, which emphasises the importance of a high degree of concordance between preclinical models and the clinical setting of the disease. Despite the significant contribution of all of the currently available animal models to the understanding of the pathophysiology of PD as well as the development of novel therapeutic strategies, none of these models are able to exactly reproduce the complexity of the human disease, primarily because they lack some specific neuropathological and/or behavioural features (Blesa and Przedborski, 2014). In particular, the investigation and recapitulation of non-motor symptoms in experimental animal models of PD are less well defined.

There is a pressing need for novel animal models of PD encompassing many characteristics of the disease with high validity. In this regard, it is urgently required that the existing animal models of the disorder are continuously refined and optimised. Then, animal models attain a state of good transferability from their preclinical aspect to clinical efficacy and allow investigation of novel therapeutic strategies as a prerequisite for the testing in humans.
1.4 Aim of the study

Demographic changes towards an aging population will increase the prevalence of PD in the future. In addition to the typical motor phenotype, PD patients also suffer from multiple non-motor symptoms such as olfactory dysfunction, which largely contribute to the reduced quality of life and disability associated with the disease. Currently, there is no therapy available which can halt or slow the progression of PD. The development of neuroprotective and disease-modifying drugs requires the presence of reliable biomarkers to monitor disease progression and to identify potential treatment effects.

Animal models of PD are central tools for gaining an improved understanding of the pathophysiology of the disease as well as testing potential therapeutics. However, the success rate of translating preclinical results into the clinical situation relies substantially on the extent to which the experimental models recapitulate the disease condition as seen in humans. Unfortunately, there is no ideal animal model of PD which is able to exactly reproduce all features of the human disease, including motor and non-motor symptoms. Therefore, it is of utmost importance to create new as well as improve existing animal models of the disease.

The overriding goal of my thesis was the investigation of in vivo animal models of PD in view of supporting translational research in medicine and to uncover the role of olfactory dysfunction as an early biomarker in a genetic adeno-associated virus (AAV)-based rat model of PD.

In particular, the following specific aims defined the basis of the present work. I validated and refined the symptomatic animal models of haloperidol-induced catalepsy in rats as well as reserpine-induced akinesia in mice by behavioural and neurochemical measurements. These models have also been used to study the concept of CDS as a treatment strategy for PD. Furthermore, I investigated the pharmacokinetic (PK)-pharmacodynamic (PD) relationship of amantadine in the 6-OHDA dyskinesia as well as haloperidol-induced catalepsy model in rats.

Regarding biomarker evaluation, I generated an AAV-α-syn-overexpression model in rats which demonstrates olfactory dysfunction as a premotor biomarker for progressive PD. Moreover, I developed two liquid chromatography tandem mass
spectrometry (LC-MS/MS) methods for the quantification of GABA and glutamate as well as glycine as biomarkers in brain microdialysis and CSF samples.

Figure 7 shows the relationship of the studies in my PhD thesis in a graphical abstract.

**Figure 7:** Graphical abstract showing the relationship of the studies in my PhD thesis. GABA (γ-aminobutyric acid), AAV (adeno-associated virus), 6-OHDA (6-hydroxydopamine).
2. Materials and methods

2.1 In vivo experiments

2.1.1 Animals

The present studies were conducted in male Wistar rats (RjHan:WI, Janvier, Le Genest St Isle, France), weighing 220-250 g at the beginning of the experiments. The animals were housed in groups of four per cage under a 12 h light/dark cycle (lights on 06:00 - 18:00) in temperature (22 ± 2 °C) and humidity (55 ± 10 %) controlled rooms with free access to food (GLP Vitamin fortified, Provimi Kliba AG, Kaiseraugst, Switzerland) and water throughout the experiments, except during olfactory testing (see below).

Experiments concerning the reserpine mouse model were conducted in male C57BL/6 mice (C57BL/6JRj, Janvier, Le Genest St Isle, France), weighing 22-25 g at the beginning of the study. The housing conditions for mice were the same as for rats.

All in vivo studies were approved by the appropriate institutional governmental agency (Regierungspraesidium Tuebingen, Germany, permission reference number 35/9185.83) 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.

2.1.2 Exposure measurements in plasma, brain and CSF samples

The exposure of haloperidol, reserpine, PPX and amantadine to plasma, brain and CSF was investigated in rats. Each compound was tested in a separate experiment.

Haloperidol (3 mg/kg, intraperitoneally (i.p.)), reserpine (3 mg/kg, i.p.), PPX (3 mg/kg, i.p.) and amantadine (3 and 30 mg/kg, i.p.) were administered to naïve rats, respectively, and the exposure to plasma, brain and CSF was determined at different time points (n=5 for each compound/dose and time point). 0.5 h, 1 h, 2.5
h and 5 h following administration, 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 Instruments, Tujunga, CA, USA). Subsequently, samples of CSF, blood and brain tissue were taken. CSF was firstly collected via puncture of the cisterna magna followed by removal of blood via punctuation of the heart. Blood samples were taken using S-Monovette® containing 1.6 mg ethylenediaminetetraacetic acid (EDTA)/ml blood (Sarstedt AG & Co., Nümbrecht, Germany) and gently shaken. Plasma was obtained by centrifugation of the blood at 3200 x g for 15 min at 4 °C (Centrifuge 5810R, Eppendorf, Hamburg, Germany). Afterwards, the rats were transcardially perfused with Ringer's solution for 3 min. After decapitation, the brain was removed, transferred into plastic tubes and weighed. Brain tissue was homogenised in water (1:7) using sonication (Bandelin Sonopuls, Bandelin electronic, Berlin, Germany).

Ten µl aliquots of CSF, plasma and brain tissue samples, respectively, were diluted with 10 µl of a mixture of acetonitrile/water (80:20) and 80 µl of a mixture of methanol/acetonitrile (50:50) in order to precipitate proteins. The samples were mixed and kept at -20 °C for at least 15 min to improve precipitation. Subsequently, samples were centrifuged at 3200 x g for 15 min at 4 °C and the supernatants were frozen at -80 °C prior to LC-MS/MS analysis.

2.1.3 Stereotaxic surgeries and in vivo microdialysis

2.1.3.1 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 on a flat-skull position. Anaesthesia was maintained by using 0.2-2 % isoflurane in O₂. Meloxicam (0.2 mg/kg, subcutaneously (s.c.)) was used for peri- and post-operative analgesia. 6-OHDA hydrobromide (3 µg/µl of free base) was dissolved in 0.02 % ascorbate solution and injected with a flow rate of 0.3 µl/min (UltraMicroPump, World Precision Instruments Germany GmbH, Berlin, Germany) into the left MFB at the following coordinates relative to bregma and the dural surface, according to the rat brain atlas of Paxinos and Watson (1998): (1) anteroposterior (AP): - 4.4 mm, mediolateral (ML): + 1.2 mm, dorsoventral (DV): - 7.8 mm (2.5 µl); (2) AP: - 4.0 mm, ML: + 0.7 mm, DV: - 8.0 mm (2 µl) (Cenci et al., 1998).
Materials and methods

2.1.3.2 AAV-mediated overexpression of α-syn

A viral vector-based model was used to overexpress human wild-type α-syn in the BO of rats.

Adeno-associated virus 5 (AAV5) was utilised for transgene expression of human (h) wild-type (WT) α-syn under the control of the chicken beta actin (CBA) or the human synapsin-1 promoter. To enhance gene expression, a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was included into one virus construct (AAV5-CBA-h-α-syn-WT and AAV5-synapsin-1-WPRE-h-α-syn-WT). The injected titers for AAV5-CBA-h-α-syn-WT and AAV5-synapsin-1-WPRE-h-α-syn-WT were 1.0 x 10^{13} and 3.1 x 10^{13} vector genomes (vg)/ml, respectively. The effects of these viral vectors were compared to an AAV5-CBA-green fluorescent protein (GFP) control vector (9.5 x 10^{12} vg/ml). Phosphate-buffered saline (PBS) served as an overall control.

Rats were anaesthetised with a mixture of medetomidine (150 µg/kg, intramuscularly (i.m.)), midazolam (2 mg/kg, i.m.) and fentanyl (5 µg/kg, i.m.) and mounted in a stereotaxic frame on a flat-skull position. Meloxicam (0.2 mg/kg, s.c.) was used for peri- and post-operative analgesia. The viral vector solutions or PBS (n=5) were injected into the BO using the following coordinates relative to bregma, according to the rat brain atlas of Paxinos and Watson (1998): AP: + 6.5 mm, ML: ± 1.0 mm, DV: - 4.0 mm. Injections were carried out bilaterally (2 x 1 µl, 0.1 µl/15 sec), and the needle was left in place for additional 5 min before it was slowly retracted. The anaesthesia was antagonised with a mixture of atipamezole (750 µg/kg, s.c.), flumazenil (200 µg/kg, s.c.) and naloxone (120 µg/kg, s.c.).

2.1.3.3 In vivo microdialysis surgeries and procedures

The first in vivo microdialysis experiment was performed in the reserpine mouse model in order to investigate the effect of reserpine on extracellular DA levels in the striatum of mice. The animals were anaesthetised with a mixture of ketamine (50 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) and mounted in a stereotaxic frame on a flat-skull position. Anaesthesia was maintained by using 0.2-2 % isoflurane in O_2. Meloxicam (0.2 mg/kg, s.c.) was used for peri- and post-operative analgesia. An intracerebral guide cannula (MAB 4.9.IC, Microbiotech, Stockholm, Sweden) was
implanted unilaterally aiming at the striatum at the following coordinates relative to bregma: AP: + 1.0 mm, ML: + 2.0 mm, DV: -2.0 mm (from dura), according to the mouse brain atlas of Paxinos and Franklin (1997). A hole was drilled for the placement of the guide cannula, which was fixed to the skull with two stainless steel screws and dental cement (PermaCem, DMG Chemisch-Pharmazeutische Fabrik GmbH, Hamburg, Germany). Following surgery, mice were allowed to recover for at least 24 h before performing the in vivo microdialysis procedure.

On the day of the experiment, concentric microdialysis probes (MAB 4.9.2.Cu, 2 mm membrane length, 0.24 mm diameter, Microbiotech, Stockholm, Sweden) were inserted into the guide cannulae of awake mice. The probes were perfused with artificial cerebrospinal fluid (aCSF) containing 147 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 0.85 mM MgCl₂ and 1 mM Na₂HPO₄, pH 7.0-7.4, at a constant flow rate of 1 µl/min. After an equilibration period of 2 h, four 30 min baseline samples were collected into a vial containing 0.1 M HCl (30 µl + 10 µl 0.1 M HCl), which were regarded as 100 % baseline. Afterwards, mice were treated with reserpine (2 mg/kg, s.c. (n=7)) or vehicle (0.5 % acetic acid in saline, s.c. (n=8)), and 60 min dialysates were collected (60 µl + 20 µl 0.1 M HCl) for 16 h. All samples were frozen at -80 °C prior to analysis using high performance liquid chromatography (HPLC) coupled to electrochemical detection (ECD). The reported data were not corrected for the in vitro recovery which was 10 % for DA. To measure the in vitro recovery, the microdialysis probe was placed in aCSF containing a defined concentration of DA (1 µmol/l). After an equilibration period of 60 min, three 20 min samples were collected from the microdialysis probe effluent and analysed by HPLC-ECD. The mean of the three samples was compared with a sample taken from the medium and expressed as % relative recovery of the medium.

The second in vivo microdialysis experiment was performed in rats and focused on the investigation of PPX and DA following PPX immediate release (PPX-IR) or PPX continuous release (PPX-CR) treatment. 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 on a flat-skull position. Anaesthesia was maintained by using 0.2-2 % isoflurane in O₂. Meloxicam (0.2 mg/kg, s.c.) was used for peri- and post-operative analgesia. An intracerebral guide cannula (MAB 4.9.IC) was implanted unilaterally aiming at the striatum at the following coordinates relative to bregma:
Materials and methods

AP: + 0.7 mm, ML: + 3.0 mm, DV: - 3.0 mm (from skull), according to the rat brain atlas of Paxinos and Watson (1998). A hole was drilled for the placement of the guide cannula, which was fixed to the skull with two stainless steel screws and dental cement. Subsequently, an ALZET® osmotic minipump (model 1007D, DURECT Corporation, Cupertino, CA) filled with PPX solution was implanted s.c. 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 two days before performing the in vivo microdialysis procedure.

On the day of the experiment, concentric microdialysis probes (MAB 4.9.4.Cu, 4 mm membrane length) were introduced into the guide cannulae. The probes were perfused with aCSF at a constant flow rate of 2 µl/min. After an equilibration period of 2 h, dialysis samples were collected every 30 min into a vial containing 10 µl of 0.1 M HCl. During the night, the sampling interval was prolonged to 60 min (20 µl of 0.1 M HCl). Fractions 1 to 4 (0 to 2 h) were used for calculation of the basal levels. After 2 h, 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 h up to the next morning. All samples were frozen at -80 °C prior to analysis using LC-MS/MS and HPLC-ECD. The reported data were not corrected for the in vitro recovery which was 12-14 % for DA and 8 % for PPX. After the experiment, the localisation of the probes was verified and only rats with appropriate probe placement were included in the experiment.

The next in vivo microdialysis experiment was performed in rats of the GABA/glutamate study. 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 on a flat-skull position. Anaesthesia was maintained by using 0.2-2 % isoflurane in O₂. Meloxicam (0.2 mg/kg, s.c.) was used for peri- and post-operative analgesia. An intracerebral guide cannula (MAB 4.9.IC) was implanted aiming at the globus pallidus (GP) at the following coordinates relative to bregma: AP: - 1.2 mm, ML: + 3.0 mm, DV: - 5.2 mm (from skull), according to the rat brain atlas of Paxinos and Watson (1998). A hole was drilled for the placement of the guide cannula, which was fixed to the skull with two stainless steel screws and dental cement. Following surgery, rats were allowed to recover for three days before performing the in vivo microdialysis procedure.
Materials and methods

The evening before the microdialysate sampling experiment, a microdialysis probe (MAB 4.9.2.Cu, 2 mm membrane length) was inserted into the guide cannula. The rats (n=4) were slightly anaesthetised with isoflurane to protect the membrane from damage. The probes were perfused with aCSF 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 min. The mean of fractions 1 to 4 (0 to 80 min) was used for calculation of the basal levels which were regarded as 100 %. During fraction 5 (80 to 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$_2$HPO$_4$, pH 7.0-7.4) was perfused for 20 min into the GP. The sampling was then continued up to fraction 14 (100 to 280 min). All samples were frozen at -80 °C prior to analysis using LC-MS/MS. The reported data were not corrected for the in vitro recovery which was 9 % for GABA and 7 % for glutamate. After the experiment, the localisation of the probes was verified and only rats with appropriate probe placement were included in the experiment.

To assess the effect of the GlyT1 inhibitor LY 2365109 on extracellular glycine levels in the rat striatum, another in vivo microdialysis experiment was performed. Rats were anaesthetised with a mixture of medetomidine (150 µg/kg, i.m.), midazolam (2 mg/kg, i.m.) and fentanyl (5 µg/kg, i.m.) and mounted in a stereotaxic frame on a flat-skull position. Meloxicam (0.2 mg/kg, s.c.) was used for peri- and post-operative analgesia. An intracerebral guide cannula (MAB 4.9.IC) was implanted unilaterally aiming at the striatum at the following coordinates relative to bregma: AP: + 0.7 mm, ML: - 3.0 mm, DV: - 3.0 mm (from dura), according to the rat brain atlas of Paxinos and Watson (1998). A hole was drilled for the placement of the guide cannula, which was fixed to the skull with two stainless steel screws and dental cement. The anaesthesia was antagonised with a mixture of atipamezole (750 µg/kg, s.c.), flumazenil (200 µg/kg, s.c.) and naloxone (120 µg/kg, s.c.). Following surgery, rats were allowed to recover for one week before performing the in vivo microdialysis procedure.

The evening before the microdialysate sampling experiment, a microdialysis probe (MAB 4.9.2.Cu, 2 mm membrane length) was inserted into the guide cannula. The rats were slightly anaesthetised with isoflurane to protect the membrane from damage. The probes were perfused with aCSF at a constant flow rate of 2 µl/min.
up to the end of the experiment. Starting on the following morning, dialysis samples were collected every 20 min. The mean of fractions 1 to 4 (-80 to -20 min) was used for calculation of the basal levels which were regarded as 100 %. After collection of the last baseline sample (-20 min), the rats were treated with the GlyT1 inhibitor LY 2365109 (10 mg/kg, perorally (p.o.), n=9) or vehicle solution (p.o., n=6). The sampling was then continued up to fraction 20 (0 to 300 min). All samples were frozen at -80 °C prior to analysis using LC-MS/MS. The reported data were not corrected for the in vitro recovery which was 10 % for glycine. After the experiment was finished, the localisation of the probes was verified and only rats with appropriate probe placement were included in the experiment.

2.1.4 Post mortem tissue preparation and CSF removal

2.1.4.1 Post mortem tissue preparation

To assess the effect of reserpine on striatal tissue DA levels as well as DA turnover in mice, animals were treated with reserpine (2 mg/kg, s.c. (n=5)) and 0 h, 1 h, 4 h, 6 h, 8 h, 16 h and 24 h later, they were sacrificed by cervical dislocation. Subsequently, the left and right striata were dissected out on an ice-cooled plate and pooled. The striata were transferred into plastic tubes, weighed, homogenised by sonication in 1000 µl 0.4 M HClO₄ and centrifuged at 3200 x g for 20 min at 4 °C. The supernatants were passed through a 0.2 µm filter (Minisart RC4, Sartorius AG, Goettingen, Germany) and frozen at -80 °C until analysis using HPLC and ECD.

The rats of the AAV study were analysed for striatal tissue DA levels as well as DA turnover. Three and 11 weeks after injection of viral vector solution or PBS into the BO, rats were sacrificed by decapitation under isoflurane anaesthesia. From three animals per group (n=3), the left striatum was dissected out on an ice-cooled plate, weighed, homogenised by sonication in 1000 µl 0.4 M HClO₄ and centrifuged at 3200 x g for 20 min at 4 °C. The supernatants were passed through a 0.2 µm filter and frozen at -80 °C until analysis using HPLC coupled to ECD. From two animals per group (n=2), the whole brain was removed and immersion-fixed in 4 % formaldehyde solution for four days at 4 °C.
2.1.4.2 CSF removal

The GlyT1 inhibitor LY 2365109 was investigated for its effect on glycine concentrations in rat CSF. The animals received either LY 2365109 (10 mg/kg, p.o., n=3) or vehicle solution (p.o., n=3). One h, 2 h and 4 h following administration, 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. CSF samples were collected by punctuation of the cisterna magna and frozen at -80 °C until analysis was performed.

2.1.5 Behavioural experiments

2.1.5.1 Catalepsy

Haloperidol-induced catalepsy is used as an animal model of extrapyramidal side effects and for screening antiparkinsonian drugs (Lorenc-Koci et al., 1996). Cataleptic immobility is regarded as an animal equivalent of akinesia and is demonstrated by an animal which cannot correct an abnormal or unusual posture back to its normal position (Sanberg et al., 1988).

In a first experiment, different routes of administration of haloperidol were compared concerning their ability to induce catalepsy in rats. The animals were treated with haloperidol (0.5 mg/kg) s.c. (n=10) and i.p. (n=10). Thirty, 60, 90 and 120 min later, cataleptic response was tested by means of the bar test. The rats were placed with their forelimbs on a horizontal bar elevated 5.5 cm from the floor and the time taken for them to move their forelimbs was measured (cut-off period 60 sec).

Additionally, plasma samples were taken from these rats. 150 min after haloperidol treatment, rats were anaesthetised with isoflurane and blood was removed by retrobulbar puncture using K₂EDTA containing capillaries (Sarstedt AG & Co., Nuembrecht, Germany). The blood was filled into microtubes also containing K₂EDTA (Sarstedt AG & Co., Nuembrecht, Germany) and gently shaken. Plasma was obtained by centrifugation of the blood at 3200 x g for 15 min at 4 °C. Plasma samples were frozen at -80 °C prior to LC-MS/MS analysis.

In a second experiment, the effect of PPX-IR and PPX-CR on haloperidol-induced catalepsy in rats was tested. Catalepsy was induced by treatment of rats with
haloperidol (0.5 mg/kg, i.p.) and maintained for 12 h by administration of haloperidol (0.1 mg/kg, i.p.) every 4 h. Three treatment groups were chosen. In the PPX-CR group (n=9), ALZET® osmotic minipumps (model 2004 or 1007D, DURECT Corporation, Cupertino, CA) filled with PPX solution were implanted s.c. under isoflurane anaesthesia the day before the catalepsy experiment. PPX was delivered continuously at a dose of 1 mg/kg/day. The PPX-IR group (n=9) was treated with PPX (1 mg/kg, s.c.) three 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 h after the bolus injection of haloperidol using the bar test. Subsequently, the PPX-IR and vehicle group (n=9) were treated with PPX (1 mg/kg, s.c.) and vehicle (saline), respectively. Catalepsy was measured 2 h, 4 h, 6 h, 8 h, 10 h and 12 h later.

In a third experiment, amantadine was investigated for its effect on haloperidol-induced catalepsy in rats. In order to induce catalepsy, rats were treated with haloperidol (0.5 mg/kg, s.c.). Sixty min later, amantadine was administered (i.p.) at doses of 3 mg/kg (n=6), 10 mg/kg (n=6) and 30 mg/kg (n=6), respectively. The control group received an i.p. injection of saline (n=6). Thirty, 60, 90 and 120 min after amantadine/saline treatment, the cataleptic response was tested using the bar test.

2.1.5.2 Akinesia and motor performance

Reserpine-induced akinesia is a valuable tool for the screening of potential symptomatic PD medications (Ferger et al., 2010; Maj et al., 1997).

Here, reserpine was investigated on its ability to provoke akinesia and impairment of motor coordination in mice. The animals were treated with reserpine (2 mg/kg, s.c. (n=5)) and 0 h, 1 h, 4 h, 6 h and 8 h after injection, they were tested for motor performance on a rotarod system with five 3 cm diameter drums (UGO Basile, Gemonio, Italy). The mice were placed onto the drums and the rotarod was accelerated from 4 to 40 rotations per min over 5 min. The latency to fall (time in sec) was recorded for each mouse. Five mice were tested in parallel. Before the experiment, the mice were trained to run on the rotarod for two days. Each mouse performed five runs with a 10 min interval between each run.
2.1.5.3 Locomotor activity

Locomotor activity measurement in the open field system Actimot™ (TSE Systems GmbH, Bad Homburg, Germany) was used to investigate the effect of PPX-IR and PPX-CR on reserpine-induced akinesia in rats. The animals were placed individually in the centre of the activity box (46.5 x 46.5 cm) and horizontal motor activity (m) was recorded in 10 min 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) filled with PPX solution were implanted s.c. under isoflurane anaesthesia three days before the measurement of akinesia. PPX was delivered continuously at a dose of 2 mg/kg/day. The PPX-IR group (n=6) was treated with PPX (0.3 mg/kg, s.c.) three times (morning, midday, evening) on the day before the akinesia measurement. The vehicle group (n=6) was injected with saline. 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 h later, motor activity was measured in the open field system for 60 min (early morning akinesia).

2.1.5.4 Body temperature

Body temperature of mice was measured 0 h, 1 h, 2 h, 4 h, 6 h, 8 h and 24 h after injection of reserpine (2 mg/kg, s.c. (n=5)) or vehicle (0.5 % acetic acid in saline, s.c. (n=5)) by inserting a lubricated rectal probe thermometer (Physitemp Instruments Inc., Clifton, NJ, USA) 2.5 cm within the rectum.

2.1.5.5 Dyskinesia

Three weeks after the 6-OHDA lesion surgery, rats were treated chronically with L-DOPA (6 mg/kg, p.o.) plus benserazide (15 mg/kg, p.o.) once daily for three weeks (monday - friday). This treatment led to an induction of stable dyskinetic movements, which were quantified every 20 min using a previously described rating scale (Cenci et al., 1998). The total observation time was 180 min.
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 = non-existent; 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 ratings per time point was expressed as a total dyskinesia score.

Amantadine was investigated for its effect on L-DOPA-induced dyskinesia in rats. The animals were treated with L-DOPA/benserazide (6/15 mg/kg, p.o.) once daily to maintain stable dyskinetic behaviour. Before the testing day, the rats were matched according to their total dyskinesia score. Amantadine was administered (i.p.) 30 min prior to L-DOPA/benserazide (6/15 mg/kg, p.o.) at doses of 3 mg/kg (n=6), 10 mg/kg (n=6) and 30 mg/kg (n=6), respectively. The control group received an i.p. injection of saline (n=6). Dyskinesia including axial, limb and orolingual subtype was monitored every 20 min for 180 min following administration of L-DOPA/benserazide by an “experimentally blinded” observer not aware of the experimental group alignment.

2.1.5.6 Smelling behaviour

The rats of the AAV study were tested for olfactory dysfunction. Three and nine weeks after injection of viral vector solution or PBS into the BO of rats, smelling behaviour was assessed using the buried food pellet test. The experiment was performed in test chambers of the Actimot™ system (46.5 x 46.5 x 46.5 cm) which were filled with a layer of clean bedding (3 cm). For three consecutive days before the test, rats (n=5) received a daily training session of 2 h. They were put into the test chambers which contained multiple food pellets (Kellogg’s® Froot Loops) allowing the rats to become familiar with the food stimulus. To ensure motivation, rats were food restricted 20 h prior to testing. The test day started with a habituation period of 5 min in which the rat was put into the test chamber without
food pellets. Then the rat was withdrawn from the test chamber and transferred to a clean temporary holding cage. In the meantime, one food pellet was buried in a random corner of the test chamber approximately 1 cm beneath the bedding. The rat was reintroduced into the centre of the test chamber and the latency to retrieve the food pellet was measured. If a rat failed to locate the food pellet within 15 min, it was removed and given a score of 15 min. Additionally, horizontal motor activity (s) of the rat was recorded during the observation period of 15 min as described above. A time line of the buried food pellet test is depicted in Figure 8.

![Figure 8: Time schedule of the buried food pellet test.](image)

The rats were trained to become familiar with the food stimulus at day 1, 2 and 3 (2 h per day). At day 4, the rats were food restricted for 20 h ensuring motivation to find the hidden food pellet at the testing day (day 5).

### 2.2 In vitro experiments

#### 2.2.1 Receptor binding

Amantadine was evaluated against a set of 68 targets by Eurofins Panlabs (formerly MDS Pharma Services, Taipei, Taiwan). The LeadProfilingScreen® was performed according to Eurofins specifications and standard protocols and comprised 68 primary molecular targets and receptors. Amantadine was tested at 10 µmol/l concentrations.
2.3 Neurochemical and histological analyses

2.3.1 LC-MS/MS analysis

2.3.1.1 General LC-MS/MS setup

The LC-MS/MS system consisted of an HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), an Agilent 1200 Binary Pump, an Agilent 1200 Micro Vacuum Degasser and an Agilent 1200 Thermostatted Column Compartment (Agilent Technologies, Morges, Switzerland). Eluates were detected using an API 4000™ triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) in the positive electrospray ionisation (ESI) mode. The ion spray voltage was set at 4500 V and the source temperature at 500 °C. Data were acquired and analysed using Analyst® 1.4.2. software (MDS Sciex, Ontario, Canada).

2.3.1.2 LC-MS/MS analysis of CSF, plasma and brain samples for haloperidol, reserpine and amantadine

CSF, plasma and brain samples were analysed for haloperidol, reserpine and amantadine, respectively, using LC-MS/MS.

Mobile phase “A” and “B” consisted of 0.1 % formic acid in water and acetonitrile, respectively. For the analysis of haloperidol and reserpine, the same gradient was chosen: 0.00 min: 95 % A, 0.20 min: 95 % A, 0.70 min: 0 % A, 1.50 min: 0 % A, 1.60 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. For the analysis of amantadine, the gradient was chosen as follows: 0.00 min: 98 % A, 0.10 min: 98 % A, 0.50 min: 0 % A, 1.00 min: 0 % A, 1.10 min: 98 % A, 1.50 min: 98 % A and delivered at 0.5 ml/min onto a reversed-phase column (XTerra® MS C18, 50 x 2.1 mm i.d., 5 µm particles, Waters Corporation, Milford, MA, USA) at 20 °C. Different column switching valves were applied: haloperidol and amantadine: 0.00 min: waste, 0.90 min: mass spectrometer; reserpine: 0.00 min: waste, 1.00 min: mass spectrometer. For each compound, three transitions were measured, whilst one transition was used for quantification (Table 1). The specific parameters of the mass spectrometer for each transition are shown in Table 1.
Table 1: Specific parameters of the API 4000™ mass spectrometer for the measured transitions of haloperidol, reserpine and amantadine. The transition which is presented in bold was used for quantification.

<table>
<thead>
<tr>
<th></th>
<th>dwell time [ms]</th>
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<th>collision energy [V]</th>
<th>cell exit potential [V]</th>
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<td>150</td>
<td>76</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>376 - 123</td>
<td>150</td>
<td>76</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>376 - 95</td>
<td>150</td>
<td>76</td>
<td>103</td>
</tr>
<tr>
<td>reserpine</td>
<td>609 - 195</td>
<td>150</td>
<td>121</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>609 - 174</td>
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<td>59</td>
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<td></td>
<td>609 - 397</td>
<td>150</td>
<td>121</td>
<td>39</td>
</tr>
<tr>
<td>amantadine</td>
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<td>76</td>
<td>25</td>
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<tr>
<td></td>
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<td>39</td>
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<tr>
<td></td>
<td>152 - 76</td>
<td>150</td>
<td>76</td>
<td>59</td>
</tr>
</tbody>
</table>

2.3.1.3 LC-MS/MS analysis of CSF, plasma, brain and microdialysis samples for PPX

CSF, plasma, brain and microdialysis samples were analysed for PPX using LC-MS/MS.

Mobile phase “A” and “B” consisted of 0.1 % formic acid in 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 ºC. 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. For the determination of PPX, three transitions were measured, whilst one transition was used for quantification (Table 2). For the internal standard [D7]-PPX, one transition was selected. The specific parameters of the mass spectrometer for each transition are shown in Table 2.
Materials and methods

Table 2: Specific parameters of the API 4000™ mass spectrometer for the measured transitions of PPX. The transition which is presented in bold was used for quantification. PPX (pramipexole).

<table>
<thead>
<tr>
<th></th>
<th>dwell time [ms]</th>
<th>declustering potential [V]</th>
<th>collision energy [V]</th>
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<td>21</td>
</tr>
<tr>
<td></td>
<td>212 - 111</td>
<td>150</td>
<td>56</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>212 - 126</td>
<td>150</td>
<td>56</td>
<td>39</td>
</tr>
<tr>
<td>[D$_7$]-PPX</td>
<td>219 - 153</td>
<td>150</td>
<td>86</td>
<td>21</td>
</tr>
</tbody>
</table>

2.3.1.4 LC-MS/MS analysis of microdialysis samples for GABA and glutamate

Microdialysis samples were analysed for GABA and glutamate using LC-MS/MS. 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 water at a concentration of 1 mmol/l. 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. Ten µl of the microdialysis or standard sample, respectively, were diluted with 10 µl of internal standard solution (500 nmol/l of [D$_6$]-GABA and [D$_5$]-glutamate in acetonitrile). Ten µl of this solution were injected into the LC-MS/MS.

Mobile phase “A” and “B” consisted of 0.1 % formic acid in water and acetonitrile, respectively. The gradient elution profile was chosen as follows: 0.00 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 0.00 min to the waste, at 0.70 min to the mass spectrometer and at 2.50 min to the waste again.
2.3.1.5 LC-MS/MS analysis of microdialysis and CSF samples for glycine

CSF and microdialysis samples were analysed for glycine using LC-MS/MS.

Glycine stock solutions were prepared by dissolving the compound in water at a concentration of 1 mmol/l. All solutions were subsequently frozen at -80 °C. All further standard samples were obtained by diluting the stock solutions with aCSF. Ten µl of the microdialysis, CSF or standard sample were diluted 1:4 with a mixture of acetonitrile/methanol/water 65/25/10. Fifteen µl of this solution were injected into the LC-MS/MS.

Mobile phase “A” and “B” consisted of 0.1 % formic acid in water and methanol, respectively. The gradient elution profile at a constant flow rate of 400 µl/min was chosen as follows: 0.0 min: 80 % A, 0.2 min: 80 % A, 3.0 min: 40 % A, 4.0 min: 40 % A, 4.2 min: 80 % A, 5.0 min: 80 % A. Chromatographic retention was obtained using a Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm, Agilent Technologies, Morges, Switzerland) at 40 °C. The column switching valve was set at 0.0 min to the waste, at 3.0 min to the mass spectrometer and at 4.9 min to the waste again.

2.3.2 HPLC analysis

2.3.2.1 HPLC analysis of microdialysis and striata samples for DA and metabolites

Microdialysis samples of the reserpine mouse study and the PPX experiment as well as striata samples of the reserpine mouse and the AAV study were analysed for DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT) using HPLC and ECD under isocratic conditions. The HPLC system consisted of an HPLC pump (P680, Thermo Scientific Dionex, Dreieich, Germany), an automated sample injector (ASI-100, Thermo Scientific Dionex, Dreieich, Germany) and an electrochemical detector (Decade II, Antec, Zoeterwoude, Netherlands). The detector potential was set at +650 mV using a glassy carbon electrode and an Ag/AgCl reference electrode (Antec VT-03, Zoeterwoude, Netherlands). 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 Na₂EDTA x 2 H₂O, 8.0 mM NaCl, 100.0 mM NaH₂PO₄ x 2 H₂O, adjusted to pH 3.80 with H₃PO₄, filtered through a 0.22 µm filter (Merck Millipore, Darmstadt, Germany), mixed up with 9.3 % acetonitrile, and was delivered at a flow rate of 0.4 ml/min. Aliquots were injected by an autosampler with a cooling module set at 4 °C (ASI-100, Thermo Scientific Dionex, Dreieich, Germany). Data were calculated using an external five-point standard calibration. Data acquisition and calculation were carried out using Chromeleon™ 7.1 software (Thermo Scientific Dionex, Dreieich, Germany).

2.3.2.2 HPLC analysis of microdialysis and CSF samples for amino acids

Microdialysis and CSF samples were analysed for amino acids including GABA, glutamate and glycine using HPLC coupled to fluorescence detection (FD). The HPLC system consisted of an HPLC pump (P680), an automated sample injector (ASI-100) and a fluorescence detector (RF 2000) (all Thermo Scientific Dionex, Dreieich, Germany). Fifteen µl of the sample were mixed with 3 µl o-phthalialdehyde/mercaptoethanol (OPA/MCE) derivatisation reagent and allowed to incubate for 3 min before injection onto the analytical column (Nucleosil 120 C18, 5 µm, 60.0 x 4.0 mm, MZ Analysentechnik GmbH, Mainz, Germany). In order to protect the column from contamination and to extend its lifetime, a pre-column was installed (Nucleosil 120 C18, 5 µm, 5.0 x 4.0 mm, MZ Analysentechnik GmbH, Mainz, Germany). The system was maintained at a stable temperature of 30 °C using a column oven. Chromatographic separation of a standard mix containing 20 amino acids was achieved within 30 min using the following gradient at a constant flow rate of 1.2 ml/min: 0.0 min: 100 % A, 6.0 min: 100 % A, 8.0 min: 90 % A, 11.0 min: 90 % A, 15.0 min: 75 % A, 18.0 min: 60 % A, 21.0 min: 60 % A, 23.0 min: 40 % A, 25.5 min: 15 % A, 25.8 min: 0 % A, 27.5 min: 0 % A, 28.5 min: 100 % A, 30.0 min: 100 % A. For the determination of GABA and glutamate, the gradient elution profile could be shortened to 20.0 min and for the determination of glycine, which elutes at 10.0 min, it could be shortened to 15.5 min. Mobile phase “A” was composed of 92.5 % 0.1 M sodium acetate buffer (pH 6.95), 5 % methanol and 2.5 % tetrahydrofuran. Mobile phase “B” consisted of 97.5 % methanol and 2.5 %
tetrahydrofuran. Data acquisition and calculation were carried out using Chromeleon™ 7.1 software (Thermo Scientific Dionex, Dreieich, Germany).

2.3.3 Immunohistochemical staining

In the AAV study, immunohistochemistry was performed to stain for human α-syn as marker for α-syn overexpression. After fixation, rat brains were processed for paraffinisation (Tissue-Tek® VIP® 6, Sakura Finetek Germany GmbH, Staufen, Germany) and embedded in paraffin (Leica EG1150 H, Leica Biosystems Nussloch GmbH, Nussloch, Germany). Three and 6 µm sections of the BO (6 µm) and striatum (3 µm) were prepared on a microtome (Microm HM 355S, Thermo Scientific, Dreieich, Germany) and mounted onto glass slides (Superfrost Ultra Plus®, Gerhard Menzel GmbH, Braunschweig, Germany). Immunohistochemical staining was performed using an antibody against human α-syn (LB509, 1:8000, mouse, Covance). The staining was carried out on a Leica BOND-MAX™ autostainer (Leica Biosystems Nussloch GmbH, Nussloch, Germany) using a standard immunohistochemistry protocol incorporating heat-induced antigen retrieval with citrate buffer, followed by a peroxide blocking step and primary antibody incubation for 30 min. The reaction was developed with the biotin-free Bond Polymer Refine Detection system (Leica Biosystems, Newcastle, United Kingdom). 3,3'-diaminobenzidine (DAB) was used as the chromogen substrate for visualisation of the reaction. Nuclei were counterstained with haematoxylin. Finally, sections were mounted with Aquatex® (RCM 7000 Glass Coverslipper, Medite GmbH, Burgdorf, Germany). Immunohistochemically stained slides were scanned using a ZEISS Axio Scan.Z1 combined with ZEN 2 software (Carl Zeiss Microscopy GmbH, Jena, Germany).
2.4 Drugs and chemicals

All drugs were calculated as free bases. The injection volume was 1 ml/kg for rats and 10 ml/kg for mice. The drugs were dissolved in saline if not otherwise stated. Reserpine was dissolved in 0.5 or 1 % acetic acid solution.

Drugs and chemicals were purchased from different companies as listed in Table 3. AAV5-CBA-h-α-syn-WT and AAV5-CBA-GFP were provided by the Michael J. Fox Foundation for Parkinson’s Research. AAV5-synapsin-1-WPRE-h-α-syn-WT, PPX dihydrochloride as well as [D7]-PPX dihydrochloride were produced at Boehringer Ingelheim Pharma GmbH & Co. KG.

Table 3: List of drugs and chemicals used for the present experiments.

<table>
<thead>
<tr>
<th>drugs/chemicals</th>
<th>company</th>
</tr>
</thead>
<tbody>
<tr>
<td>isoflurane (Forene®)</td>
<td>AbbVie GmbH &amp; Co KG (Ludwigshafen, Germany)</td>
</tr>
<tr>
<td>Ringer’s solution</td>
<td>B. Braun Melsungen AG (Melsungen, Germany)</td>
</tr>
<tr>
<td>saline, 0.9 % NaCl</td>
<td>Fresenius Kabi GmbH (Bad Homburg, Germany)</td>
</tr>
<tr>
<td>haloperidol (Haldol®-Janssen), fentanyl citrate (Fentanyl®-Janssen)</td>
<td>Janssen-Cilag GmbH (Neuss, Germany)</td>
</tr>
<tr>
<td>ketamine hydrochloride (Ketavet®), medetomidine hydrochloride (Dorbene®), atipamezole hydrochloride (Alzane®)</td>
<td>Pfizer Pharma GmbH (Berlin, Germany)</td>
</tr>
<tr>
<td>xylazine hydrochloride (Rompun®)</td>
<td>Bayer Vital GmbH (Leverkusen, Germany)</td>
</tr>
<tr>
<td>flumazenil</td>
<td>Hikma Pharma GmbH (Graefelfing, Germany)</td>
</tr>
<tr>
<td>naloxone hydrochloride (Naloxon-ratiopharm®)</td>
<td>ratiopharm GmbH (Ulm, Germany)</td>
</tr>
<tr>
<td>midazolam (Dormicum®)</td>
<td>Roche Pharma AG (Grenzach-Wyhlen, Germany)</td>
</tr>
</tbody>
</table>

(continued on page 41)
## Materials and methods

### Table 3 (continued).

<table>
<thead>
<tr>
<th>drugs/chemicals</th>
<th>company</th>
</tr>
</thead>
<tbody>
<tr>
<td>meloxicam (Metacam®)</td>
<td>Boehringer Ingelheim Vetmedica GmbH (Ingelheim, Germany)</td>
</tr>
<tr>
<td>LY 2365109 hydrochloride</td>
<td>Tocris Bioscience (Bristol, United Kingdom)</td>
</tr>
<tr>
<td>KCl, Na₂HPO₄, formaldehyde solution 4 %, Aquatex®, paraffin (Histosec® pastilles)</td>
<td>Merck KGaA (Darmstadt, Germany)</td>
</tr>
<tr>
<td>reserpine, amantadine hydrochloride, L-DOPA methyl ester hydrochloride, benserazide hydrochloride, 6-OHDA hydrobromide, NaCl, CaCl₂ x 2 H₂O, MgCl₂ x 6 H₂O, ascorbic acid, HClO₄, acetic acid, formic acid, HCl, acetonitrile, methanol, 1-octanesulfonic acid sodium salt, Na₂EDTA x 2 H₂O, NaH₂PO₄ x 2 H₂O, H₃PO₄, DA hydrochloride, DOPAC, HVA, 3-MT, GABA, glutamate, glycine, amino acid standard mix, phthaldialdehyde reagent solution, sodium acetate trihydrate</td>
<td>Sigma-Aldrich Chemie GmbH (Steinheim, Germany)</td>
</tr>
<tr>
<td>tetrahydrofuran Rotisolv®, 2-mercaptoethanol</td>
<td>Carl Roth GmbH + Co. KG (Karlsruhe, Germany)</td>
</tr>
<tr>
<td>[D₆]-GABA, [D₅]-glutamate, [D₅]-glycine</td>
<td>Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA)</td>
</tr>
<tr>
<td>PBS (Gibco® PBS)</td>
<td>Life Technologies GmbH (Darmstadt, Germany)</td>
</tr>
<tr>
<td>α-syn (LB509) monoclonal antibody</td>
<td>Covance Inc. (Princeton, NJ, USA)</td>
</tr>
<tr>
<td>reagents for the Leica BOND-MAX™ autostainer</td>
<td>Leica Biosystems (Newcastle, United Kingdom)</td>
</tr>
<tr>
<td>Kellogg's® Froot Loops</td>
<td>Kellogg GmbH (Hamburg, Germany)</td>
</tr>
</tbody>
</table>
2.5 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5.04 software (GraphPad software, La Jolla, CA, USA). All values are expressed as mean ± standard error of the mean (SEM). A p-value < 0.05 was considered as statistically significant (#p < 0.05, *p < 0.05, **p < 0.01, ***p < 0.001).

An unpaired, two-tailed Student’s t-test was used to compare two separate sets of independent data. For comparison of more than two data sets with one different variable, a one-way analysis of variance (ANOVA) was carried out. To compare data sets with two different variables, a two-way ANOVA was performed. For multiple comparisons, a Bonferroni or Dunnett’s Multiple Comparison post hoc analysis was applied. Repeated measures (RM) ANOVA was used if parameters were measured in the same animal at different time points.

The statistical tests applied for the analysis of the different experiments are noted in the corresponding figures.
3. Results

3.1 Behavioural and neurochemical investigations in the haloperidol-induced catalepsy model in rats and reserpine-induced akinesia model in mice

3.1.1 Haloperidol experiments

3.1.1.1 Exposure measurement of haloperidol in plasma, brain and CSF

Haloperidol (3 mg/kg, i.p.) was injected into naïve rats and 0.5 h, 1 h, 2.5 h and 5 h later, the concentration of haloperidol was determined in CSF, plasma and brain (Table 4). Maximum levels ($c_{\text{max}}$) of haloperidol in all three sample matrices were observed 0.5 h following administration and were found to be $20.38 \pm 4.46$ nmol/l (mean ± SEM), $306.50 \pm 53.93$ nmol/l (mean ± SEM) and $4671.00 \pm 903.44$ nmol/kg (mean ± SEM) for CSF, plasma and brain, respectively. The brain/plasma ratio at $t_{\text{max}}$, calculated as quotient between the concentration in brain and plasma, was found to be 15.24.

Table 4: Exposure of haloperidol to CSF, plasma and brain of rats. CSF, plasma and brain levels of haloperidol 0.5 h (n=5), 1 h (n=5), 2.5 h (n=5) and 5 h (n=5) following injection (3 mg/kg, i.p.) into naïve rats. Data are expressed as mean ± SEM. CSF (cerebrospinal fluid), i.p. (intraperitoneal), SEM (standard error of the mean).

<table>
<thead>
<tr>
<th>time</th>
<th>CSF nmol/l (mean ± SEM)</th>
<th>plasma nmol/l (mean ± SEM)</th>
<th>brain nmol/kg (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 h</td>
<td>20.38 ± 4.46</td>
<td>306.50 ± 53.93</td>
<td>4671.00 ± 903.44</td>
</tr>
<tr>
<td>1 h</td>
<td>14.16 ± 1.27</td>
<td>170.20 ± 6.75</td>
<td>3312.00 ± 212.57</td>
</tr>
<tr>
<td>2.5 h</td>
<td>8.18 ± 0.43</td>
<td>68.94 ± 1.43</td>
<td>1486.80 ± 109.76</td>
</tr>
<tr>
<td>5 h</td>
<td>3.38 ± 0.73</td>
<td>34.85 ± 8.08</td>
<td>655.50 ± 80.53</td>
</tr>
</tbody>
</table>
3.1.1.2 Haloperidol-induced catalepsy in rats

Different routes of administration of haloperidol (0.5 mg/kg, s.c. and i.p.) were investigated on their ability to induce catalepsy in rats (Figure 9). Subcutaneous administration of haloperidol resulted in a significantly higher degree of catalepsy recorded over the whole observation period of 120 min (163.3 ± 11.8 s (mean ± SEM), p < 0.05), in comparison to the i.p. route (90.7 ± 26.2 s (mean ± SEM)) (Figure 9A). Regarding the time course, rats treated with haloperidol s.c. showed significantly increased catalepsy 90 min (52.7 ± 3.3 s (mean ± SEM), p < 0.05) and 120 min (56.3 ± 2.6 s (mean ± SEM), p < 0.05) after haloperidol injection, compared to i.p. treatment (Figure 9B).

![Figure 9: Investigation of different routes of administration of haloperidol on their ability to induce catalepsy in rats. Haloperidol (0.5 mg/kg) was injected s.c. (n=10) and i.p. (n=10). Catalepsy was monitored every 30 min. Data are expressed as mean ± SEM of the time spent by animals on the bar. Cumulative data (A) were analysed by an unpaired t-test (*p < 0.05). The time course (B) was analysed by RM two-way ANOVA followed by a Bonferroni post hoc test (*p < 0.05). s.c. (subcutaneous), i.p. (intraperitoneal), SEM (standard error of the mean), RM (repeated measures), ANOVA (analysis of variance).]

3.1.1.3 Analysis of haloperidol in plasma samples

Plasma samples were taken 150 min after haloperidol injection and were analysed for haloperidol. Subcutaneous administration resulted in significantly higher haloperidol levels (24.70 ± 1.63 nmol/l (mean ± SEM), p < 0.001) compared to i.p. application (14.50 ± 1.98 nmol/l (mean ± SEM)) (Figure 10).
Results

Figure 10: Analysis of haloperidol in plasma samples of rats. Haloperidol (0.5 mg/kg) was injected s.c. (n=10) and i.p. (n=10). Plasma samples were taken 150 min after administration and analysed for haloperidol levels. Data are expressed as mean ± SEM and were analysed by an unpaired t-test (**p < 0.001). s.c. (subcutaneous), i.p. (intraperitoneal), SEM (standard error of the mean).

3.1.2 Reserpine experiments

3.1.2.1 Exposure measurement of reserpine in plasma, brain and CSF

Reserpine (3 mg/kg, i.p.) was injected into naïve rats and 0.5 h, 1 h, 2.5 h and 5 h later, the concentration of reserpine was determined in CSF, plasma and brain (Table 5). Maximum levels (c\text{max}) of reserpine in all three sample matrices were observed 0.5 h following administration and were found to be 1.26 ± 0.14 nmol/l (mean ± SEM), 126.28 ± 23.59 nmol/l (mean ± SEM) and 101.86 ± 20.86 nmol/kg (mean ± SEM) for CSF, plasma and brain, respectively. The brain/plasma ratio at t\text{max}, calculated as quotient between the concentration in brain and plasma, was found to be 0.81.

3.1.2.2 Measurement of extracellular DA levels in the mouse striatum via in vivo microdialysis

The effect of reserpine (2 mg/kg, s.c.) or vehicle (0.5 % acetic acid in saline, s.c.) treatment on extracellular DA levels in the striatum of mice is displayed in Figure 11. Basal levels of DA were found to be 3.81 ± 0.26 nmol/l (mean ± SEM) and 2.90 ± 0.45 nmol/l (mean ± SEM) in the reserpine and vehicle group, respectively. Following administration of reserpine, extracellular DA levels significantly decreased 5 h after injection and remained at that level until the end of the
Results

Vehicle treated mice did not show a decrease in extracellular DA levels.

Table 5: Exposure of reserpine to CSF, plasma and brain of rats. CSF, plasma and brain levels of reserpine 0.5 h (n=5), 1 h (n=5), 2.5 h (n=5) and 5 h (n=5) following injection (3 mg/kg, i.p.) into naïve rats. Data are expressed as mean ± SEM. CSF (cerebrospinal fluid), i.p. (intraperitoneal), SEM (standard error of the mean).

<table>
<thead>
<tr>
<th>time</th>
<th>CSF nmol/l (mean ± SEM)</th>
<th>plasma nmol/l (mean ± SEM)</th>
<th>brain nmol/kg (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 h</td>
<td>1.26 ± 0.14</td>
<td>126.28 ± 23.59</td>
<td>101.86 ± 20.86</td>
</tr>
<tr>
<td>1 h</td>
<td>1.07 ± 0.21</td>
<td>54.16 ± 10.76</td>
<td>59.21 ± 11.16</td>
</tr>
<tr>
<td>2.5 h</td>
<td>0.92 ± 0.07</td>
<td>22.64 ± 4.80</td>
<td>39.35 ± 6.28</td>
</tr>
<tr>
<td>5 h</td>
<td>0.75 ± 0.14</td>
<td>3.99 ± 1.71</td>
<td>19.94 ± 4.86</td>
</tr>
</tbody>
</table>

Figure 11: In vivo microdialysis in the mouse striatum after reserpine treatment. Effect of reserpine (2 mg/kg, s.c. (n=7)) or vehicle (0.5 % acetic acid in saline, s.c. (n=8)) treatment on extracellular DA levels in the striatum of mice. Data are expressed as mean ± SEM and were analysed by RM two-way ANOVA followed by a Bonferroni post hoc test (**p < 0.001, **p < 0.01, *p < 0.05). s.c. (subcutaneous), DA (dopamine), SEM (standard error of the mean), RM (repeated measures), ANOVA (analysis of variance).
3.1.2.3 Measurement of DA and DA metabolites in the mouse striatum

To investigate the effect of reserpine (2 mg/kg, s.c.) treatment on striatal tissue DA levels as well as DA turnover in mice, a post mortem analysis of DA, DOPAC and HVA in the striatum was performed (Figure 12). DA turnover was expressed as DOPAC+HVA/DA. Statistical analysis yielded a significant reduction of striatal tissue DA levels 1 h (p < 0.05), 4 h, 6 h, 8 h, 16 h and 24 h (p < 0.001) after reserpine treatment (Figure 12A). DA turnover showed a significant increase 16 h after reserpine injection (p < 0.05) (Figure 12B).

![Figure 12: Measurement of DA and DA metabolites in the mouse striatum after reserpine treatment.](image)

**Figure 12: Measurement of DA and DA metabolites in the mouse striatum after reserpine treatment.** Effect of reserpine (2 mg/kg, s.c. (n=5)) treatment on striatal tissue DA levels (A) and DA turnover (DOPAC+HVA/DA) (B) in mice. Data are expressed as mean ± SEM. Statistical comparison was performed using a one-way ANOVA followed by a Dunnett’s Multiple Comparison test (**p < 0.001, *p < 0.05 versus time point 0 h). s.c. (subcutaneous), DA (dopamine), DOPAC (3,4-dihydroxyphenylacetic acid), HVA (homovanillic acid), SEM (standard error of the mean), ANOVA (analysis of variance).

3.1.2.4 Reserpine-induced akinesia in mice

Reserpine was investigated on its ability to provoke akinesia and impairment of motor coordination in mice (Figure 13). Rotarod behaviour was recorded at several time points following reserpine treatment (2 mg/kg, s.c.). Considering the whole observation period of 8 h, there was a decrease in rotarod performance which reached statistical significance 1 h (p < 0.05), 4 h, 6 h as well as 8 h (p < 0.01) after reserpine injection.
Figure 13: Reserpine-induced akinesia in mice. Investigation of reserpine (2 mg/kg, s.c. (n=5)) treatment on its ability to provoke akinesia and impairment of motor coordination in mice. Data are expressed as mean ± SEM and were analysed by a one-way ANOVA followed by a Dunnett’s Multiple Comparison test (**p < 0.01, *p < 0.05 versus time point 0 h). s.c. (subcutaneous), SEM (standard error of the mean), ANOVA (analysis of variance).

3.1.2.5 Body temperature of mice after reserpine treatment

Figure 14 shows the effect of reserpine (2 mg/kg, s.c.) or vehicle (0.5 % acetic acid in saline, s.c.) treatment on the body temperature of mice. In the vehicle treated group, there was no change in body temperature which remained constant at 38.00 ± 0.04 °C (mean ± SEM) throughout the experiment. In the reserpine treated group, body temperature significantly decreased in a time-dependent manner, ranging from 34.12 ± 0.25 °C (mean ± SEM) 4 h after injection (p < 0.001) to 24.88 ± 0.91 °C (mean ± SEM) 24 h after injection of reserpine (p < 0.001).

Figure 14: Body temperature of mice after reserpine treatment. Effect of reserpine (2 mg/kg, s.c. (n=5)) or vehicle (0.5 % acetic acid in saline, s.c. (n=5)) treatment on the body temperature of mice. Data are expressed as mean ± SEM. The time course of the body temperature was analysed by RM two-way ANOVA followed by a Bonferroni post hoc test (***p < 0.001). s.c. (subcutaneous), SEM (standard error of the mean), RM (repeated measures), ANOVA (analysis of variance).
Results

3.2 Investigation of CDS as treatment strategy for PD

3.2.1 Exposure measurement of PPX in plasma, brain and CSF

PPX (3 mg/kg, i.p.) was injected into naïve rats and 0.5 h, 1 h, 2.5 h and 5 h later, the concentration of PPX was determined in CSF, plasma and brain (Table 6). Maximum levels (c_{max}) of PPX in CSF and plasma were observed 0.5 h following administration and were found to be 1377.20 ± 265.51 nmol/l (mean ± SEM) and 2926.00 ± 330.54 nmol/l (mean ± SEM), respectively. Brain c_{max} levels of PPX were observed 1 h following injection and were found to be 4530.00 ± 364.35 nmol/kg (mean ± SEM). The brain/plasma ratio at t_{max}, calculated as quotient between the concentration in brain and plasma, was found to be 2.95.

Table 6: Exposure of PPX to CSF, plasma and brain of rats. CSF, plasma and brain levels of PPX 0.5 h (n=5), 1 h (n=5), 2.5 h (n=5) and 5 h (n=5) following injection (3 mg/kg, i.p.) into naïve rats. Data are expressed as mean ± SEM. PPX (pramipexole), CSF (cerebrospinal fluid), i.p. (intraperitoneal), SEM (standard error of the mean).

<table>
<thead>
<tr>
<th>time</th>
<th>CSF (mean ± SEM)</th>
<th>plasma (mean ± SEM)</th>
<th>brain (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 h</td>
<td>1377.20 ± 265.51</td>
<td>2926.00 ± 330.54</td>
<td>4104.00 ± 677.61</td>
</tr>
<tr>
<td>1 h</td>
<td>941.40 ± 96.3</td>
<td>1534.00 ± 108.29</td>
<td>4530.00 ± 364.35</td>
</tr>
<tr>
<td>2.5 h</td>
<td>354.00 ± 49.97</td>
<td>439.00 ± 50.64</td>
<td>2974.80 ± 224.83</td>
</tr>
<tr>
<td>5 h</td>
<td>62.44 ± 6.70</td>
<td>89.08 ± 6.94</td>
<td>760.80 ± 55.07</td>
</tr>
</tbody>
</table>

3.2.2 Haloperidol-induced catalepsy

Figure 15 shows the effect of PPX on haloperidol-induced catalepsy in rats (Ferger et al., 2010). Time spent on the bar in the PPX-CR group was significantly decreased in comparison to the vehicle group during the whole experiment (0 h, 2 h, 8 h, 10 h (p < 0.001), 4 h (p < 0.05), 6 h, 12 h (p < 0.01)) (Figure 15A). The PPX-IR and vehicle group did not display a significant difference in catalepsy 2 h after the bolus injection of haloperidol (time point 0; pre-test before PPX/vehicle
Results

injection), indicating that pre-treatment with PPX the day before did not show an effect on haloperidol-induced catalepsy the next morning. Following injection with PPX, time spent on the bar significantly decreased in the PPX-IR group at time points 2 h, 4 h (p < 0.001) and 6 h (p < 0.05). Regarding the cumulative data (Figure 15B), PPX-CR (p < 0.001) and PPX-IR (p < 0.05) showed an improvement of haloperidol-induced catalepsy, while PPX-CR revealed a significantly higher effect compared to PPX-IR (p < 0.05).

Figure 15: Effect of PPX on haloperidol-induced catalepsy in rats. Effect of PPX-IR (1 mg/kg, s.c. (n=9)), PPX-CR (1 mg/kg/day, s.c. (n=9)) and vehicle (s.c. (n=9)) on the time course (A) and cumulative data (B) of haloperidol-induced catalepsy in rats. The PPX-IR group was treated with PPX three times on the day before the catalepsy experiment. Haloperidol (0.5 mg/kg, i.p.) was injected 2 h prior to the first catalepsy measurement. Catalepsy was maintained for 12 h by administration of haloperidol (0.1 mg/kg, i.p.) every 4 h. Data are expressed as mean ± SEM. The time course was analysed by RM two-way ANOVA followed by a Bonferroni post hoc test (**p < 0.001, *p < 0.01, *p < 0.05 versus vehicle). Cumulative data were analysed using RM one-way ANOVA followed by a Bonferroni post hoc test (**p < 0.001, *p < 0.05 versus vehicle; #p < 0.05 PPX-IR versus PPX-CR). PPX (pramipexole), PPX-IR (pramipexole immediate release), PPX-CR (pramipexole continuous release), s.c. (subcutaneous), i.p. (intraperitoneal), SEM (standard error of the mean), RM (repeated measures), ANOVA (analysis of variance). Reprinted from Ferger et al. (2010), Synapse, Vol. 64, Pages No. 533-541, Copyright (2015), with permission from John Wiley and Sons.
3.2.3 Reserpine-induced akinesia

The effect of PPX on reserpine-induced early morning akinesia in rats is shown in Figure 16 (Ferger et al., 2010). No significant differences were observed between the vehicle and the PPX-IR group, indicating that pre-treatment with PPX the day before did 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) (Figure 16A) as well as considering the whole experiment over 60 min (p < 0.05) (Figure 16B). Here, PPX-CR revealed a significantly higher effect compared to PPX-IR (p < 0.05).

Figure 16: Effect of PPX on reserpine-induced akinesia in rats. Effect of PPX-IR (0.3 mg/kg, s.c. (n=6)), PPX-CR (2 mg/kg/day, s.c. (n=7)) and vehicle (s.c. (n=6)) on the time course (A) and cumulative data (B) of reserpine-induced akinesia in rats. The PPX-IR group was treated with PPX three times on the day before the akinesia measurement. Reserpine (1 mg/kg, s.c.) was injected 17 h prior to the experiment. Data are expressed as mean ± SEM. The time course was analysed by RM two-way ANOVA followed by a Bonferroni post hoc test (***p < 0.001, *p < 0.05 versus vehicle). Cumulative data were analysed using RM one-way ANOVA followed by a Bonferroni post hoc test (*p < 0.05 versus vehicle; #p < 0.05 PPX-IR versus PPX-CR). PPX (pramipexole), PPX-IR (pramipexole immediate release), PPX-CR (pramipexole continuous release), s.c. (subcutaneous), SEM (standard error of the mean), RM (repeated measures), ANOVA (analysis of variance). Adapted from Ferger et al. (2010), Synapse, Vol. 64, Pages No. 533-541, Copyright (2015), with permission from John Wiley and Sons.
3.2.4 Measurement of extracellular DA levels

The effect of PPX on extracellular DA levels in the rat striatum is displayed in Figure 17A (Ferger et al., 2010). Pre-dose basal levels of DA in the PPX-IR group were found to be 1.86 nmol/l. 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 group, stable DA levels of approximately 0.07 nmol/l were obtained, which did not vary over time implicating steady state conditions. In comparison to the pre-dose basal levels in the PPX-IR group, DA levels in the PPX-CR group were reduced by 96.2 %. The maximum reduction of DA levels in the PPX-IR group was observed 90 min after PPX treatment (44.4 % in comparison to basal DA levels).

3.2.5 Measurement of PPX in microdialysis samples

Extracellular PPX levels in the rat striatum are displayed in Figure 17B (Ferger et al., 2010). As mentioned before, no pre-dose levels in the PPX-CR group were obtained. However, basal PPX levels of the PPX-CR group were found to be 1.86 nmol/l. These concentrations remained at that level (2.46 nmol/l) until the end of the experiment. 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 nmol/l).
Figure 17: In vivo microdialysis in the rat striatum after PPX treatment. Effect 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 (A) and PPX (B) in the rat striatum. Data are expressed as mean ± SEM. PPX (pramipexole), DA (dopamine), PPX-IR (pramipexole immediate release), PPX-CR (pramipexole continuous release), s.c. (subcutaneous), SEM (standard error of the mean). Adapted from Ferger et al. (2010), Synapse, Vol. 64, Pages No. 533-541, Copyright (2015), with permission from John Wiley and Sons.
3.3 Investigation of amantadine in the 6-OHDA dyskinesia and haloperidol-induced catalepsy model in rats

3.3.1 Exposure measurement of amantadine in plasma, brain and CSF

Amantadine (3 and 30 mg/kg) was injected into naïve rats and 0.5 h, 1 h, 2.5 h and 5 h later, the concentration of amantadine was determined in CSF, plasma and brain (Table 7). Amantadine at a dose of 3 mg/kg achieved maximum levels in CSF, plasma and brain 0.5 h following administration. Maximum CSF and plasma levels were also detected 0.5 h following administration of amantadine at a dose of 30 mg/kg, whereas the brain \( c_{\text{max}} \) value was observed 1 h post dosing. \( c_{\text{max}} \) values for CSF, plasma and brain following amantadine 3 mg/kg were found to be 691.80 ± 25.81 nmol/l (mean ± SEM), 2944.00 ± 312.66 nmol/l (mean ± SEM) and 16092.00 ± 1873.63 nmol/kg (mean ± SEM), respectively. Amantadine 30 mg/kg produced maximum levels in CSF, plasma and brain of 13440.00 ± 524.02 nmol/l (mean ± SEM), 28480.00 ± 749.27 nmol/l (mean ± SEM) and 135360.00 ± 7586.15 nmol/kg (mean ± SEM), respectively. The brain/plasma ratio at \( t_{\text{max}} \), calculated as quotient between the concentration in brain and plasma, was found to be 5.47 for amantadine 3 mg/kg and 8.22 for amantadine 30 mg/kg.

3.3.2 Receptor binding

In order to better understand its pharmacological mode of action, amantadine was evaluated against a set of 68 primary molecular targets and receptors by Eurofins Panlabs (Taipei, Taiwan). Amantadine was tested at a concentration of 10 µmol/l in a LeadProfilingScreen® comprising several radio ligand binding assays. An inhibition or stimulation greater than 50 % was considered as significant response. No significant responses were obtained at the 68 receptor targets of the screen. However, for sigma-1 receptors (35 % inhibition) and glutamatergic NMDA receptors (phencyclidine (PCP) binding site) (27 % inhibition), a weak antagonism was observed (Table 8).
Table 7: Exposure of amantadine to CSF, plasma and brain of rats. CSF, plasma and brain levels of amantadine 0.5 h (n=5), 1 h (n=5), 2.5 h (n=5) and 5 h (n=5) following injection (3 and 30 mg/kg, i.p.) into naïve rats. Data are expressed as mean ± SEM. CSF (cerebrospinal fluid), i.p. (intraperitoneal), SEM (standard error of the mean).

<table>
<thead>
<tr>
<th>time</th>
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<th>plasma (mean ± SEM)</th>
<th>brain (mean ± SEM)</th>
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<tr>
<td></td>
<td>nmol/l</td>
<td>nmol/l</td>
<td>nmol/kg</td>
</tr>
<tr>
<td></td>
<td>(mean ± SEM)</td>
<td>(mean ± SEM)</td>
<td>(mean ± SEM)</td>
</tr>
<tr>
<td>amantadine 3 mg/kg</td>
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<tr>
<td>0.5 h</td>
<td>691.80 ± 25.81</td>
<td>2944.00 ± 312.66</td>
<td>16092.00 ± 1873.63</td>
</tr>
<tr>
<td>1 h</td>
<td>544.00 ± 84.90</td>
<td>1166.88 ± 323.50</td>
<td>11208.00 ± 1726.75</td>
</tr>
<tr>
<td>2.5 h</td>
<td>203.20 ± 32.10</td>
<td>505.60 ± 138.52</td>
<td>5389.20 ± 690.92</td>
</tr>
<tr>
<td>5 h</td>
<td>48.70 ± 14.72</td>
<td>92.30 ± 20.94</td>
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</tr>
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<td>amantadine 30 mg/kg</td>
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<tr>
<td>0.5 h</td>
<td>13440.00 ± 524.02</td>
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<tr>
<td>1 h</td>
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<td>5 h</td>
<td>924.60 ± 104.71</td>
<td>1335.20 ± 171.54</td>
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</table>

Table 8: Assessment of amantadine in 68 radio ligand binding assays. The receptors and species (human, rat, mouse, rabbit or hamster) are listed. Amantadine was tested at 10 μmol/l concentrations. The % inhibition is shown for each receptor. Negative values correspond to stimulation of binding. Significant responses are defined as % inhibition or stimulation > 50 %.

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<th>target</th>
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<th>% inhibition</th>
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<th>% inhibition</th>
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(continued on page 56)
Table 8 (continued).

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<th>species</th>
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3.3.3 Effect of amantadine on haloperidol-induced catalepsy

Three doses of amantadine (3 mg/kg, 10 mg/kg and 30 mg/kg, i.p.) were tested for their effects on haloperidol-induced catalepsy in rats (Figure 18). Injection of haloperidol (0.5 mg/kg, s.c.) plus vehicle treatment led to an induction of stable cataleptic behaviour as indicated by the total time spent on the bar during the whole observation period of 180 min (142.8 ± 16.4 s (mean ± SEM)) (Figure 18A). Administration of amantadine at a dose of 30 mg/kg significantly reduced haloperidol-induced catalepsy (81.0 ± 11.5 s (mean ± SEM), p < 0.05) compared to vehicle treatment. The lower doses of amantadine (3 mg/kg and 10 mg/kg) had no significant effect on haloperidol-induced catalepsy. Regarding the time course (Figure 18B), amantadine 30 mg/kg displayed a significant reduction in catalepsy 150 min (23.2 ± 3.7 s (mean ± SEM), p < 0.01) and 180 min (33.3 ± 8.8 s (mean ± SEM), p < 0.05) after haloperidol injection, compared to vehicle treatment. Again, the lower doses of amantadine (3 mg/kg and 10 mg/kg) did not show any significant effect.

Figure 18: Effect of amantadine on haloperidol-induced catalepsy in rats. Amantadine (3 mg/kg, i.p. (n=6), 10 mg/kg, i.p. (n=6) and 30 mg/kg, i.p. (n=6)) and vehicle (saline, i.p. (n=6)) were administered 60 min after haloperidol injection (0.5 mg/kg, s.c.). Catalepsy was monitored every 30 min. Data are expressed as mean ± SEM of the time spent by animals on the bar. Cumulative data (A) were analysed by RM one-way ANOVA followed by a Dunnett's Multiple Comparison test (*p < 0.05 versus vehicle). The time course (B) was analysed by RM two-way ANOVA followed by a Bonferroni post hoc test (**p < 0.01, *p < 0.05 versus vehicle). i.p. (intraperitoneal), s.c. (subcutaneous), SEM (standard error of the mean), RM (repeated measures), ANOVA (analysis of variance).
3.3.4 Effect of amantadine on L-DOPA-induced dyskinesia

The effect of amantadine on L-DOPA-induced dyskinesia in rats was investigated using three different doses (3 mg/kg, 10 mg/kg and 30 mg/kg, i.p.) (Figure 19). Compared to vehicle, administration of amantadine at a dose of 30 mg/kg significantly decreased axial (p < 0.001, p < 0.01) (Figure 19B), limb (p < 0.001, p < 0.05) (Figure 19C) and total dyskinesia score (p < 0.001, p < 0.01) (Figure 19A) from 20 to 100 min as well as orolingual dyskinesia score (p < 0.001, p < 0.01, p < 0.05) (Figure 19D) from 20 to 120 min after L-DOPA treatment. Comparison of amantadine 10 mg/kg and vehicle showed a significant effect in axial (20 min, p < 0.05), orolingual (60 min, p < 0.05) and total dyskinesia score (60 min, p < 0.05), but no statistically significant effect in limb dyskinesia score. The lowest dose of amantadine (3 mg/kg) did not show any significant effect. Regarding the cumulative data, treatment with L-DOPA/benserazide (6/15 mg/kg, p.o.) plus vehicle led to an induction of stable dyskinetic behaviour as indicated by axial (16.2 ± 1.1 (mean ± SEM)) (Figure 19F), limb (18.8 ± 2.2 (mean ± SEM)) (Figure 19G), orolingual (19.7 ± 2.2 (mean ± SEM)) (Figure 19H) as well as total dyskinesia score (54.7 ± 4.7 (mean ± SEM)) (Figure 19E). Amantadine 30 mg/kg significantly reduced the single dyskinesia scores of axial (2.7 ± 1.5 (mean ± SEM), p < 0.001), limb (3.8 ± 1.4 (mean ± SEM), p < 0.01) and orolingual (4.7 ± 1.3 (mean ± SEM), p < 0.01) dyskinesia as well as total dyskinesia score (11.2 ± 4.0 (mean ± SEM), p < 0.001), compared to vehicle. The lower doses of amantadine (3 mg/kg and 10 mg/kg) did not show a significant alleviation of dyskinesia.

Figure 19: Effect of amantadine on L-DOPA-induced dyskinesia in rats. L-DOPA-induced dyskinesia is indicated by total dyskinesia score (A and E), axial dyskinesia score (B and F), limb dyskinesia score (C and G) and orolingual dyskinesia score (D and H). Amantadine (3 mg/kg, i.p. (n=6), 10 mg/kg, i.p. (n=6) and 30 mg/kg, i.p. (n=6)) and vehicle (saline, i.p. (n=6)) were administered 30 min prior to L-DOPA/benserazide (6/15 mg/kg, p.o.). Dyskinesia was monitored every 20 min. Time course data (A, B, C and D) are expressed as mean ± SEM and were analysed by RM two-way ANOVA followed by a Bonferroni post hoc test (**p < 0.001, *p < 0.01, p < 0.05 versus vehicle). The cumulative dyskinesia data of the total observation period of 180 min (E, F, G and H) are expressed as mean ± SEM and were analysed by RM one-way ANOVA followed by a Dunnett's Multiple Comparison test (**p < 0.001, *p < 0.01 versus vehicle). i.p. (intraperitoneal), p.o. (peroral), L-DOPA (L-3,4-dihydroxyphenylalanine), SEM (standard error of the mean), RM (repeated measures), ANOVA (analysis of variance). Please see Figure 19 next page.
Figure 19: Effect of amantadine on L-DOPA-induced dyskinesia in rats.
3.4 Establishment of an AAV-\(\alpha\)-syn-overexpression model in rats for the investigation of olfactory dysfunction

3.4.1 Overexpression of \(\alpha\)-syn

Expression of human wild-type \(\alpha\)-syn was assessed in specific brain areas three and 11 weeks after injection of viral vector solution or PBS into the BO of rats. Immunohistochemical staining with an antibody specific to human \(\alpha\)-syn showed that bulbar injection of AAV5-CBA-h-\(\alpha\)-syn-WT and AAV5-synapsin-1-WPRE-h-\(\alpha\)-syn-WT resulted in high expression of human wild-type \(\alpha\)-syn in the BO of rats, which was already visible at three weeks and preserved and even increased at 11 weeks (Figure 20). No staining was observed after injection of AAV5-CBA-GFP or PBS (Figure 20).

![Image](image-url)

**Figure 20: Overexpression of \(\alpha\)-syn in the BO of rats.** Immunostaining was carried out with an antibody specific to human \(\alpha\)-syn (brown stain). Immunohistochemical staining shows the expression of human wild-type \(\alpha\)-syn in the BO of rats three and 11 weeks after bulbar injection of AAV5-CBA-h-\(\alpha\)-syn-WT (A and E) and AAV5-synapsin-1-WPRE-h-\(\alpha\)-syn-WT (B and F). No staining was observed after injection of AAV5-CBA-GFP (C and G) or PBS (D and H). \(\alpha\)-syn (\(\alpha\)-synuclein), BO (bulbus olfactorius), AAV5 (adeno-associated virus 5), CBA (chicken beta actin), h (human), WT (wild-type), WPRE (woodchuck hepatitis virus post-transcriptional regulatory element), GFP (green fluorescent protein), PBS (phosphate-buffered saline). Scale bar: 100 \(\mu\)m.
High levels of α-syn immunoreactivity were also seen in the striatum of rats 11 weeks, but not three weeks, after bulbar injection of AAV5-CBA-h-α-syn-WT and AAV5-synapsin-1-WPRE-h-α-syn-WT (Figure 21). This indicates that α-syn was transported in a caudal-rostral manner over time, which is consistent with the Braak staging scheme (Braak et al., 2003). Again, no staining was observed after injection of AAV5-CBA-GFP or PBS (Figure 21).

![Figure 21: Overexpression of α-syn in the striatum of rats.](image)

**Figure 21: Overexpression of α-syn in the striatum of rats.** Immunostaining was carried out with an antibody specific to human α-syn (brown stain). Immunohistochemical staining shows the expression of human wild-type α-syn in the striatum of rats 11 weeks, but not three weeks, after bulbar injection of AAV5-CBA-h-α-syn-WT (A and E) and AAV5-synapsin-1-WPRE-h-α-syn-WT (B and F). No staining was observed after injection of AAV5-CBA-GFP (C and G) or PBS (D and H). α-syn (α-synuclein), AAV5 (adeno-associated virus 5), CBA (chicken beta actin), h (human), WT (wild-type), WPRE (woodchuck hepatitis virus post-transcriptional regulatory element), GFP (green fluorescent protein), PBS (phosphate-buffered saline). Scale bar: 100 µm.

### 3.4.2 Effect of α-syn overexpression on smelling behaviour

To study the impact of α-syn overexpression on the function of the olfactory system, smelling behaviour was assessed using the buried food pellet test three and nine weeks after injection of viral vector solution or PBS into the BO of rats (Figure 22). At both time points, AAV5-CBA-h-α-syn-WT- and AAV5-synapsin-1-WPRE-h-α-syn-WT-injected rats took significantly longer to retrieve the buried food pellet compared to AAV5-CBA-GFP- or PBS-injected rats (p < 0.001). This
suggests that α-syn overexpression in the BO led to olfactory dysfunction which was significant from three weeks after viral vector injection and persisted at nine weeks. At both time points, there was no significant difference in total motor activity between all tested groups, indicating that the different smelling behaviour was not caused by decreased locomotion (Figure 23).

Figure 22: Effect of α-syn overexpression on olfactory function of rats. Smelling behaviour of rats was assessed using the buried food pellet test three (A) and nine (B) weeks after injection of viral vector solution or PBS into the BO of rats (n=5). Data are expressed as mean ± SEM and were analysed by a one-way ANOVA followed by a Bonferroni post hoc test (**p < 0.001 versus PBS and AAV5-CBA-GFP). α-syn (α-synuclein), BO (bulbus olfactorius), AAV5 (adeno-associated virus 5), CBA (chicken beta actin), WPRE (woodchuck hepatitis virus post-transcriptional regulatory element), GFP (green fluorescent protein), PBS (phosphate-buffered saline), SEM (standard error of the mean), ANOVA (analysis of variance).
Results

Figure 23: Effect of α-syn overexpression on locomotor activity of rats. During the smell test, locomotor activity of rats was recorded three (A) and nine (B) weeks after injection of viral vector solution or PBS into the BO of rats (n=5). Data are expressed as mean ± SEM and were analysed by a one-way ANOVA followed by a Bonferroni post hoc test. α-syn (α-synuclein), BO (bulbus olfactorius), AAV5 (adeno-associated virus 5), CBA (chicken beta actin), WPRE (woodchuck hepatitis virus post-transcriptional regulatory element), GFP (green fluorescent protein), PBS (phosphate-buffered saline), SEM (standard error of the mean), ANOVA (analysis of variance).

3.4.3 Effect of α-syn overexpression on nigrostriatal neurons

In order to investigate the effect of α-syn overexpression on the function of nigrostriatal dopaminergic neurons, striatal tissue DA levels as well as DA turnover were analysed three and 11 weeks after injection of viral vector solution or PBS into the BO of rats. DA turnover was expressed as DOPAC+HVA+3-MT/DA. Statistical analysis yielded a significant reduction of DA levels in the striatum 11 weeks after bulbar injection of AAV5-CBA-h-α-syn-WT and AAV5-synapsin-1-WPRE-h-α-syn-WT (p < 0.001) (Figure 24B). There was no significant difference in striatal DA concentrations between all groups at the early time point of three weeks (Figure 24A). Striatal DA turnover did not show any significant differences between all tested groups at both time points (Figure 24C and D).
Results

Figure 24: Effect of α-syn overexpression on DA concentrations as well as DA turnover in the rat striatum. DA levels as well as DA turnover in the striatum were analysed three (A and C) and 11 (B and D) weeks after injection of viral vector solution or PBS into the BO of rats (n=3). Data are expressed as mean ± SEM and were analysed by a one-way ANOVA followed by a Bonferroni post hoc test (***p < 0.001 versus PBS and AAV5-CBA-GFP). α-syn (α-synuclein), BO (bulbus olfactorius), AAV5 (adeno-associated virus 5), CBA (chicken beta actin), WPRE (woodchuck hepatitis virus post-transcriptional regulatory element), GFP (green fluorescent protein), PBS (phosphate-buffered saline), DA (dopamine), SEM (standard error of the mean), ANOVA (analysis of variance).
3.5 LC-MS/MS analysis of GABA and glutamate

3.5.1 Optimisation of mass spectrometric conditions

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. For GABA and glutamate, positively charged ions with a mass-to-charge ratio (m/z) of 104 and 148 were generated, respectively. Using multiple reaction monitoring (MRM), several fragment ions were produced (Figure 25) (Buck et al., 2009).

![Figure 25: ESI-MS/MS positive ion scanning spectra for (A) GABA and (B) glutamate. ESI-MS/MS (electrospray ionisation tandem mass spectrometry), GABA (γ-aminobutyric acid), cps (counts per second), m/z (mass-to-charge ratio). Reprinted from Buck et al. (2009), Journal of Neuroscience Methods, Vol. 182, Pages No. 78-84, Copyright (2015), with permission from Elsevier.]
For the determination of GABA and glutamate, three transitions were measured, whilst one transition was used for quantification (Table 9). For the internal standards [D₆]-GABA and [D₅]-glutamate, one transition was selected. The specific parameters of the mass spectrometer for each transition are shown in Table 9 (Buck et al., 2009). Mass spectrometric conditions were optimised using the flow injection analysis (FIA) program provided by the “Quantitative Optimisation” function of the Analyst® software.

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

Stable deuterated internal standards of both amino acids were used. Thereby, a compensation of potentially confounding matrix effects was obtained resulting in a reliable quantification of the analytes. The internal standards were added at concentrations of 500 nmol/l, which did not interfere with the quantification of the analytes.

Table 9: Specific parameters of the API 4000™ mass spectrometer for the measured transitions of GABA and glutamate. The transition which is presented in bold was used for quantification. GABA (γ-aminobutyric acid). Reprinted from Buck et al. (2009), Journal of Neuroscience Methods, Vol. 182, Pages No. 78-84, Copyright (2015), with permission from Elsevier.

<table>
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<tr>
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<th>dwell time [ms]</th>
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<td>150</td>
<td>23</td>
<td>6</td>
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3.5.2 Optimisation of chromatographic conditions

GABA and glutamate are not retainable in a simple fashion on reversed-phase 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, a HILIC column was chosen 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 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 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, a flow rate of 200 µl/min was chosen. However, at the end of the run, a higher flow rate of 1000 µl/min was applied to achieve a faster equilibration of the system.

3.5.3 Linearity, limit of quantitation, reproducibility and accuracy

Linearity was given in the tested range of 1 nmol/l to 10 µmol/l for GABA and 10 nmol/l to 10 µmol/l for glutamate. The calibration curve was analysed with a weighting of 1/x². Over the considered range of concentrations, a correlation coefficient of 0.9981 for GABA and 0.9940 for glutamate was obtained. The limit of quantitation (LOQ) for GABA and glutamate was 1 nmol/l and 10 nmol/l (injection volume 10 µl), respectively, with a signal-to-noise ratio of 10:1 (Figure 26) (Buck et al., 2009). The intra- and inter-batch reproducibility was measured using a GABA and glutamate concentration of 50 nmol/l and 500 nmol/l, respectively. The intra-
batch reproducibility for ten repeated injections was 2.7 % (relative standard deviation (RSD)) for GABA and 4.0 % (RSD) for glutamate. The accuracy for GABA and glutamate was 112.2 ± 3.2 % (mean ± standard deviation (SD)) and 102.7 ± 4.0 % (mean ± SD), respectively. The inter-batch reproducibility for ten repeated injections in four batches was found to be 5.0 % (RSD) and 5.3 % (RSD) for GABA and glutamate, respectively.

Figure 26: Representative LC-MS/MS chromatograms of GABA and glutamate standard samples. (A) GABA (1 nmol/l), (B) [D₆]-GABA (500 nmol/l), (C) glutamate (10 nmol/l) and (D) [D₅]-glutamate (500 nmol/l). LC-MS/MS (liquid chromatography tandem mass spectrometry), GABA (γ-aminobutyric acid), cps (counts per second). Adapted from Buck et al. (2009), Journal of Neuroscience Methods, Vol. 182, Pages No. 78-84, Copyright (2015), with permission from Elsevier.
3.5.4 GABA and glutamate monitoring in microdialysis samples

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 which depolarises neurons and facilitates 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). Figure 27 shows representative chromatograms of basal levels of GABA and glutamate in the GP of rats (Buck et al., 2009). The basal levels were found to be 12.72 ± 1.31 nmol/l (mean ± SEM) and 189.02 ± 14.76 nmol/l (mean ± SEM) for GABA and glutamate, respectively. Stimulation with potassium enriched aCSF resulted in a 14- and 8-fold increase in extracellular GABA and glutamate levels, respectively (Figure 28) (Buck et al., 2009).

![Figure 27](image)

**Figure 27**: LC-MS/MS chromatograms of a basal microdialysis sample from the GP of rats. (A) GABA (12.8 nmol/l) and (B) glutamate (201.0 nmol/l). LC-MS/MS (liquid chromatography tandem mass spectrometry), GABA (γ-aminobutyric acid), cps (counts per second), GP (globus pallidus). Reprinted from Buck et al. (2009), Journal of Neuroscience Methods, Vol. 182, Pages No. 78-84, Copyright (2015), with permission from Elsevier.
Figure 28: Time course of potassium-evoked (A) GABA and (B) glutamate release in the GP of rats. From time point 80-100 min, aCSF was switched to potassium enriched aCSF to stimulate neurotransmitter release (n=4). Data are presented as mean ± SEM. GABA (γ-aminobutyric acid), GP (globus pallidus), aCSF (artificial cerebrospinal fluid), SEM (standard error of the mean). Adapted from Buck et al. (2009), Journal of Neuroscience Methods, Vol. 182, Pages No. 78-84, Copyright (2015), with permission from Elsevier.

To verify the present LC-MS/MS method, a HPLC-FD method was applied for the analysis of GABA and glutamate (Figure 29) (Buck et al., 2009). This approach requires a pre-column derivatisation step with OPA and MCE which improves retention on reversed-phase columns and forms fluorescing molecules that can be detected by FD. A chromatographic separation of GABA and glutamate in standard samples was achieved within 20 min. The LOQ of the HPLC-FD method
was found to be 5 nmol/l for both GABA and glutamate (injection volume 10 µl), which is in the same range as the LOQ of the present LC-MS/MS method.

Figure 29: Analysis of GABA and glutamate using HPLC-FD compared to LC-MS/MS. Chromatograms of the same standard sample containing GABA (100 nmol/l) and glutamate (100 nmol/l) analysed by (A) HPLC-FD, (B) LC-MS/MS (GABA) and (C) LC-MS/MS (glutamate). HPLC-FD (high performance liquid chromatography and fluorescence detection), LC-MS/MS (liquid chromatography tandem mass spectrometry), GABA (γ-aminobutyric acid), cps (counts per second). Adapted from Buck et al. (2009), Journal of Neuroscience Methods, Vol. 182, Pages No. 78-84, Copyright (2015), with permission from Elsevier.
3.6 LC-MS/MS analysis of glycine

3.6.1 Optimisation of mass spectrometric conditions

Glycine was detected by MS/MS using positive ESI generating the positively charged ion m/z 75.9. Using MRM, several fragment ions were observed (Figure 30) (Voehringer et al., 2013). For the determination of glycine, two transitions were measured, whilst one transition was used for quantification (Table 10). The mass spectrometric conditions for each transition were optimised using the “Quantitative Optimisation” program of the Analyst® software and are shown in Table 10.

![Figure 30: ESI-MS/MS positive ion scanning spectrum for glycine.](image)

**Figure 30:** ESI-MS/MS positive ion scanning spectrum for glycine. ESI-MS/MS (electrospray ionisation tandem mass spectrometry), cps (counts per second), m/z (mass-to-charge ratio). Reprinted from Voehringer et al. (2013), Journal of Chromatography B, Vol. 939, Pages No. 92-97, Copyright (2015), with permission from Elsevier.

![Table 10: Specific parameters of the API 4000™ mass spectrometer for the measured transitions of glycine.](image)

**Table 10:** Specific parameters of the API 4000™ mass spectrometer for the measured transitions of glycine. The transition which is presented in bold was used for quantification.

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<td>75.9 - 30.2</td>
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<td>41</td>
<td>19</td>
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When analysing microdialysis or CSF samples, there is a high potential for matrix effects which could compromise a reliable quantification of the analyte. Using stable deuterated internal standards is one possibility to compensate for potentially confounding matrix effects. In the present work, studies using [D$_5$]-glycine as internal standard were performed. However, no matrix effects interfering with the analysis of glycine were observed. Therefore, [D$_5$]-glycine was not included in the following experiments.

### 3.6.2 Optimisation of chromatographic conditions

For the chromatographic retention of glycine, a Zorbax Eclipse XDB-C18 column was chosen. Furthermore, different mobile phases, flow rates as well as column temperatures were optimised. Using acetonitrile as the organic solvent of the mobile phase gave a high background signal. A column temperature of 20 °C resulted in low signal intensity. The best sensitivity was obtained with 0.1 % formic acid in water and 100 % methanol as components of the mobile phase which was applied at a constant flow rate of 400 µl/min and a column temperature of 40 °C. An elevation of signal intensity was also obtained by using a column switching valve to avoid unnecessary salt contamination of the mass spectrometer.

### 3.6.3 Linearity, limit of quantitation, precision and accuracy

Linearity was given in the range from 100 nmol/l to 100 µmol/l. Considering these limits, the calibration curve was analysed using a linear fit with a weighting of $1/x^2$, resulting in a correlation coefficient of 0.9977. The limit of detection (LOD), as defined by a signal-to-noise ratio of $> 3:1$, was determined as 50 nmol/l. The LOQ, as defined by a signal-to-noise ratio of $> 10:1$, was found to be 100 nmol/l. Figure 31 shows the chromatogram of a standard sample containing 5 µmol/l of glycine which represents the physiological glycine level (Voehringer et al., 2013). The intra- and inter-batch precisions were measured using a defined glycine concentration of 1 µmol/l. The intra-batch precision for ten repeated injections was found to be 2.4 % (RSD). The accuracy was 109.5 ± 2.6 % (mean ± SD). The inter-batch precision for ten repeated injections in three batches was found to be 7.4 % (RSD) with an accuracy of 112.9 ± 4.6 % (mean ± SD).
**3.6.4 In vivo application of the LC-MS/MS method**

In order to validate the present LC-MS/MS method in vivo, glycine concentrations were determined in rat CSF and striatal in vivo microdialysates following administration of the GlyT1 inhibitor LY 2365109. This compound is known to selectively inhibit GlyT1 (half maximal inhibitory concentration ($IC_{50}$) = 15.8 nmol/l) and to increase extracellular glycine levels by blocking its reuptake (Perry et al., 2008). Figure 32 shows the chromatograms of basal levels of glycine in both rat CSF and striatal microdialysate. The basal levels were found to be 10.38 ± 0.83 µmol/l (mean ± SEM) and 1.52 ± 0.14 µmol/l (mean ± SEM) in CSF and striatum, respectively (Voehringer et al., 2013).

An increase in glycine concentration was observed in rat CSF 1 h (p < 0.05), 2 h (p < 0.01) as well as 4 h (p < 0.001) after administration of LY 2365109 (10 mg/kg, p.o.) compared to vehicle treatment (Figure 33A) (Voehringer et al., 2013). This rise was similar to the elevation in extracellular glycine levels in the rat striatum after injection of LY 2365109 (10 mg/kg, p.o.) as determined by in vivo microdialysis, which reached statistical significance between 100 and 300 min post-
dosing (100-180 min (p < 0.001), 200-260 min (p < 0.01), 280-300 min (p < 0.05)) (Figure 33B) (Voehringer et al., 2013). Administration of vehicle solution did not alter extracellular concentrations of striatal glycine, which sustained at basal levels throughout the experiment.

Figure 32: LC-MS/MS chromatograms of basal CSF and microdialysis samples of rats. The chromatograms show a basal CSF sample containing 10 µmol/l of glycine (A) and a basal striatal microdialysis sample containing 1.5 µmol/l of glycine (B). LC-MS/MS (liquid chromatography tandem mass spectrometry), CSF (cerebrospinal fluid), cps (counts per second). Adapted from Voehringer et al. (2013), Journal of Chromatography B, Vol. 939, Pages No. 92-97, Copyright (2015), with permission from Elsevier.

In order to demonstrate the reliability of the present LC-MS/MS method, glycine was additionally analysed by routinely used HPLC-FD. A chromatographic separation of a standard mix containing 20 amino acids was achieved within 30 min (Figure 34) (Voehringer et al., 2013). For the analysis of glycine, the LOD and LOQ were found to be 10 and 30 nmol/l, respectively. Linearity was given in the range from 30 nmol/l to 1 µmol/l. The intra-batch precision for ten repeated injections (1 µmol/l) was found to be 0.04 % (RSD) with an accuracy of 100.2 ± 0.5 % (mean ± SD). The inter-batch precision for ten repeated injections in three batches (1 µmol/l) was found to be 4.3 % (RSD) with an accuracy of 94.7 ± 4.0 % (mean ± SD).
Results

Figure 3: Time course of glycine increase in rat CSF and striatum. Effect of the GlyT1 inhibitor LY 2365109 (10 mg/kg, p.o.) on glycine levels in rat CSF (n=3) (A) and on extracellular glycine levels in the rat striatum (n=6-9) (B). Data are expressed as mean ± SEM and were analysed by RM two-way ANOVA followed by a Bonferroni post hoc test (**p < 0.01, *p < 0.05). GlyT1 (glycine transporter 1), p.o. (peroral), CSF (cerebrospinal fluid), SEM (standard error of the mean), RM (repeated measures), ANOVA (analysis of variance). Reprinted from Voehringer et al. (2013), Journal of Chromatography B, Vol. 939, Pages No. 92-97, Copyright (2015), with permission from Elsevier.
Figure 34: Representative HPLC-FD chromatogram of a standard sample containing 20 amino acids. The sample contained 100 nmol/l of the following amino acids: 1 aspartic acid, 2 glutamic acid, 3 asparagine, 4 serine, 5 histidine, 6 glutamine, 7 glycine, 8 threonine, 9 arginine, 10 alanine, 11 taurine, 12 GABA, 13 tyrosine, 14 methionine, 15 valine, 16 tryptophan, 17 phenylalanine, 18 isoleucine, 19 leucine, 20 lysine. HPLC-FD (high performance liquid chromatography and fluorescence detection), GABA (γ-aminobutyric acid). Reprinted from Voehringer et al. (2013), Journal of Chromatography B, Vol. 939, Pages No. 92-97, Copyright (2015), with permission from Elsevier.
4. Discussion

One of the greatest challenges facing PD research is the inability to translate preclinical findings into clinically relevant outcomes. Several putative neuroprotective treatment strategies have been described in preclinical animal studies of PD. However, most of them failed, and no proven therapy has yet been identified that can slow the rate of neurodegeneration or stop the progression of PD (Kalia et al., 2015). This may largely be due to the lack of an appropriate animal model mimicking the progressive course of the disease. Indeed, there is no ideal animal model of PD which is able to exactly reproduce all features of the human disorder, including pathology, symptoms and pathogenic mechanisms (Blesa and Przedborski, 2014). Especially the recapitulation of premotor changes such as olfactory dysfunction, which is regarded as an important premotor biomarker for PD (Haas et al., 2012; Kranick and Duda, 2008; Morley and Duda, 2010; Postuma et al., 2012; Xiao et al., 2014), may be an essential factor for screening neuroprotective strategies intended for early disease-modification. Obviously, there is an urgent need for novel and improved animal models of PD.
4.1 Behavioural and neurochemical investigations in the haloperidol-induced catalepsy model in rats and reserpine-induced akinesia model in mice

Currently, several animal models of PD are available which mimic many aspects of the human disease and provide insight into its pathogenesis (Blesa and Przedborski, 2014; Le et al., 2014). Two common functional models of PD are based on treatment with haloperidol or reserpine and are important to predict the efficacy of novel symptomatic therapies (Duty and Jenner, 2011).

Based on preclinical models, experimental results are translated into the clinical situation. Thus, it is crucial to consider to which extent the animal model reflects the disease state. This comparison can be evaluated using the concepts of face, construct and predictive validity (Duty and Jenner, 2011; Jenner, 2008a). To achieve a considerable concordance between animal models and the clinic, it is of fundamental importance that experimental models are not considered as fixed frames. Rather, they have to undergo a permanent amelioration and optimisation process to eventually reach a good transferability from their preclinical aspect to clinical efficacy. In this regard, the pharmacokinetic profiles of haloperidol and reserpine, including brain, plasma as well as CSF levels, are important parameters which can be used in combination with behavioural observations.

Improved animal models of high validity allow themselves to be used as precursors in the development and refinement of therapeutics before clinical trials. Moreover, the better the animal models are validated, the fewer animals are needed to translate preclinical results into clinical effects, which is in favour of the 3R (reduction, refinement, replacement) principle (Tannenbaum and Bennett, 2015).

The first animal model presented in this study is the haloperidol model in rats which is known to induce PD-like symptoms such as catalepsy and muscular rigidity by blocking the nigrostriatal DA transmission (Alvarez-Fischer et al., 2002; Lorenc-Koci et al., 1996).

The analysis of the exposure to the three compartments plasma, brain and CSF identified haloperidol to be an appropriate central acting agent as indicated by a
brain/plasma ratio of 15.24. Additionally, haloperidol achieved a high CSF exposure of 20 nmol/l. CSF concentrations do not always exactly reflect brain extracellular fluid concentrations (Shen et al., 2004). However, as suggested by preclinical and clinical studies, CSF levels appear to give reasonably accurate estimates of the active, free drug concentration in the brain. This is of importance concerning the assumption that predominantly the free drug concentration is crucial for interaction with its receptor target. Hence, CSF exposure is an appropriate surrogate marker for the in vivo assessment of potential central acting drugs (Lin, 2008). Interestingly, comparison of the pharmacokinetic profile of haloperidol in plasma and CSF suggests that higher haloperidol plasma levels are related to higher exposure to CSF, allowing plasma levels to be used for the evaluation of pharmacological effects of haloperidol.

In the present study, haloperidol was investigated regarding two different routes of administration (subcutaneous and intraperitoneal injection) on the ability to induce cataleptic movement behaviour in rats. Haloperidol administered subcutaneously led to a significantly higher degree of catalepsy compared to intraperitoneal treatment. Furthermore, the analysis of plasma samples of the same rats showed that subcutaneous injection of haloperidol resulted in significantly higher haloperidol plasma levels than intraperitoneal administration. This higher exposure of haloperidol to plasma after subcutaneous treatment may be a possible reason for the higher catalepsy time. Thus, in order to induce stable cataleptic behaviour in rats, the subcutaneous route of administration of haloperidol has to be favoured over the intraperitoneal route due to emergent variations in haloperidol plasma levels. In fact, Lindgren et al. reported that dose-failure episodes can be attributed to the route of drug administration (Lindgren et al., 2007). Especially the occurrence of dose-failing after intraperitoneal injection can be explained by the erratic peripheral absorption of the drug from the abdominal cavity. In contrast, dose-failure episodes are completely abolished when the drug is administered subcutaneously. Consequently, fewer animals are needed to detect a specific treatment effect, which is in accordance with the 3R reduction principle (Tannenbaum and Bennett, 2015).

Concerning the haloperidol model, the route of administration was changed from intraperitoneally to subcutaneously, which leads to the induction of stable motor impairment in rats with a high degree of reliability. Indeed, the model of
haloperidol-induced catalepsy is widely accepted and validated in the research area of PD and various DA receptor agonists such as PPX have thereby been established as anticataleptic pharmacotherapy (Ferger et al., 2010; Maj et al., 1997).

In the second symptomatic animal model of PD presented here, treatment with reserpine leads to PD-resembling motor behaviour symptoms such as akinesia, tremor and rigidity by blocking the uptake of monoamines into synaptic vesicles (Betarbet et al., 2002; Colpaert, 1987; Lorenc-Koci et al., 1995). This inhibition unselectively affects the storage of several monoamine neurotransmitters including DA in the brain as well as in the periphery (Carlsson et al., 1957; Dolphin et al., 1976).

The analysis of the exposure of reserpine to plasma, brain and CSF revealed that the compound achieves similar levels in plasma and brain tissue, which is indicative of its peripheral as well as central effects. Additionally, reserpine was available in CSF (1.26 nmol/l), although to a relatively low level.

In the past, doses of reserpine used to induce parkinsonian symptoms were fairly high, ranging from 10 mg/kg to 40 mg/kg (Colpaert, 1987; Lorenc-Koci et al., 1995). Additionally, scientists often use a combination of reserpine and α-methyl-p-tyrosine (AMPT), a selective inhibitor of the amine synthesis enzyme tyrosine hydroxylase (TH), which is resulting in a more distinct loss of DA in the striatum (Dolphin et al., 1976; Rech et al., 1968). However, the present study demonstrated that the sole administration of reserpine at a relatively low dose is sufficient to induce a significant neurochemical as well as behavioural effect in mice. Using in vivo microdialysis, it has been shown that reserpine at a dose of 2 mg/kg reduced extracellular DA levels in the striatum of mice 5 h following injection. Similarly, striatal tissue DA levels were markedly decreased 4 h after reserpine administration. These neurochemical changes were accompanied by severe akinesia and impaired motor coordination, which could be observed within the same time period.

In many studies using the reserpine akinesia model, potential effects of antiparkinsonian drugs are investigated 16-24 h following reserpine administration (Carlsson et al., 1957; Dolphin et al., 1976; Gerlach and Riederer, 1996; Gossel et al., 1995; Maj et al., 1997). However, the present study clearly showed that
reserpine treatment of mice led to a significant reduction of DA levels in the striatum as well as inhibition of motor activity after a much shorter time period. Therefore, 5 h after reserpine injection is an optimal latency time for performing experiments in the reserpine model.

Administration of reserpine is complicated by the occurrence of adverse events such as diarrhoea and hypothermia resulting from depletion of peripheral monoamine stores (Jenner, 2008a). Here, injection of reserpine significantly reduced the body temperature of mice by 13 °C within 24 h. This massive decrease requires attention concerning animal welfare. In accordance with the 3R refinement principle (Tannenbaum and Bennett, 2015), mice should be used earlier, approximately 5 h after reserpine injection, when the body temperature is still in a more physiological range. Furthermore, solubility properties of reserpine recommend addition of acetic acid to the vehicle solution, which is not tolerated well by mice. For animal protection, the use of 0.5 % acetic acid in saline instead of 1 % or more is advisable.

Regarding the reserpine model, it has been concluded that interventions such as decreasing the dose of reserpine, reducing the latency time after injection as well as lowering the portion of acetic acid in the vehicle solution not only act in animals interest but also result in highly reproducible behavioural as well as neurochemical features, enabling the refined model to be used in PD research. Recently, a report by Leao et al. highlighted the reserpine model still to be a useful tool to screen candidate drugs for the treatment of PD (Leao et al., 2015).

**Conclusion**

The present study demonstrated that already existing animal models of PD can be improved and optimised by performing only marginal alterations. The symptomatic PD models of haloperidol-induced catalepsy in rats and reserpine-induced akinesia in mice have been modified concerning route of administration as well as dosage, latency time after injection and vehicle solution, resulting in animal models of high predictive and face validity, enhanced animal welfare as well as a reduction in animal numbers.
4.2 Investigation of CDS as treatment strategy for PD

PD patients are usually treated several times a day with short-acting DA receptor agonists, resulting in fluctuating plasma and brain levels which induce non-physiological, intermittent stimulation of striatal DA receptors (Jenner, 2008c; Senek and Nyholm, 2014). Particularly at night and in the early morning, constant plasma levels of short-acting DA receptor agonists cannot be maintained due to drug clearance. Of note, the therapeutic efficacy of DA receptor agonists is closely related to sufficient drug exposure levels. As a matter of fact, cardinal PD symptoms appear if dopaminergic receptor stimulation cannot be maintained. The concept of CDS postulates that it is desirable to avoid the pulsatile and achieve continuous, more physiological stimulation of DA receptors in the striatum (Jenner, 2008c; Olanow et al., 2006). This strategy could translate into prolonged therapeutic efficacy by alleviating nocturnal disturbances as well as early morning akinesia and would result in a lower propensity to develop motor fluctuations and dyskinesia as demonstrated in parkinsonian cynomolgus monkeys (Bibbiani et al., 2005). Furthermore, the reversal of motor deficits without induction of dyskinesia in MPTP-treated common marmosets argues for the concept of CDS (Stockwell et al., 2008).

In the present study, the effects of continuous versus acute exposure of the DA D3/D2 receptor agonist PPX were compared in the haloperidol-induced catalepsy as well as reserpine-induced akinesia model in rats. Continuous release of PPX was achieved by subcutaneous implantation of Alzet® minipumps filled with PPX solution, whereas subcutaneous PPX injections were used to mimic PPX immediate release. In order to relate brain PPX pharmacokinetics with behavioural outcomes as well as levels of the biomarker DA, PPX as well as DA levels were measured in the striatum of rats using in vivo microdialysis.

In both animal models of PD, it has been shown that the effects of PPX are dependent on PPX exposure in the brain (Ferger et al., 2010). In particular, the day following acute PPX pre-treatment of rats, symptomatic effects of PPX were no longer present, which resulted in early morning akinesia as well as catalepsy. In contrast, continuous PPX exposure in rats prevented early morning akinesia as well as catalepsy. In vivo microdialysis revealed that continuous PPX release produced significantly lower extracellular DA levels in the striatum of rats.
compared to the peak decrease obtained after acute PPX administration (Ferger et al., 2010). Similarly, acute PPX treatment produced a peak increase in extracellular PPX levels in the rat striatum, whereas PPX-CR resulted in a constant PPX exposure during the whole experiment (Ferger et al., 2010).

Neither haloperidol- nor reserpine-induced behavioural effects are associated with neurodegeneration as indicated by no loss of dopaminergic neurons in the nigrostriatal pathway. Therefore, these animal models of PD can only be considered to study symptomatic effects of drugs such as DA receptor agonists with predictive validity. The advantages of these symptomatic models are the robustness, clear behavioural readouts and simple procedures which do not require stereotaxic surgery as for non-brain-penetrating dopaminergic neurotoxins or safety constrictions as for MPTP which needs to be handled with extreme care not to be harmful for the experimenter (Ferger et al., 2010).

Haloperidol is able to induce parkinsonian-like symptoms such as catalepsy and muscular rigidity (Alvarez-Fischer et al., 2002; Lorenc-Koci et al., 1996). Haloperidol-induced catalepsy is considered as an animal model of parkinsonian akinesia reflecting impaired postural stability and the inability to actively initiate phasic movements (Sanberg et al., 1988). Haloperidol-induced catalepsy is a result of the blockade of DA D$_2$ receptors in the striatum (Ellenbroek et al., 1985; Sanberg, 1980).

In the present study, pre-treatment of rats with PPX the day before the catalepsy experiment did not show an effect on haloperidol-induced catalepsy the next morning. However, acute treatment of rats with PPX exerted a pronounced effect on haloperidol-induced catalepsy which was reversible, declined after 6 h and was absent at 8 h (Ferger et al., 2010). Previous experiments have shown that a single haloperidol injection led to significant catalepsy for approximately 6 h. In order to study the effect of PPX-IR and PPX-CR over a longer observation period, the catalepsy model was adapted. Using multiple injections of lower haloperidol doses, stable cataleptic behaviour could be maintained for 12 h. In contrast to PPX-IR, PPX-CR was able to antagonise haloperidol-induced catalepsy in rats in the morning and over the whole observation period of 12 h. This is in agreement with the present microdialysis data which demonstrated that PPX-CR produced a constant PPX exposure during the whole experiment (Ferger et al., 2010). In terms
of maximum efficacy, the PPX-IR and PPX-CR groups did not differ. The duration of the anticataleptic effect was longer in the PPX-CR group. The present data are in line with a previous study on haloperidol-induced catalepsy in rats, 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 haloperidol-induced muscular rigidity in rats (Lorenc-Koci and Wolfarth, 1999).

In the second symptomatic animal model of PD, PPX-IR and PPX-CR were investigated on their effects on reserpine-induced akinesia in rats (Ferger et al., 2010). In comparison to haloperidol which offers high affinity for DA D2-like receptors (D2, D3, D4: inhibition constant (k_i) value 1.2, 7, 2.3 nmol/l, respectively) (Seeman and Van Tol, 1994), reserpine acts presynaptically by blocking the uptake of monoamines by the VMAT-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 (Betarbet et al., 2002; Ferger et al., 2010; Gossel et al., 1995; Leao et al., 2015; Maj et al., 1997) as well as to study non-dopaminergic mechanisms of the disease (Kreitzer and Malenka, 2007; Niswender et al., 2008).

As seen in the haloperidol-induced catalepsy model, PPX-CR antagonised the motor impairment in reserpine-treated rats and was effective over the whole observation period including the first measurement on early morning akinesia. Conversely, pre-treatment of rats with PPX the day before the akinesia experiment did not improve reserpine-induced akinesia the next morning (Ferger et al., 2010). Using a higher dose of reserpine (5 mg/kg) in combination with AMPT (250 mg/kg, i.p.) to additionally block DA biosynthesis, Maj et al. showed that a single subcutaneous injection of PPX (0.3 and 1 mg/kg) increased locomotor activity in rats (Maj et al., 1997). The effect of PPX was even higher than obtained in the vehicle+vehicle control group. Under the present conditions, hyperactivity in rats was not observed, neither in the PPX-IR nor in the PPX-CR group (Ferger et al., 2010).
In the present study, only single doses of PPX-IR and PPX-CR were selected after pilot dose finding studies. PPX-IR has been repeatedly tested over the last years serving as a positive control in the haloperidol and reserpine rat model. Reproducible effects were found in a dose range from 1 to 3 mg/kg in the haloperidol model and using a slightly different dose range from 0.3 to 3 mg/kg in the reserpine model. The doses for PPX-CR were selected by measurements of PPX exposure being in a similar range as after PPX-IR treatment.

Most of the pharmacokinetic data on PPX exposure rely on studies measuring PPX plasma levels. For example, in healthy volunteers, plasma concentrations of PPX were found to be proportional to the dose under steady state conditions (elimination half-life $t_{1/2}$ 8-12 h, $t_{\text{max}}$ 1-3 h, $c_{\text{max}}$ 0.375-4.5 ng/ml) (Kvernmo et al., 2006; Wright et al., 1997). In the present study, the exposure of PPX to the three compartments plasma, brain and CSF was analysed in rats after intraperitoneal administration. These data clearly show that PPX accumulated in the brain as indicated by a brain/plasma ratio of 2.95. Additionally, PPX achieved a high CSF exposure of > 1 μmol/l, highlighting PPX to be a suitable central acting drug. This 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 site in the same sample. Accordingly, PPX as well as DA levels were measured in the striatum of rats using in vivo microdialysis (Ferger et al., 2010). The PPX exposure in the rat striatum was maximum 90 min (3.48 nmol/l) following injection in the PPX-IR group and declined over a period of 3 h. Animals continuously treated with PPX showed lower maximum PPX levels and revealed an almost constant striatal PPX exposure of 2.46 nmol/l over the whole experiment. Both PPX-IR and PPX-CR decreased extracellular DA levels in the rat striatum. This 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 the impact on regulation of DA synthesis as well as exocytotic DA release by a 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 affecting DA release.
(Gainetdinov et al., 1996) as well as DA synthesis (Wolf and Roth, 1990). Additionally, it has been demonstrated that DA $D_3$ preferring compounds modulate DA uptake in vitro and in vivo, suggesting that DA $D_3$ receptor activation increases DA uptake by modulating DA transporter activity (Zapata and Shippenberg, 2002).

Indeed, systemic PPX administration caused a long-lasting reduction of extracellular DA and DA metabolite levels in the rat striatum (Carter and Muller, 1991; Robertson et al., 1993). This effect was reversed by the DA $D_2$ receptor antagonist sulpiride but not by the DA $D_1$ receptor antagonist SCH 23390 (Carter and Muller, 1991). Moreover, local PPX administration reduced the 6-OHDA-induced increase of extracellular DA concentrations in the striatum of rats (Ferger et al., 2000). Cumulative evidence underlines that extracellular DA levels measured by in vivo microdialysis are 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. Extracellular DA levels in the rat striatum were consistently lower in the PPX-CR group compared to the PPX-IR group, which speaks against desensitisation and a tolerance effect concerning regulation of extracellular DA levels after PPX-CR (Ferger et al., 2010). Chernoloz et al. performed an electrophysiological experiment in anaesthetised rats which were subcutaneously implanted with osmotic minipumps delivering PPX at a dose of 1 mg/kg/day for two or 14 days (Chernoloz et al., 2009). They demonstrated a decrease in the spontaneous firing rate of dopaminergic neurons by 40 % after two days of treatment, whereas after 14 days of PPX treatment, the firing rate of DA neurons had recovered. They implicated desensitisation of DA $D_2/D_3$ autoreceptors after long-lasting continuous PPX treatment to be responsible for this effect.

As demonstrated in the present study, continuous DA receptor stimulation using DA receptor agonists delivered by osmotic minipumps is useful in preclinical animal experiments offering beneficial effects on early morning akinesia. Clinically used approaches to achieve CDS comprise direct delivery of a L-DOPA intestinal gel into the duodenum or subcutaneous infusion of the DA receptor agonist apomorphine (Deleu et al., 2004; Olanow et al., 2014). However, for patients suffering from PD, the most convenient route of administration is oral. Moreover, a drawback of long-term subcutaneous infusion could be adverse events such as skin reactions and nodules accompanied by variable drug absorption (Deleu et al.,
2004). These disadvantages prompted the development of oral sustained or extended release formulations of dopaminergic agents in several drug development programs. In the meantime, a once-daily oral PPX extended release formulation is available (Jenner et al., 2009; Schapira et al., 2011). Additionally, a novel extended release oral L-DOPA formulation has received FDA approval for use in PD (Hauser et al., 2013). In the 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 (Elshoff et al., 2012; Steiger, 2008).

**Conclusion**

The present study highlighted that CDS using PPX-CR may offer a clear therapeutic benefit compared to PPX-IR regarding early morning akinesia, as demonstrated in the haloperidol-induced catalepsy as well as reserpine-induced akinesia model in rats. The behavioural effects correspond to in vivo microdialysis measurements in the striatum of rats showing a continuous decrease of extracellular DA levels and a constant PPX exposure following continuous PPX release.
4.3 Investigation of amantadine in the 6-OHDA dyskinesia and haloperidol-induced catalepsy model in rats

In the present study, amantadine was investigated regarding its PK-PD relationship in the L-DOPA-induced dyskinesia as well as haloperidol-induced catalepsy model in rats. It could be demonstrated that high CSF levels of amantadine are necessary to achieve a significant effect. L-DOPA-induced dyskinesia as well as haloperidol-induced catalepsy was reduced by amantadine at a dose of 30 mg/kg. This effective dose resulted in CSF levels of amantadine comparable to those observed in humans at therapeutic doses.

Amantadine, originally launched as a prophylactic agent against influenza A, has been used as treatment for PD for more than 40 years (Schwab et al., 1969). The drug is indicated for symptomatic treatment of PD as monotherapy as well as in combination with L-DOPA or DA receptor agonists. Moreover, infusion of amantadine remains the first line therapy during acute akinesia (Onofrj and Thomas, 2005). After discovery of its antidyskinetic properties, amantadine is now regarded as the gold standard medication for ameliorating L-DOPA-induced dyskinesia (Fahn, 2008; Pilleri and Antonini, 2015; Schaeffer et al., 2014). A recent study by Pahwa et al. reported a long-acting extended release formulation of amantadine as the most promising dyskinesia therapy for the near future (Pahwa et al., 2015). However, amantadine is not registered as an antidyskinetic drug. It is rather used off label for the treatment of L-DOPA-induced dyskinesia.

During the last decades, amantadine was extensively studied in various animal models of PD including 6-OHDA-lesioned rats, MPTP-treated monkeys or haloperidol-induced cataleptic rats (Blanchet et al., 1998; Danysz et al., 1994; Danysz et al., 1997). Additionally, there are several studies investigating amantadine in animal models of L-DOPA-induced dyskinesia (Blanchet et al., 1998; Dekundy et al., 2007; Lundblad et al., 2002). These preclinical results translate well into PD patients, where amantadine is reported to be the only drug considered clinically useful to treat L-DOPA-induced dyskinesia (Ferreira et al., 2013; Fox et al., 2011).
In order to bridge the gap between preclinical and clinical studies as well as guide dose finding studies, animal models should achieve high predictive validity suggesting that treatment strategies that are effective in the model will also be successful in human patients. In this context, brain, plasma as well as CSF levels of amantadine are important pharmacokinetic parameters which can be used in combination with behavioural observations.

Amantadine binds to the PCP binding site of the NMDA receptor with a \( k_i \) value of 10 µmol/l as well as the sigma-1 receptor (Kornhuber et al., 1991; Kornhuber et al., 1993; Kornhuber et al., 1995). Although the antagonistic potency at the NMDA receptor is considered weak, there is growing evidence that amantadine alleviates dyskinesia by blockade of NMDA receptors within excitatory pathways of the basal ganglia motor loops (Brotchie, 2005; Chase et al., 2000; Verhagen Metman et al., 1998a; Verhagen Metman et al., 1998b).

In the present study, amantadine was analysed for potential effects at various target sites by evaluating its binding profile against a selected set of 68 targets. No significant responses were obtained, although for sigma-1 receptors and glutamatergic NMDA receptors (PCP binding site), a weak antagonism of 35 % and 27 %, respectively, was observed. Theoretically, the effect of amantadine could be mediated in part by sigma-1 receptors. Indeed, a study by Paquette et al. revealed that sigma ligands such as the sigma-1 antagonist BMY-14802 reduced L-DOPA-induced dyskinesia in rats, but not the NMDA antagonist MK-801 (Paquette et al., 2008). However, a follow-up study of the same group showed that BMY-14802 suppressed dyskinesia via a 5-HT\(_{1A}\) receptor agonistic mechanism (Paquette et al., 2009).

In order to evaluate the pharmacokinetic profile of amantadine, the exposure to CSF, plasma as well as brain tissue was analysed using LC-MS/MS. Amantadine 30 mg/kg resulted in plasma and brain levels which were about 10-fold higher than following amantadine 3 mg/kg. Furthermore, brain/plasma ratios of 5.47 and 8.22 for amantadine 3 mg/kg and 30 mg/kg, respectively, reflect the good CNS availability of the compound. Indeed, maximum CSF levels, which serve as a good surrogate marker for the active, free drug concentration in the brain (Lin, 2008), were found to be 692 nmol/l for amantadine 3 mg/kg and 13440 nmol/l for amantadine 30 mg/kg. Particularly, amantadine at a dose of 30 mg/kg achieved a
Discussion

The CSF concentration of 13 µmol/l, which is in the range of the published kᵢ value of amantadine at the PCP binding site of the NMDA receptor (10 µmol/l) (Kornhuber et al., 1995). A kᵢ value of 10 µmol/l indicates a low affinity to the respective receptor target. The weak binding of amantadine at NMDA receptors has also been confirmed in the presented data of the LeadProfilingScreen®. Obviously, amantadine needs to achieve a high CSF exposure to ensure antagonism at NMDA receptors and thus enable a pharmacological effect.

In order to assess the pharmacodynamic profile of amantadine, the compound was investigated in two rat models of PD, namely the haloperidol-induced catalepsy as well as the L-DOPA-induced dyskinesia model. Amantadine at a dose of 30 mg/kg was able to attenuate haloperidol-induced catalepsy in rats. This corresponds well to the work of Danysz et al. who found that amantadine led to a dose-dependent decrease of haloperidol-induced catalepsy in rats with a threshold dose of 25 mg/kg (Danysz et al., 1994). In the L-DOPA-induced dyskinesia model, administration of L-DOPA to 6-OHDA-lesioned rats causes debilitating abnormal involuntary movements which resemble dyskinesia as seen in humans (Cenci et al., 1998). In the present study, amantadine at a dose of 30 mg/kg was shown to be effective in alleviating dyskinetic movements of rats decreasing axial, limb as well as orolingual dyskinesia by 80 %. These data are in agreement with results reported by other groups observing a 50 % reduction of dyskinesia in rats following administration of amantadine (20-40 mg/kg) (Dekundy et al., 2007; Lundblad et al., 2002).

Multiple clinical studies investigated the efficacy of amantadine in PD patients (da Silva-Júnior et al., 2005; Luginger et al., 2000; Snow et al., 2000; Verhagen Metman et al., 1999). The common therapeutic dose which yielded a clinical effect ranged from 200 mg/day to 300 mg/day which on average is equivalent to a dose of 3 mg/kg calculated for a body weight of 70 kg. The maximum serum levels in patients were found to be 5.7 µmol/l for amantadine 200 mg/day and 13.5 µmol/l for amantadine 300 mg/day (Kornhuber et al., 1995). These patients achieved a CSF exposure in the range of 4.3 and 9.5 µmol/l, respectively. In addition, other studies reported plasma concentrations of 5-7 µmol/l (Rizzo et al., 1973) and 10 µmol/l (Verhagen Metman et al., 1999) after treatment with amantadine 300 mg/day. The reported human plasma levels do not match the preclinical plasma.

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levels (28 µmol/l) of the behavioural effective dose in the present study. However, human CSF levels fit well to preclinical CSF levels of the present study (13 µmol/l), indicating that CSF levels might be a better parameter to predict clinical efficacy, at least in the case of amantadine. Since amantadine enters the brain by passive carrier-mediated processes (Spector, 1988), a possible reason for the fact that different plasma levels of amantadine result in approximately equivalent CSF levels may be species differences between rats and humans concerning these carrier molecules.

**Conclusion**

The present study demonstrated that the haloperidol-induced catalepsy as well as L-DOPA-induced dyskinesia model in rats shows predictive validity in terms of the antiparkinsonian and antidyskinetic effect of amantadine. It has been revealed that it is of fundamental importance to consider the behavioural as well as pharmacokinetic analysis of the drug. This becomes apparent when considering the minimal behaviourally effective dose of amantadine in rats which is about 10-fold higher than in humans. Additionally, the therapeutic doses of amantadine in preclinical and clinical studies result in considerably different plasma levels. However, albeit these discrepancies in plasma levels, CSF exposure in rats corresponds well to therapeutic CSF levels in the clinic. These data indicate that measurement of plasma levels is not sufficient to translate preclinical effects into clinical efficacy in the case of amantadine. CSF exposure is a better parameter to predict therapeutic efficacy, since CSF levels reflect the unbound drug concentration in the brain which is assumed to evoke the pharmacological response by interaction with its receptor target. High levels of amantadine in CSF are necessary to demonstrate a significant behavioural effect which can be translated from rats to humans and backwards.
4.4 Establishment of an AAV-α-syn-overexpression model in rats for the investigation of olfactory dysfunction

PD has been traditionally considered as a neurodegenerative movement disorder. However, clinical studies have evidenced that PD patients also suffer from non-motor symptoms including olfactory deficits, sleep disturbances, mood disorders as well as constipation (Chaudhuri et al., 2011; Chaudhuri and Naidu, 2008; Schapira and Tolosa, 2010; Visanji and Marras, 2015). These symptoms frequently predate the onset of the classical motor phenotype by years and indicate the so-called premotor phase of PD.

Olfactory dysfunction is becoming increasingly recognised as a symptom of PD and comprises impairments in odour detection, identification and discrimination (Doty et al., 1988; Doty, 2012b; Tissingh et al., 2001). The smell deficit may occur as early as four years before the onset of motor disturbances (Ross et al., 2008). Therefore, an early detection of hyposmia may serve as a predictive preclinical biomarker for PD, allowing the identification of patients with an increased risk for future disease (Haas et al., 2012; Kranick and Duda, 2008; Morley and Duda, 2010; Postuma et al., 2012; Xiao et al., 2014). An early diagnosis is crucial for neuroprotective and disease-modifying therapies which require early intervention to achieve their maximum effects.

The molecular mechanisms responsible for the smell deficit remain largely elusive. There is convincing evidence that dopaminergic modulation plays an important role in odour information processing, since DA is known to inhibit the olfactory transmission within the olfactory glomeruli of the BO (Davila et al., 2003; Doty, 2012a; Hsia et al., 1999). Interestingly, Huisman et al. found a 100 % increase of dopaminergic neurons in the olfactory bulb of parkinsonian patients, which may be due to a compensatory mechanism against the loss of dopaminergic neurons in the basal ganglia (Huisman et al., 2004). This altered DA homeostasis in olfaction-related brain regions is suggested to result in hyposmia, and may also explain why olfactory dysfunction does not respond to dopaminergic treatment strategies (Doty et al., 1992).
Neuropathological studies by Braak and colleagues demonstrated that PD pathology is supposed to start in olfactory regions, such as the BO and AON, as revealed by the presence of Lewy bodies and α-syn aggregates (Braak et al., 2003; Daniel and Hawkes, 1992; Pearce et al., 1995). Lewy pathology in the AON was accompanied by a reduction in the total number of AON neurons, which strongly correlated with disease duration (Pearce et al., 1995). Moreover, premature deposition of α-syn aggregates in the BO is reported to impair the synaptic efficiency in the olfactory glomeruli (Mundinano et al., 2011). These early neuropathological changes in structures involved in the sense of smell may result in the appearance of smell loss very early in the course of PD. This idea is further supported by studies concerning incidental Lewy body disease which demonstrate that olfactory deficits are strongly associated with the presence of incidental Lewy bodies in brains of patients without parkinsonism (Del Tredici et al., 2002; Driver-Dunckley et al., 2014; Ross et al., 2006).

Although the Braak model suggests a pathogenic process for PD that begins in the lower brainstem and olfactory structures, it is not clear if these regions really represent the induction sites of the disease (Visanji et al., 2013). In fact, it is known that PD extends far beyond the CNS and involves the peripheral nervous system as well as the enteric nervous system. This corresponds to clinical observations in PD patients showing additional non-motor symptoms related to gastrointestinal disturbances which often precede the motor symptoms of PD.

The present study demonstrated that AAV5-mediated overexpression of human wild-type α-syn in the BO of rats is able to induce olfactory deficits reminiscent of those observed in parkinsonian patients. Immunohistochemical analysis in α-syn-overexpressing rats shows the accumulation of human wild-type α-syn throughout the olfactory bulb. Here, human α-syn could already be detected three weeks after virus injection, whereas the striatum was free from human α-syn at this early time point. However, 11 weeks after virus injection, α-syn immunoreactivity could also be seen in the nigrostriatal system as indicated by positive α-syn staining in the striatum. This is reminiscent of human PD brains, where α-synucleinopathy is reported to initially appear in the BO followed by progression in a predictable pattern to regions located more rostrally (Braak et al., 2003). Lerner and Bagic reviewed anatomical connections
between the olfactory system and brainstem nuclei and proposed a possible transmission pathway of Lewy pathology through the brainstem nuclei to finally reach the SN (Lerner and Bagic, 2008). Moreover, a recent report of Höglinger et al. demonstrated the existence of a direct axonal dopaminergic projection from the SNpc to the olfactory bulb (Höglinger et al., 2015). Accordingly, there are several studies suggesting a spreading mechanism by which α-syn pathology may be transferred between anatomically interconnected brain regions (Recasens and Dehay, 2014; Rey et al., 2013; Ubeda-Banon et al., 2014; Ulusoy et al., 2013).

The current study examined the impact of α-syn overexpression in the BO of rats on the function of their olfactory system by assessing smelling behaviour using the buried food pellet test of odour detection/identification. This test relies on the animal’s natural tendency to use olfactory cues for foraging and can be used to investigate the ability to smell volatile odours (Yang and Crawley, 2009). In the buried food pellet test, AAV5-h-α-syn-WT-injected rats demonstrated impaired smelling behaviour compared to control rats. The olfactory deficits were already detectable three weeks after injection and persisted at nine weeks. These results are in line with the immunohistochemistry data of the present study, suggesting that overexpression of α-syn in the BO is sufficient to induce olfactory dysfunction which manifests promptly and continues over time.

To ensure that these findings were not caused by differences in motor behaviour, total activity of the rats was additionally recorded. There was no significant difference between all tested animals, indicating that rats overexpressing α-syn did not show any deficits in locomotion which could have affected smelling behaviour analysis.

In addition to this behavioural readout, the effect of human wild-type α-syn overexpression in the BO of rats on the integrity of dopaminergic nerve terminals in the striatum was investigated. Compared to control animals, AAV5-h-α-syn-WT-injected rats demonstrated a significant reduction in striatal DA levels 11 weeks after injection. No difference could be detected at the early time point of three weeks. Again, these data are in accordance with the immunohistochemical analysis of the present study, indicating that the later occurring α-synucleinopathy in the nigrostriatal system is linked to disturbances in the function of dopaminergic neurons.
Another interesting fact of the present study is that the smell loss observed in α-syn-overexpressing rats was detected at early time points when no DA depletion in the striatum was visible yet. This temporal evolution is in line with observations in human PD, suggesting that hyposmia predates the cardinal motor symptoms of the disease which are pathologically linked to striatal DA depletion (Ross et al., 2008).

Interestingly, a significant reduction of DA levels in the striatum was observed 11 weeks after AAV5-h-α-syn-WT injection into the BO of rats. However, the animals did not show any changes in locomotor behaviour. This can be explained by the fact that the magnitude of nigral degeneration and striatal DA depletion at this time point is still below the motor symptoms threshold. Indeed, striatal DA concentrations have to be reduced by about 80 % before manifestation of motor disturbances (Hornykiewicz and Kish, 1987).

Olfactory impairment is suggested to be an early premotor sign of PD useful to enable detection of patients that are at high risk for developing the disease. Experimental research concerning this early premotor phase of PD has attracted considerable interest, particularly in assessing the therapeutic effect of future disease-modifying strategies. While motor aspects of PD have been strongly investigated in experimental models, studies regarding the non-motor features are much less mature. Indeed, there are only a few PD animal models that are able to develop olfactory dysfunction (Bezard and Fernagut, 2014; McDowell and Chesselet, 2012).

One toxin-based model related to intranasal administration of MPTP led to an early disruption in olfactory discrimination abilities in rats and mice (Prediger et al., 2009; Prediger et al., 2010). Additionally, olfactory deficits could also be observed in mouse models based on unilateral or bilateral injection of 6-OHDA into the striatum (Bonito-Oliva et al., 2014; Valle-Leija and Drucker-Colín, 2014).

Similar defects in smelling behaviour have also been reported in transgenic mouse models of dopaminergic dysfunction, which is consistent with the view that DA may influence olfactory function. Mice with reduced expression of the VMAT-2, as well as mice deficient for the DA transporter or the DA D2 receptor, showed progressive deficits in olfactory discrimination skills (Taylor et al., 2009; Tillerson et al., 2006).
However, since abnormal accumulation of α-syn might play a crucial role in the neuropathology underlying olfactory dysfunction in PD, other animal models mostly rely on overexpression of this protein in wild-type or mutant forms (Löw and Aebischer, 2012). Bacterial artificial chromosome (BAC) transgenic rats expressing human wild-type α-syn were reported to show early smell deficits and later occurring locomotor impairments (Nuber et al., 2013). Lelan et al. found that transgenic rats with a double mutation in the human α-syn gene (A30P and A53T) display olfactory disturbances in the absence of motor impairments, as observed in most early PD cases (Lelan et al., 2011).

Additionally, there are different transgenic α-syn mouse lines found to recapitulate some of the non-motor symptoms of PD including hyposmia. In most of them, olfactory dysfunction was associated with the appearance of α-syn pathology within the olfactory system. This is in line with the Braak staging hypothesis suggesting the occurrence of α-syn inclusions in the BO during early premotor stages of PD (Braak et al., 2003). Mice overexpressing human wild-type α-syn showed olfactory impairments in aspects of multiple olfactory tests prior to the loss of nigrostriatal dopaminergic neurons (Fleming et al., 2008). Another human wild-type α-syn GFP mouse model was developed by Hansen et al. in 2013. These mice mimic highly relevant features of PD pathology and progressively exhibit deficits in olfactory functions (Hansen et al., 2013). In a similar transgenic mouse model of PD expressing human wild-type α-syn, rasagiline was reported to ameliorate olfactory deficits in terms of detection and discrimination abilities (Petit et al., 2013). Recently, Farrell et al. showed that aging mice expressing human A53T mutant α-syn display olfactory impairment that was paralleled by α-syn aggregation in olfactory structures (Farrell et al., 2014). A similar model was presented by Zhang and colleagues. Their studies indicated that human A53T α-syn transgenic mice have early deficits in odour discrimination and odour detection before motor symptoms occur (Zhang et al., 2015).

Another successful tool to model PD in rodents and non-human primates is viral vector-mediated overexpression of α-syn. This technology has been successfully applied for many years to mimic some of the core symptoms of the disease and has the advantage that overexpression of α-syn can be restricted to the area of interest (Bezard et al., 2013). However, currently, there is no study using AAV-
mediated overexpression of α-syn which could demonstrate olfactory impairment as an early and premotor symptom of PD.

Since the BO is thought to be a key structure in the pathology of olfactory dysfunction, a rat model based on targeted overexpression of α-syn in this important region was developed in the present study. α-syn accumulation in the BO was already visible at early time points and persisted over time. Later on, α-syn accumulation could also be seen in the striatum. This caudal-rostral transport of α-syn to deeper located brain regions resulted in DA depletion in the striatum, indicating a delayed α-syn progression in the pathogenesis of PD. The results of α-syn accumulation and DA depletion correlated well with behavioural deficits in olfaction which could be detected as soon as α-synucleinopathy in the BO occurred.

Conclusion

The present study demonstrated that AAV5-mediated overexpression of human wild-type α-syn in the BO represents an excellent tool to model olfactory dysfunction in rats. Additionally, the present data confirm that olfactory deficits occur early in the course of PD before the clinical presentation of motor impairment. These findings underpin the important role of olfactory dysfunction to be a premotor biomarker which reflects PD pathogenesis at early stages of the disease and can be used to follow PD progression. Thus, the present model may provide a valuable tool for screening neuroprotective and disease-modifying therapies in the early premotor stages of PD.
4.5 LC-MS/MS analysis of GABA and glutamate

GABA and glutamate are the most prominent amino acid neurotransmitters in the CNS. Monitoring GABA and glutamate in biomarker studies is of great importance for the investigation of neurochemical alterations in experimental models of PD and L-DOPA-induced dyskinesia. This requires a fast and reliable method for the analysis of the two amino acids.

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-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). The amino acids GABA and glutamate are polar molecules which offer low affinity to reversed-phase columns. However, their hydrophobic character and molecular size can be increased using pre-column derivatisation with OPA and MCE (Lindroth and Mopper, 1979; Tossman et al., 1983). Subsequently, the amino acid derivative can be analysed by FD or ECD. However, especially for the analysis of GABA in microdialysis samples, HPLC conditions are critical (Rea et al., 2005). It has been 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 such as a very long running time of 60 min. In addition to classical HPLC, 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 (Buck et al., 2009). This was achieved using a HILIC column which avoids time consuming sample pre-treatment. The total running time of the novel method was 3 min. Linearity was given in the range
from 1 nmol/l to 10 µmol/l for GABA and from 10 nmol/l to 10 µmol/l for glutamate. The LOQ was found to be 1 nmol/l for GABA and 10 nmol/l for glutamate.

In order to demonstrate the feasibility of the LC-MS/MS method in vivo, alterations of extracellular GABA and glutamate levels in the GP of rats following stimulation with potassium were determined using in vivo microdialysis. Basal levels of GABA and glutamate in the GP of rats were found to be 12.72 ± 1.31 nmol/l (mean ± SEM) and 189.02 ± 14.76 nmol/l (mean ± SEM), respectively (Buck et al., 2009). These results are consistent with previous studies in which basal levels of GABA and glutamate in the GP ranged from 12 to 48 nmol/l and 100 to 650 nmol/l, respectively (Grimm and See, 2000; Mela et al., 2007; Ochi et al., 2004; Windels et al., 2005). Stimulation with potassium enriched aCSF resulted in a 14- and 8-fold increase in extracellular GABA and glutamate levels, respectively (Buck et al., 2009).

Additionally, the present LC-MS/MS method was compared to the widely used HPLC-FD analysis of GABA and glutamate (Buck et al., 2009). A chromatographic separation of GABA and glutamate in standard samples was achieved within 20 min. The LOQ of the HPLC-FD method was found to be 5 nmol/l for both GABA and glutamate, which is in the same range as the LOQ of the present LC-MS/MS method. However, the HPLC-FD method required a pre-column derivatisation step and was more time consuming due to the 7-fold longer running time. Moreover, GABA could not be reliably analysed in microdialysis samples of the GP using HPLC-FD, since unknown peaks of biological origin coelute with GABA in some of the samples. Other groups have 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 differing effects of pharmacological compounds may arise due to the difficult chromatographic separation of GABA using HPLC. However, under optimised conditions such as keeping an exact pH value of the mobile phase and extending the retention time up to 60 min, 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 (Buck et al., 2009). It is rather a rapid method which allows detection of the analytes in a short running time of 3 min. Moreover, pre-treatment of the samples is not required due
Discussion

to the usage of a HILIC column which enables separation of the polar analytes from the matrix. In addition, the LC-MS/MS technique provides superior selectivity. Indeed, a triple quadrupole mass spectrometer 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 fluorophore agent naphthaline-2,3-dicarboxaldehyde and separated within 10 min by micellar electrokinetic chromatography followed by detection with laser-induced fluorescence. The LOD was found to be 3 nmol/l for GABA and 15 nmol/l for glutamate. Moreover, a novel technique was developed for the detection of glutamate by implantation of ceramic-based multisite microelectrodes achieving a LOD of 500 nmol/l (Burmeister et al., 2002). Similarly, an enzyme-based microelectrode array with fast response times and low detection limits for glutamate has been established (Hascup et al., 2008). Additionally, a novel biosensor based on quartz crystal microbalance was designed for the analysis of GABA with a LOD of 42 µmol/l (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 the usage of derivatisation reagents, which is a complex and time consuming procedure. These prerequisites are necessary since the amino acids are very hydrophilic and poorly retained on reversed-phase columns. Ma et al. investigated GABA and glutamate in rat brain tissue using atmospheric pressure chemical ionisation LC-MS (Ma et al., 1999). Preparation of the sample was performed using a cation-exchange column and a subsequent evaporation step. The LOD for GABA and glutamate was 24 µmol/l and 34 µmol/l, respectively. Song et al. developed a capillary LC-MS/MS method for the quantification of GABA in human plasma and CSF (Song et al., 2005). 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 48 nmol/l was achieved. Eckstein and colleagues analysed GABA and glutamate levels in CSF using positive ESI LC-MS/MS (Eckstein et al., 2008). In this study, heptafluorobutyric acid was used as ion-pairing agent. For GABA and glutamate a LOQ of 75 nmol/l and 53 nmol/l was achieved, respectively. Another approach was developed by Zhang et al. who applied a capillary LC-MS/MS method for the determination of six neurotransmitters including GABA and glutamate in the extracellular brain fluid of monkeys obtained by the push-pull sampling method (Zhang et al., 2007). Using a fused silica capillary tubing packed with polyhydroxyethyl aspartamide particles as HILIC stationary phase, a running time of 26 min including reconditioning was required. The LOD for GABA and glutamate was reported to be 4 nmol/l and 20 nmol/l, respectively, and linearity ranged from 20 to 4000 nmol/l for GABA and from 100 to 100000 nmol/l 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 (Buck et al., 2009). In contrast, the LOQ of the LC-MS/MS method presented here was found to be 1 nmol/l for GABA and 10 nmol/l for glutamate requiring a sample volume of 10 µl. Taking advantage of this method, a higher time resolution for the microdialysis experiment may be achieved due to the requirement of lower sample volumes, which in turn allows shorter sampling intervals.

**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 min. 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 nmol/l for GABA and 10 nmol/l for glutamate, which is sensitive to quantify extracellular levels of GABA and glutamate in microdialysis samples of the GP.
4.6 LC-MS/MS analysis of glycine

Glycine is an important amino acid neurotransmitter in the CNS and its measurement in CSF and brain microdialysis samples is of great interest to neuropharmacological and biomarker studies. Therefore, an accurate, precise and preferably fast quantification method is essential.

In addition to capillary electrophoresis (Bowser and Kennedy, 2001; Klinker and Bowser, 2007; Li et al., 2008), one conventional technique for the determination of amino acids in biological samples is reversed-phase HPLC coupled to FD which involves pre-column derivatisation with OPA and MCE (Biermann et al., 2013; Lindroth and Mopper, 1979; Piepponen and Skujins, 2001). Alternatively, HPLC methods combined with electrochemical (Qu et al., 1998; Rowley et al., 1995) or ultraviolet (Kang et al., 2006; Zhang et al., 2012) detection have been described. However, these methods have some limitations due to their challenging separation, long running times and the use of potentially toxic derivatisation reagents such as OPA, MCE, dabsyl chloride, dansyl chloride or 2,4-dinitrofluorobenzene as well as ninhydrin (Moore et al., 1958) or fluorescamine (Udenfriend et al., 1972).

To overcome these problems, mass spectrometric approaches have been developed. Among these are gas chromatography mass spectrometry (GC-MS) (Wood et al., 2006), capillary electrophoresis mass spectrometry (CE-MS) (Soga and Heiger, 2000; Williams et al., 2007), LC-MS (Özcan and Senyuva, 2006) and LC-MS/MS (Dalluge et al., 2004; Johnson, 2011; Petritis et al., 2000; Piraud et al., 2003; Qu et al., 2002a) techniques, which became a valuable tool for amino acid analysis. Most of these methods applied a butylation step in the sample preparation procedure (Casetta et al., 2000; Dietzen et al., 2008; Harder et al., 2011) or required confounding ion-pairing reagents. Amino acid analysis including glycine without derivatisation was either time consuming (Qu et al., 2002b) or suffered from a loss of sensitivity (Piraud et al., 2005a).

Thus, the aim of the present study was to develop a fast and sensitive method for the quantification of glycine in CSF and in vivo microdialysis samples using LC-MS/MS (Voehringer et al., 2013). This was achieved without any derivatisation step. The total running time of the novel method was 5 min. The LOQ was
determined as 100 nmol/l, while linearity was given in the range from 100 nmol/l to 100 µmol/l.

In order to demonstrate the feasibility of the LC-MS/MS method in vivo, the effect of the GlyT1 inhibitor LY 2365109 on glycine concentrations in rat CSF was measured. Furthermore, changes of extracellular glycine levels in the rat striatum following administration of LY 2365109 were determined using in vivo microdialysis. Basal levels of glycine were found to be 10.38 ± 0.83 µmol/l (mean ± SEM) and 1.52 ± 0.14 µmol/l (mean ± SEM) in CSF and striatum, respectively (Voehringer et al., 2013). This is consistent with previous studies which reported similar values of basal glycine ranging from 8.8 to 12 µmol/l in CSF and from 1.6 to 10.5 µmol/l in the striatum of rats (Alberati et al., 2012; Bannai et al., 2011; Halonen et al., 1990; Johnson et al., 2003; Kawai et al., 2012; Kennedy et al., 2002; Klinker and Bowser, 2007; Nagy et al., 2010). A 3-fold increase in glycine concentration was observed in rat CSF 4 h after administration of LY 2365109 compared to vehicle treatment (Voehringer et al., 2013). This rise was similar to the 2-fold elevation in extracellular glycine levels in the rat striatum after injection of LY 2365109 as determined by in vivo microdialysis (Voehringer et al., 2013).

There are several studies reporting an increase in CSF and striatal glycine levels following treatment with GlyT1 inhibitors (Alberati et al., 2012; Nagy et al., 2010; Perry et al., 2008; Pinard et al., 2010).

The present study showed that GlyT1 inhibitor treatment leads to an increase of glycine levels in CSF that is very similar to the rise in extracellular glycine levels in the striatum. This suggests that CSF glycine levels are a readily accessible surrogate marker to study target engagement of GlyT1 inhibitors in the clinic.

In order to demonstrate the reliability of the present LC-MS/MS method, glycine was additionally analysed by commonly used HPLC-FD. When comparing the analytical properties of the HPLC-FD method and the LC-MS/MS technique, both methods showed similar sensitivity in a range which allowed the analysis of glycine in CSF and microdialysis samples (Voehringer et al., 2013). In addition, each method demonstrated accuracy and precision characteristics within limits which are defined to be < 15 % by the FDA guideline on validation of bioanalytical methods. In both assays, the CSF or microdialysate matrix did not interfere with the analysis of glycine (Voehringer et al., 2013).
Importantly, the LC-MS/MS method shows two advantages compared to the HPLC-FD method as it first provides an almost 4-fold shorter running time allowing a significantly higher sample throughput. Second, no pre-column derivatisation step with potentially toxic or strong-smelling reagents is needed offering benefits in safety aspects and the day-to-day handling of the procedure. Another disadvantage of the derivatisation step is the decrease in fluorescence intensity over time due to the instability of the OPA/MCE reagent, which requires its replacement every 12 h. The LC-MS/MS technique offers another important advantage by enabling the parallel measurement of drug exposure levels and therefore allowing the direct correlation of a compound effect to altered neurotransmitter levels.

Analysis of amino acid neurotransmitters in biological fluids via LC-MS (Ma et al., 1999) and LC-MS/MS (Armenta et al., 2010; Bourcier et al., 2006; Cai et al., 2010; Eckstein et al., 2008; Piraud et al., 2005a; Piraud et al., 2005b; Qu et al., 2002b; Shimbo et al., 2009; Waterval et al., 2009) has been reported previously. These methods often utilise derivatisation or ion-pairing reagents resulting in complicated and time demanding sample pre-treatment procedures. Especially for the analysis of the very polar molecules GABA and glutamate, the combination of HILIC and mass spectrometric detection is a useful approach avoiding derivatisation (Buck et al., 2009; Zhang et al., 2007). However, there are limited studies on LC-MS or LC-MS/MS analysis of glycine in biological matrices such as CSF or microdialysates.

Fuchs et al. investigated glycine in human CSF using a LC-MS method including derivatisation with Marfey’s reagent (1-fluoro-2,4-dinitrophenyl-5-alanine amide) which achieved a LOQ of 140 nmol/l (Fuchs et al., 2008). The development of a LC-MS/MS method for the quantification of glycine in CSF, plasma and urine samples was reported by Fonteh and colleagues. In this study, glycine was converted to a chloroformate derivative and analysed with a LOD of 0.05 pmol/l (Fonteh et al., 2007). Uutela et al. compared different amino acid derivatives using LC-MS/MS analysis (Uutela et al., 2009). In a second part, they analysed glycine in rat brain microdialysates after derivatisation with propyl chloroformate achieving linearity ranging from 500 to 4000 nmol/l. In another approach, Wilson et al. determined glycine in human CSF samples using pre-column derivatisation with phenylisothiocyanate followed by LC-MS/MS. They reported a LOQ of 666 nmol/l.
(Wilson et al., 2011). Furthermore, Tang et al. employed a HILIC column and ESI-MS/MS to quantify glycine released from embryonal carcinoma stem cells with a LOQ of 532 nmol/l (Tang et al., 2012). In 2012, Song et al. published a LC-MS/MS method utilising benzoyl chloride derivatisation for the analysis of glycine out of brain microdialysates. This assay achieved a LOD of 500 nmol/l (Song et al., 2012).

When evaluating these preceding methods, it has to be considered that due to the analysis of more than one analyte some compromises have to be made. But summarised, these methods either require a derivatisation step or ion-pairing reagents resulting in a very long analysis time or fail to reach the LOQ of the LC-MS/MS method presented here (Voehringer et al., 2013).

**Conclusion**

A fast and reliable LC-MS/MS method for the quantification of glycine as biomarker in CSF and in vivo microdialysis samples was developed. This novel method shows several advantages compared to already established techniques such as no sample pre-treatment and a short running time allowing high sample throughput. The method was characterised in terms of linearity, precision and accuracy, which are all within FDA-approved limits. The procedure achieves a LOQ of 100 nmol/l, which makes the quantification of glycine in CSF samples and brain microdialysates possible as demonstrated in this study.
5. Summary

Parkinson’s disease (PD) is a progressive neurodegenerative disorder which manifests with a broad range of symptoms including motor and non-motor disturbances. The development of neuroprotective and disease-modifying drugs requires the presence of reliable biomarkers to monitor disease progression and to identify potential treatment effects. Furthermore, reliable animal models of PD recapitulating the human disease condition with high validity are urgently needed. Based on pharmacokinetic, neurochemical and behavioural observations, the symptomatic PD models of haloperidol-induced catalepsy in rats and reserpine-induced akinesia in mice have been modified. Interventions such as changing the route of administration of haloperidol as well as decreasing the dose of reserpine, reducing the latency time after reserpine injection and lowering the portion of acetic acid in the reserpine vehicle solution resulted in animal models of high predictive and face validity as well as enhanced animal welfare.

The concept of continuous dopaminergic stimulation (CDS) was evaluated in the haloperidol-induced catalepsy as well as reserpine-induced akinesia model in rats. It has been shown that continuous dopamine (DA) receptor stimulation using pramipexole (PPX) offered a clear therapeutic benefit compared to acute PPX treatment regarding early morning akinesia. The behavioural effects corresponded to in vivo microdialysis measurements in the striatum of rats showing a continuous decrease of extracellular DA levels and a constant PPX exposure following continuous PPX release.

Amantadine is the most commonly used drug to alleviate L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia in PD patients. Amantadine was investigated in the L-DOPA-induced dyskinesia as well as haloperidol-induced catalepsy model in rats. It has been demonstrated that high cerebrospinal fluid (CSF) levels of amantadine in a µmolar range were necessary to achieve antidyskinetic and anticataleptic efficacy in rats. This high CSF exposure in rats translated well into clinically effective CSF levels, highlighting the importance to combine CSF exposure measurements and behavioural observations to better bridge the gap between preclinical and clinical effects of amantadine.
Olfactory dysfunction, one of the first non-motor symptoms of PD, was evaluated as an early biomarker in a genetic adeno-associated virus (AAV)-based rat model of PD. It has been shown that AAV-mediated overexpression of human wild-type α-synuclein (α-syn) in the bulbus olfactorius (BO) of rats led to olfactory disturbances, measured by the buried food pellet test, which were detectable three weeks after viral vector injection and persisted at nine weeks. Moreover, α-syn was transported to deeper located brain regions resulting in striatal DA depletion 11 weeks after viral vector injection. These findings underpin the important role of olfactory dysfunction to be a premotor biomarker which reflects PD pathogenesis at early stages of the disease and can be used to follow PD progression.

γ-aminobutyric acid (GABA), glutamate as well as glycine are important amino acid neurotransmitters and their measurement in CSF and brain microdialysis samples is of great interest to neuropharmacological and biomarker studies, making an accurate, precise and fast quantification essential. A rapid and reliable liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of GABA and glutamate in brain microdialysates. The method provided high sensitivity and selectivity, no sample pre-treatment and a short running time of 3 min. A limit of quantitation (LOQ) of 1 nmol/l for GABA and 10 nmol/l for glutamate was achieved, which was sensitive to quantify extracellular levels of GABA and glutamate in microdialysis samples of the globus pallidus (GP). Another fast and reliable LC-MS/MS method was established for the quantification of glycine in CSF and brain microdialysis samples. This novel method showed similar advantages such as no sample pre-treatment and a short running time. The procedure achieved a LOQ of 100 nmol/l, which was sensitive for the quantification of glycine in CSF samples and brain microdialysates.

In conclusion, the present work demonstrates the face and predictive validity of PD animal models investigating symptoms such as catalepsy, akinesia or dyskinesia and evaluating PPX and amantadine as antiparkinsonian and antidyskinetic drugs. The AAV-α-syn-overexpression model recapitulating olfactory dysfunction as a premotor biomarker for progressive PD may provide a valuable tool for screening neuroprotective and disease-modifying therapies in the early premotor phase of PD. In order to support biomarker studies investigating GABA, glutamate and glycine, the rapid and sensitive LC-MS/MS methods can be used for the quantification of these compounds in brain microdialysis and CSF samples.
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Curriculum Vitae

Personal details
Patrizia Vöhringer

Date of birth 09.06.1983
Place of birth Biberach an der Riß

Work and practical experience
since 11/2013 Technical assistant
Boehringer Ingelheim Pharma GmbH & Co. KG,
Biberach
Department CNS Diseases Research

11/2012 - 10/2013 Technical assistant
Boehringer Ingelheim Pharma GmbH & Co. KG,
Biberach
Department Target Discovery Research

05/2009 - 10/2012 PhD thesis in Neuropharmacology
Boehringer Ingelheim Pharma GmbH & Co. KG,
Biberach and University of Ulm

Krankenhaus Barmherzige Brüder, Regensburg

05/2007 - 10/2007 Work placement
Maximilian-Apotheke, Regensburg

02/2004 - 03/2004 Work placement
Boehringer Ingelheim Pharma GmbH & Co. KG,
Biberach
Department Biopharmaceutical Process Science
02/2003 - 03/2003 Work placement
Apotheke am Adlerplatz, Mittelbiberach

09/2002 Work placement
Boehringer Ingelheim Pharma GmbH & Co. KG,
Biberach
Department Quality Operations

Education

2009 - 2015 University of Ulm
PhD student

10/2008 “Approbation” (license) as pharmacist

10/2002 - 03/2007 University of Regensburg, Studies of Pharmacy

08/1993 - 06/2002 Wieland-Gymnasium, Biberach
List of publications

Peer-reviewed publications


Ferger B., Buck K., Shimasaki M., Koros E., Voehringer P., Buerger E.; 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 in rats. Synapse 2010 Jul; 64(7): 533-41.


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Voehringer P., Stierstorfer B., Kreuz S., Ferger B.; *Adeno-associated virus (AAV)-mediated overexpression of α-synuclein in the olfactory bulb of rats to investigate olfactory dysfunction in Parkinson’s disease.* Manuscript prepared for publication.

**Poster presentations at national and international conferences**

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