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Experimental models of Parkinson’s disease with levodopa-induced dyskinesias and gait dysfunction: electrophysiological and behavioural measures in rats

THESIS

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To my family and friends
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
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<tr>
<td>AF64A</td>
<td>ethylcholine mustard aziridinium ion</td>
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<td>AI</td>
<td>asymmetry index</td>
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<td>AIMs</td>
<td>abnormal involuntary movements</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>AP</td>
<td>anterior-posterior</td>
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<tr>
<td>aPPTg</td>
<td>anterior pedunculopontine tegmental nucleus</td>
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<td>BG</td>
<td>basal ganglia</td>
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<td>ChAT</td>
<td>choline-acetyltransferase</td>
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<td>CnF</td>
<td>cuneiform nucleus</td>
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<td>CV</td>
<td>coefficient of variation</td>
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<td>DBS</td>
<td>deep brain stimulation</td>
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<td>ECG</td>
<td>electrocardiography</td>
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<td>ECoG</td>
<td>electrocorticogram</td>
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<td>EEG</td>
<td>electroencephalography</td>
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<td>EPN</td>
<td>entopeduncular nucleus</td>
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<tr>
<td>FFT</td>
<td>fast fourier transform</td>
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<tr>
<td>FIR</td>
<td>finite impulse response</td>
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<td>fMRI</td>
<td>functional magnetic resonance imaging</td>
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<td>FRA</td>
<td>Fos-related proteins</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>GPe</td>
<td>external segment of globus pallidus</td>
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<tr>
<td>GPI</td>
<td>internal segment of globus pallidus</td>
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<td>HP</td>
<td>hemiparkinsonian</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>---------------</td>
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<tr>
<td>HP-LID</td>
<td>hemiparkinsonian with levodopa-induced diskinesia</td>
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<td>ISI</td>
<td>inter-spike interval</td>
</tr>
<tr>
<td>L</td>
<td>lateral</td>
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<tr>
<td>L-DOPA</td>
<td>levodopa</td>
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<td>LFP</td>
<td>local field potential</td>
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<td>LIDs</td>
<td>levodopa-induced dyskinesias</td>
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<td>MCx</td>
<td>motor cortex</td>
</tr>
<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
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<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PD</td>
<td>Parkinson’s disease</td>
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<td>PDFs</td>
<td>probability density functions</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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<td>PPN</td>
<td>pedunculopontine nucleus</td>
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<td>PPNc</td>
<td>pedunculopontine nucleus pars compacta</td>
</tr>
<tr>
<td>PPNd</td>
<td>pedunculopontine nucleus pars dissipata</td>
</tr>
<tr>
<td>pPPTg</td>
<td>posterior pedunculopontine tegmental nucleus</td>
</tr>
<tr>
<td>PPTg</td>
<td>pedunculopontine tegmental nucleus</td>
</tr>
<tr>
<td>RPM</td>
<td>round per minute</td>
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<tr>
<td>s.c.</td>
<td>injected subcutaneously</td>
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<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<td>SNc</td>
<td>substantia nigra pars compacta</td>
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<tr>
<td>SNr</td>
<td>substantia nigra pars reticulata</td>
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<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
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<tr>
<td>STWA</td>
<td>spike-triggered waveform average</td>
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<tr>
<td>SU</td>
<td>single unit</td>
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<td>V</td>
<td>ventral</td>
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1 Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNC). With the progression of disease, severe motor and non-motor dysfunctions take place, such as tremor, rigidity, bradykinesia, postural instability, but also cognitive and behavioural impairments. While the mechanism underlying the neurodegeneration remains unknown, most of the current pharmacological and surgery therapies focus on relieve of clinical symptoms. The dopaminergic precursor, levodopa, remains the mainstay of therapy since the 1970s.

Chronic use of levodopa, however, often leads to therapy-related motor complications such as motor fluctuation or the “on-off” phenomenon and abnormal involuntary movements, termed levodopa-induced dyskinesias (LIDs). Although consensus has been reached that both progressive nigral denervation in the basal ganglia (BG) and pulsatile dopamine stimulation contribute to the development of LIDs (Nadjar et al., 2009), the underlying pathophysiology remain elusive. In PD patients, electrophysiology studies have reported reduced oscillatory beta band activity and enhanced theta band activity in the BG during expression of LIDs (Alonso-Frech et al., 2006; Lozano et al., 2000; Obeso et al., 2000). However, little is known with regard to the differences of neuronal single units and oscillatory activity in patients with advanced PD with or without peak-dose dyskinesias. In the first project we were interested in the neuronal firing activity of the entopeduncular nucleus (EPN, the analogue to the major output site of the BG motor loop in human, the internal segment of globus pallidus “GPi”), and its coherence with the motor cortex (MCx) field potentials in the 6-hydroxydopamine (6-OHDA) lesioned rat model of PD with or without established LIDs before and after levodopa-injection, i.e., a model for advanced PD with peak-dose dyskinesias on/off levodopa.

Gait and postural dysfunctions in advanced PD are another troublesome but frequently occurring problem, which often does not respond to either levodopa or electrical stimulation of the subthalamic nucleus (STN) or the GPi. Post mortem studies in patients with PD and non-human primate models have shown that cholinergic neurons in the pedunculopontine nucleus (PPN), which together with the cuneiform nucleus (CuF) forms the mesencephalic locomotor region (MLR), degenerate in parallel to dopaminergic neu-
rons in the SNC (Zweig et al., 1989; Jellinger, 1988). This is considered important for the pathophysiological mechanisms leading to these symptoms. Electrical stimulation of the PPN has been tested for treating these symptoms, using stimulation parameters thought to stimulate the remaining neurons, however, with variable results and with substantial controversy, where exactly the optimal site for stimulation is located (Ferraye et al., 2010; Mazzone et al., 2005; Moro et al., 2010; Plaha and Gill, 2005; Stefani et al., 2007). The PPN is heavily interconnected with the BG motor loop, and also act as an information relay site to lower motor regions in the brainstem and spinal cord (Alam et al., 2011). One recent study using a rat model observed a reduction in locomotion after lesioning of a restricted portion of the anterior but not the posterior part of the pedunculopontine tegmental nucleus (PPTg, analogue to the PPN in primates; Alderson et al., 2008). Whether the effects of the anterior PPTg (aPPTg) lesions are achieved through the effects of cholinergic neurons on descending motor projections, or through effects on the BG motor loop, possibly via the CnF as suggested by Alam et al., (2012), has not been investigated. In the second project, we examined the effects of specific cholinergic lesions of either the aPPTg or the posterior PPTg (pPPTg) on rodent gait-related behaviour and extracellular neuronal activity of the unlesioned part of the PPTg, as well as on the CnF and the EPN.

Together, these investigations utilizing electrophysiology and behaviour approaches will help us to extend our knowledge regarding the neuronal mechanisms involved in symptoms that develop in advanced PD.
2 Levodopa-induced dyskinesias and gait disturbances in Parkinson’s disease

2.1 Parkinson’s disease

PD is one of the most common neurodegenerative disorders secondly only to Alzheimer’s disease in industrialized society, with a prevalence of about 1% in the population over 60 (de Lau and Breteler, 2006). It is characterized by a number of disturbances of motor function including tremor at rest, rigidity, akinesia (or bradykinesia) and postural instability (see Fig. 2.1). These cardinal features of PD (Jankovic, 2008) are accompanied by manifestations of symptoms of different kinds and variable severity, such as autonomic disturbances, sensory alterations, sleep dysfunction, cognitive impairment. Diagnosis of PD is mainly based on the typical neurological findings, their evolution over the course of the disease and responsiveness to levodopa.

The major pathological hallmarks of PD is the presence of Lewy bodies and the loss of dopaminergic neurons in the SNc leading to dopamine depletion in the nigrostriatal pathway, which triggers a cascade of functional changes affecting the whole BG network. The dopamine depletion in the SNc and subsequent changes in the neuronal activity within the BG motor loop would then result in the aforementioned alterations in motor functions (Alves et al., 2008; Galvan and Wichmann, 2008). Electrophysiological studies have reported abnormal neuronal activities in the BG in both animal models and patients with PD, specifically a greater tendency to discharge in bursts and with a higher degree of synchronized oscillatory beta band activity (13-30 Hz; Hashimoto et al., 2003; Wichmann and DeLong, 2006). However, aside the neuronal loss in the SNc, the neurodegenerative effects in PD affects several other nuclei as well, such as the PPN, amygdala, ventral tegmental area, locus coeruleus, raphe nuclei and the vagal dorsal motor nucleus (Dauer and Przedborski, 2003; Braak et al., 2003; Lang and Lozano, 1998). This implies that other neurotransmitter, such as cholinergic, adrenergic, and serotonergic systems, are also involved in the various clinical symptoms in PD.

As the mechanism underlying this neurodegeneration remains unknown, PD is basically incurable at present. A number of symptomatic therapies have been developed for the
improvement of patient’s quality of life, among which, levodopa remains the mainstay since its first introduction into the disease. Other medications include dopamine agonists that act on the nigra-striatal dopamine pathway similar to levodopa, i.e., monoamine oxidase type B inhibitor that slows down the breakdown of dopamine in the BG, and a number of non-dopaminergic agents that act on other neurotransmitter systems involved in PD as mentioned above (Rascol et al., 2003). Deep brain stimulation (DBS), i.e., stimulation of specific brain regions using electrical impulses through implanted electrodes, is frequently been used as a neurosurgical procedure for otherwise intractable cases of PD. Other neurosurgical procedures include stereotactic ablative surgeries of certain targets like the motor thalamus and the GPi (Fasano et al., 2015; Martinez-Ramirez et al., 2015).

2.2 Levodopa-induced dyskinesias

Chronic treatment with levodopa is associated with the emergence of LIDs, defined as abnormal involuntary dyskinetic movements induced by levodopa administration. LIDs are common in late stage of PD, especially in patients with early onset of disease. Clinical studies have observed that about 53% of younger onset patients (onset age 50-59 years) develop dyskinesias at 5 years as compared to 16% with the age of onset at 70-79 years (Kumar et al., 2005). Certain mutations such as the PARK2 (parkin), PARK6 (pink-1)
and PARK7 (DJ-1) have been associated with a higher risk of levodopa-related motor complications (Penney et al., 1996; Shoulson et al., 1996; Schrag and Schott, 2006). It remains unclear whether these genetic abnormalities have a direct effect on the risk of developing LIDs or via other mechanisms consistent with the earlier age at onset. Other risk factors include female gender (Lyons et al., 1998; Zappia et al., 2005), lower body weight (Sharma et al., 2006) and history of non-smoking (Zappia et al., 2005). Besides, a negative association of resting tremor as a first sign of PD and the development of LIDs has also been reported more recently (Kipfer et al., 2011).

**Clinical Features**

The clinical manifestation of LIDs covers a broad clinical spectrum of different types of involuntary movements ranging from chorea affecting the limbs and trunk, slow dystonic movements, fixed dystonic postures or, more rarely, myoclonus or ballism (Fig. 2.2; Hametner et al., 2010). The most common phenotype is the “On” state LID or “Peak dose” dyskinesia, which occurs around the peak level of levodopa-derived dopamine in the brain in parallel with the maximal anti-parkinsonian benefit. It is usually generalized, manifesting as chorea-like movements involving the head, trunk and limbs, and sometimes even respiratory muscles (Thanvi et al., 2007). These are often exaggerated by stress or activity and are typically asymmetric (Nutt, 1990; Mones et al., 1969; Murphy, 1978; Marconi et al., 1994).

Other phenotypes of LIDs include the “Off” state LID, which is usually manifested by dystonia-like movements occurring when plasma levodopa levels are low, and the “biphasic” dyskinesia, which is characterized by stereotyped repetitive slow (< 4 Hz) movements appearing at the onset and offset of the levodopa effect.

**Pathophysiology**

The pathogenesis of LIDs remains incompletely understood. Consensus has been reached that progressive nigral denervation and chronic pulsatile dopaminergic stimulation play a critical role. A chronic dopaminergic stimulation on a denervated substantia nigra induces a process of sensitization such that each following administration modifies the response to subsequent dopaminergic treatments, which is referred to as the “priming” process (Tambasco et al., 2012). Increased responsiveness of postsynaptic dopamine receptor (possibly D1) and glutamate receptor N-methyl-D-aspartate (NMDA) have been observed in the striatum (Gerfen et al., 1990; Nash and Brotchie, 2000), which could be involved in the priming process. Both receptors are expressed along the dendritic spines of the medium size γ-aminobutyric acid (GABA)-ergic neurons. Enhanced glutamatergic
input and altered dopamine responsiveness further leads to decreased neuronal activity in the GPi and eventually to the disinhibition of the thalamus and motor cortex (Thanvi et al., 2007). Involvement of other non-dopaminergic systems, such as α2 adrenergic, serotonergic, cannabinoid and opioid have also been reported (Brotchie, 2005). Further, down-stream changes in the genes and protein synthesis, which could be involved in the neuronal plasticity during development of LIDs, are discussed (Fig. 2.3; Calon et al., 2003).

Management

As the pulsatile dopaminergic stimulation is considered to be important in the genesis of LIDs, it is anticipated that any strategies with a “dopa-sparing” technique or one that can produce smooth dopaminergic stimulations may prevent or treat LIDs. These mainly include the use of controlled-release preparations of levodopa, continuous delivery of levodopa via a duodenal infusion pump, use of dopamine receptor agonists or other medications acting on non-dopaminergic systems such as NMDA or serotonergic receptors, and also functional surgery (Thanvi et al., 2007; Manson et al., 2011; Loher et al., 2002; Jankovic et al., 1999).

In routine clinical practice, younger and biologically fit older patients are usually given a
Figure 2.3: Schematic representation of sequence of events leading to levodopa-induced dyskinesias (LIDs). FRA, Fos-related proteins; NMDA, N-methyl-D-aspartate (adapted from Thanvi et al., 2007)

dopamine receptor agonist as the initial monotherapy for the control of PD motor symptoms, in order to delay the priming of LIDs. In patients with late onset of PD, levodopa is usually not withheld since the risk of LIDs is substantially low in these patients. Once LIDs are established, levodopa dose reduction combined with adjunctive dopamine receptor agonist can be used as a strategy to reduce the use of levodopa. When this fails, amantadine, or low dose clozapine with close hematological monitoring can be the next strategy. Continuous subcutaneous infusion of apomorphine can be used as an alternative strategy for the treatment of difficult LIDs.

DBS of the STN or the GPi is also successfully used to relieve dyskinesias in addition to treating the cardinal motor symptoms of PD. The antidyskinetic effect depends to some extent on the target. Stimulation of the GPi has a direct anti-dyskinetic effect, i.e., dyskinesias are improved while the need for levodopa remains unchanged. On the other hand, STN stimulation allows reduction of levodopa, hence relieving dyskinesias, but some studies also suggested a direct anti-dyskinetic effect upon chronic stimulation (Oyama et al., 2012; Follett, 2004; Krack et al., 2002).

2.3 Gait disorders

Gait disturbances form part of the axial symptoms observed in PD and can significantly impact the quality of life for patients. These comprise the typical “Parkinsonian gait” with small shuffling steps, reduction of gait speed and a forward-leaning stance, which
is considered as one of the diagnostic criteria of PD, and the so called “freezing of gait” and postural instability, which frequently occurs in advanced PD and represents a major therapeutic challenge since it often does not respond to levodopa and DBS of the STN or GPi.

Clinical features

In the early stage of PD, the gait alterations are usually of moderate extent, characterized by a reduction of stride length (Stolze et al., 2001) and an unchanged or slightly increased cadence. Studies using imposed gait speed showed that patients were able to increase their cadence. The stride length, however, remained the same, which implies that the gait hypokinesia is mainly associated with the internal generation of adapted stride length, and that the increase of cadence could be a compensatory effect (Morris et al., 1994). After a few years of chronic levodopa medication, some patients report fluctuations of the ability to walk, which are part of the motor fluctuations related to levodopa therapy.

In the late stage of PD, severe gait disturbances, together with postural instability and postural abnormalities, constitutes the most characteristic axial motor symptoms in patients. These symptoms are very common and closely associated with increased risk of falls that could significantly impact a person’s mobility and quality of life. Freezing of gait is a special clinical phenomenon of the gait disturbance in advanced PD, which is defined as “a brief, episodic absence or a marked reduction of forward progression of the feet despite the intention to walk” (Bloem et al., 2004; Giladi and Nieuwboer, 2008). By its definition it includes episodes in which the patient cannot initiate gait and arrests in forward progression during walking (freezing episodes), as well as episodes of shuffling forward with steps that are millimeters to a couple of centimeters in length (Nutt et al., 2011). The freezing episode usually lasts a couple of seconds, but in rare cases it appears almost continuous and the patients experience complete akinesia with no limb or trunk movement. Clinical features accompanying freezing of gait include: (1) alternating knee trembling at the frequency of 3-8 Hz (knee trembling, Yanagisawa et al., 2001; Hausdorff et al., 2003); (2) hastening or an increased cadence with shuffling small steps (Nieuwboer et al., 2001); (3) can be relieved by attention focusing or external stimuli (cues); and (4) can be asymmetric, affecting only one foot or being elicit more easily by turning one direction.

Pathophysiology

The hypokinetic gait in the early stage of PD and the motor fluctuation appear to be related to the dopamine depletion following the loss of dopaminergic neurons in the SNC,
since dopaminergic therapy is effective in both conditions. However, with regard to the
gait disturbances and postural instability in the late stage of PD, traditional treatments
for PD such as the dopamine replacement therapy and physiotherapy often provide only
partial relief of the symptoms. Effects of DBS therapy targeting the STN or GPi also
remains unclear (Fasano et al., 2015). These suggest that the underlying pathophysiology
of gait disturbances in advanced PD is more complex than just dopamine depletion in
BG.
It has long been recognized that the BG are integral to the production and maintenance
of automatic motor functions. In PD, disruption of the “BG to motor supplementary
motor area” circuit impairs the central driving and the automatic “updating” of motor
programs for skilled movements such as gait (Iansek et al., 2006). One evidence is the
“sequence effect”, which describes the progressive reduction of step length, which in PD
gait eventually disintegrates into a freezing episode (Chee et al., 2009; Iansek et al., 2006).
Aside from the BG, the central pattern generator of the spinal cord could also be involved
in the impairment of automaticity (Okuma, 2014).
The impairment of central drive and automaticity put more stress on voluntary mecha-
nisms and thus increase cognitive load. It has been proposed that patients with freezing
of gait may have a frontal lobe dysfunction or a disconnection between the frontal lobe
and the BG (Okuma, 2014). Several studies have reported the induction of freezing of
gait using dual-task paradigms, where patients are required to perform cognitive tasks
while walking (Almeida, 2009; Yogev-Seligmann et al., 2008). Recent studies have reported
that increased freezing behaviour occurs when patients are denied adequate propriocep-
tive feedback, which led to a hypothesis of the impairment of the integration of visual and
proprioceptive inputs with motor output in patients with freezing of gait (Almeida et al.,
2005).
Disturbed gait has also been related to degeneration of cholinergic neurons in the PPN,
which, together with the CnF, forms the MLR. Postmortem studies in patient with PD
have shown that cholinergic neurons in the PPN degenerate in parallel to dopaminergic
neurons in the SNC (Hirsch et al., 1987; Jellinger, 1988; Zweig et al., 1989). The key roles
of the PPN in the control of gait and posture (Pahapill and Lozano, 2000), in cognition
(notably attention; Mena-Segovia et al., 2004) as well as in sensorimotor gating processes
(Diederich and Koch, 2005) have been identified. As mentioned above, these evidences
strongly suggest that the PPN may be crucially involved in the pathophysiology of gait
disturbances and postural instability in advanced PD.
Management

Clinical options for the treatment of gait disturbances in late stage of PD are limited. Dopamine replacement therapy has, at best, only a partial relieve effect. The freezing of gait is generally considered to be dopamine-resistant. Aside from that, only a few trials have tested drugs targeting extra-dopaminergic systems, such as methylphenidate (Devos et al., 2007). But the results remain controversial and some were reported to even worsen PD symptoms (Espay et al., 2011). Rehabilitation targeting gait and balance has been widely used in clinics for gait disturbances, although no consensus has been reached concerning the optimal program. Various rehabilitation approaches were evaluated in PD, and almost all types of programs showed a beneficial effect compared with non-intervention (Grabli et al., 2012).

High frequency pallidal DBS showed mild improvement of the dopa-responsive postural deficit and freezing of gait, however this effect only lasts for 3-4 years (Houeto et al., 2000). Low frequency (60 Hz) stimulation of the STN has been shown to significantly improve freezing of gait (Moreau et al., 2008), but it was less effective for the cardinal symptoms in PD than stimulation with 130 Hz, which is usually used.

The PPN has been proposed as a novel target for the treatment of PD, especially for the gait and postural disturbances in advanced stage of the disease (Fasano et al., 2015). Low frequency (5-10 Hz) electrical stimulation of the PPN in monkey model of PD has been reported to be effective in reversing akinesia symptoms (Jenkinson et al., 2004; Jenkinson et al., 2006; Mazzone et al., 2005), probably by driving the cholinergic and glutamatergic neurons in the PPN, which are probably inhibited by the altered BG output in PD (Jenkinson et al., 2004). These findings have been swiftly transferred to the clinic by two different groups in 2005. The results seemed to be promising with a significant improvement of akinesia, gait and postural disturbances and even the frequency of falls, which has not been affected by stimulation in traditional targets like the STN and GPi (Mazzone et al., 2005; Plaha and Gill, 2005). However later studies showed mixed results, and raised a fierce controversy about where exactly the optimal site for stimulation is located (Plaha and Gill, 2005; Stefani et al., 2007; Zrinzo et al., 2007). PPN DBS in patient with PD and monkey models of PD showed an additive effect to any benefits from dopaminergic therapy, suggesting that the effect was mediated via a non-dopaminergic pathway (Jenkinson et al., 2006; Plaha and Gill, 2005).
3 The basal ganglia

3.1 Anatomy and functional circuitry

The BG are a richly interconnected set of nuclei that form cortico-subcortical circuitries. The cortico-BG motor circuitry is considered to play a central role in the pathophysiology of PD (Fig. 3.1). The BG comprise two principal input nuclei, the striatum and the STN, and two principal output nuclei, the GPi and the substantia nigra pars reticulata (SNr). The external segment of globus pallidus (GPe) is an intrinsic structure that interconnects with other BG nuclei. Finally, the SNc provide the striatum with important modulatory signals.

In the classical scheme of the organization of the BG motor circuitry, signals originating in the cerebral cortex are sent to the striatum via glutamatergic projections in a topographic manner. The information is then distributed to the two intrinsic populations of striatal GABAergic projecting neurons. The neurons that express D1-type dopamine receptors contact directly with the BG output nuclei—the “direct pathway”, while the neurons that express D2-type dopamine receptors contact indirectly with the BG output nuclei via relays in the GABAergic GPe and glutamatergic STN—the “indirect pathway”. GABAergic neurons in the BG output nuclei, the GPi/SNr, project back to the cerebral cortex via glutamatergic neurons in the motor thalamus.

In addition, cortical areas that project to the striatum also send parallel glutamatergic input to the STN, which contact directly with the GPi/SNr via glutamatergic projection. This third pathway allows information to bypass the striatum and reach the BG output nuclei in a shorter latency compared to both the “direct” and “indirect” pathway (approximately 5-8 ms vs. 15-20 ms), and is thus named the “hyperdirect pathway” (Nambu et al., 2002; Nambu, 2005; von Monakow et al., 1978; Kitai and Deniau, 1981; Olszewski and Baxter, 1982).
3.2 Models of basal ganglia signaling in Parkinson’s disease

Although loss of dopaminergic neurons in the nigro-striatal system has been identified early in the 1960s, how this neurodegenerative change eventually triggers motor dysfunctions in PD is still discussed. Two major hypotheses have been developed to explain the pathophysiology of PD, i.e., the “firing rate” model and the “non-stationary oscillatory” model.

**Firing rate model**

The “firing rate model”, sometimes also referred to as the “classic Albin/DeLong model”, explains the pathophysiology of PD as follows (Albin et al., 1989; DeLong, 1990, see Figure 3.2): Loss of dopaminergic neurons in the SNc lead to the dopamine depletion in the striatum, which decreases the firing rate of the striatal neurons that express dopamine D1 receptor. This result in direct disinhibition of the neuronal activities in the BG output nuclei, the GPi/SNr, and cause an enhanced inhibitory input to the thalamus and cortical motor area. Further, striatal dopamine depletion increases the firing rate of the striatal neurons that express dopamine D2 receptor. This excites the GPi/SNr via the “indirect pathway”, which consists of a GABAergic GPe and a glutamatergic STN, and eventually leads to the inhibition of thalamus and cortex as well. Increased tonic neuronal discharging rate in the GPi and SNr as well as in the STN have been confirmed by many clinical
Figure 3.2: Simplified illustration of the basal ganglia motor circuit in normal and parkinsonian states. Red and blue arrows indicate inhibitory and excitatory projections, respectively. The changes in the thickness of the arrows in the parkinsonian state indicate the proposed increase (larger arrow) or decrease (thinner arrow) in the firing rate of specific connections. The dashed arrows used to label the dopaminergic projection from the SNc to the striatum in parkinsonism indicate partial lesion of that system in this condition. CM, centromedian nucleus; CMA, cingulate motor area; GPe, globus pallidus, external segment; GPi, globus pallidus, internal segment; M1, primary motor cortex; PMC, premotor cortex; PPN, pedunculopontine nucleus; SMA, supplementary motor area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; VA/VL, ventral anterior/ventral lateral nucleus (adapted from Smith et al., 2012)

and experimental studies, and a decreased rate of discharge has also been reported in the GPe (Tang et al., 2007; Starr et al., 2005; Bergman et al., 1994; Filion and Tremblay, 1991; Soares et al., 2004; Mallet et al., 2008). Further, lesions in the GPe have been associated with increased inhibition of the thalamus and worsening of motor symptoms in monkey models and patients with PD (Bucher et al., 1996; Zhang et al., 2006). This model also seems to be applicable to hyperkinesia conditions such as LIDs when the opposite effect takes place. Long-term plasticity triggered by chronic dopamine depletion may lead to increased dopamine receptor sensitivity in the striatum. When given dopamine agonist, the BG output nuclei receive an increased inhibitory influence via the direct pathway and a decreased excitatory influence from the indirect pathway. Both changes lead to the disinhibition of the thalamus and cortex and eventually cause hyperkinetic movements (Nambu et al., 2014).

Although the classic firing rate model has driven the field of basic and clinical BG research
for the past decades, it is still over simplified and faces many criticisms: First, some studies failed to find expected firing rate changes in the GPi or GPe, but even found opposite changes to those predicted by the model (Wichmann and Soares, 2006; Leblois et al., 2007; Galvan et al., 2010; Tachibana et al., 2011). Second, the model predicts that GPi lesions should improve akinesia by removing excessive inhibition of the motor thalamus at the expense of introducing more involuntary movements. However, GPi lesion studies using normal monkeys failed to induce involuntary movements (Inase et al., 1996; Desmurget and Turner, 2008), while pallidotomy in patients with PD showed improved LIDs (Baron et al., 1996). Third, sequential activity changes along these pathways leading to increased GPi firing rate has yet to be directly demonstrated (Nambu et al., 2014). Fourth, there are more internal connections between the components of the BG that could be involved in the pathophysiology of PD, but have not been included in this model (Bolam et al., 2004a; Bolam et al., 2004b).

Non-stationary oscillatory model

The neuronal oscillatory activity has received increasing interest more recently. The term typically refers to rhythmic amplitude fluctuations in the field potentials recorded either directly from the neural ensembles by invasive method (local field potential or LFP), or indirectly from the scalp using electroencephalography (EEG). Underlying the oscillatory appearance of the field potential are the synchronized transmembrane currents in large populations of neurons. Extremely well conserved across the evolution of mammalian brains, the temporal modulation of neuronal activity in different frequency ranges may have important functions in brain information processes rather than being just epiphenomenal (Buzsáki et al., 2013). Further, recording of field potential is considered more important than simply single unit activity. Studies comparing single unit and LFP recordings to blood-oxygen-level-dependent activations in functional magnetic resonance (fMRI) imaging showed that local blood flow is driven much more by LFP activity, which corresponds to local synaptic activity, than by single unit firing rates (Logothetis et al., 2001). There is increasing evidence that certain brain oscillatory rhythms play critical roles in processes such as perception, motor action and conscious experience. With respect to movement disorders, various abnormal oscillatory activities have been associated with specific motor symptoms throughout the motor networks, especially in the STN, the GPi and the motor thalamus (Hammond et al., 2007).

To that end, many efforts have been made in recent years focusing on the dynamic and non-stationary features of neuronal activity changes in PD, such as oscillatory bursting and synchronization of discharge among BG nuclei. The basic idea of the “non-stationary oscillatory model” can be better described with the “noise” hypothesis first proposed
by Marsden, who hypothesized that the damaged BG in PD generates uninterpretable “noise” and causes the movement disorders. Later the work of his student, Brown and others further developed the “noise” concept, and explained it is the over synchronization of BG neurons at wrong frequencies caused by uncontrolled spontaneous oscillations (Marsden et al., 2001). This hypothesis implies that the motor symptoms could be treated by drowning out the uncontrolled oscillatory activity or by replacing it with an oscillation at desirable frequency band. These could be the possible mechanisms underlying the DBS therapies in use.

**Beta oscillation and akinesia**

Electrophysiological studies in 6-OHDA rodent model of PD revealed prominent oscillatory activity in the $\beta$ band (10-30 Hz) at multiple levels of the BG cortical loops (Mallet et al., 2008; Sharott et al., 2005; Hammond et al., 2007). Similar results have been reported in PD patients as well (Brown et al., 2001; Obeso et al., 2000). Later on, Brown and his colleagues succeeded to demonstrate that maintained oscillations in the $\beta$ band in the STN and sensorimotor cortex are associated with akinesia and that driving the STN at $\beta$ frequency (20 Hz) actually made akinesia worse (Kühn et al., 2004). Studies in patient with successfully improved akinesia after use of levodopa showed a replacement of these $\beta$ oscillations by a $\mu$ rhythm (10 Hz) that precedes voluntary movements (Aziz and Stein, 2008).

**Theta oscillation and dyskinesia**

Analysis of the unit oscillatory activities and LFPs recorded in patients during surgery showed that LIDs are characterized by an enhanced $\theta$ frequency range oscillatory activity in the STN and GPi (Alonso-Frech et al., 2006; Merello et al., 1999; Papa et al., 1999; Lozano et al., 2000; Vitek and Giroux, 2000; Levy et al., 2001; Neumann et al., 2012; Liu et al., 2008). In a study carried out by our lab using free-moving 6-OHDA-induced parkinsonian rats, we also observed a significantly increased $\theta$ band (4-8 Hz) oscillatory activity in rats with LIDs (Alam et al., 2014).

Another significant oscillatory activity is the 4-9 Hz (typically 5 Hz) oscillation, which is associated with tremor symptoms, and is therefore named the “tremor frequency activity”.

The “non-stationary oscillatory model” is not as “straightforward” as the classical firing rate model (Nambu et al., 2014). The causal relationship between the abnormal spontaneous oscillations and the motor symptoms remains unclear, so that both models are relevant for understanding the pathophysiology of PD. Further investigation in this field is necessary.
4 The pedunculopontine nucleus

The PPN, or often referred to as the PPTg in rodents, has been considered important for the pathophysiology of gait disturbances in late stage PD. In the human brain, the PPN is bounded on its lateral side by fibers of the medial lemniscus and on its medial side by fibers of the superior cerebellar peduncle and its decussation. Rostrally, the anterior aspect of the PPN contacts the dorsomedial aspect of the posterolateral portion of the substantia nigra, while the retrorubral fields border it dorsally. The most dorsal aspect of the PPN is bounded caudally by the pontine cuneiform and subcuneiform nuclei and ventrally by the pontine reticular formation. The most caudal pole of the PPN is adjacent to neurons of the locus coerules (Olszewski and Baxter, 1982).

The PPN consists of two regions on the basis of cell density, a pars compacta (PPNc) located within the caudal half and a more anterior pars dissipata (PPNd; Mena-Segovia et al., 2004). The former is reported to contain > 90% of cholinergic neurons, probably with a few dopaminergic neurons intermixed (Pahapill and Lozano, 2000), while the latter contains a considerable number of glutamatergic neurons and less cholinergic neurons (Lavoie and Parent, 1994a; Mesulam et al., 1989). Both regions contain GABAergic interneurons (Stein, 2009).

The PPN has diverse synaptic connections with many areas in the brain and the spinal cord, including the BG, i.e., almost all thalamic nuclei, the limbic system (amygdala, hypothalamus, zona incerta), the ascending reticular activating system (raphe nuclei, locus coerules, laterodorsal tegmental nucleus), and cortical motor areas (von Monakow et al., 1979; Edley and Graybiel, 1983). It is involved in many functions such as control of the sleep-wake cycle, locomotor activity, muscle tone, incentive motivation, biting and gnawing, antinociception, gating of the startle reflex, and cognitive and auditory processing (Garcia-Rill, 1986; Inglis and Winn, 1995; Takakusaki et al., 2004; Benarroch, 2013).

The BG is considered more highly interconnected with the PPN than any other brain region (Mena-Segovia et al., 2004). Although it is still under debate whether the PPN should be considered part of the BG, it is obviously a significant outpost of the BG. A pedunculostrialatal projection has been reported in monkeys (Lavoie and Parent, 1994b) and the STN provides glutamatergic innervation of the PPN which, in turn, sends both cholinergic and non-cholinergic and probably excitatory projections back to the STN (Hammond
et al., 1983; Bevan and Bolam, 1995). Further the pallidum and the SNr send GABAergic inhibitory projection to the PPN (Noda and Oka, 1986; Granata and Kitai, 1991), terminating preferentially on the non-cholinergic cells of the PPNd and largely avoid the cholinergic neurons of the PPNC and PPNd (Rye et al., 1995; Shink et al., 1997; Kang and Kitai, 1990; Spann and Grofova, 1991). Anatomical studies using monkeys have shown that > 80% of GPi neurons send axons collateralistically to both the ventrolateral nucleus of the thalamus and the PPN (Harnois and Filion, 1982). In turn, the PPN sends back a mixed cholinergic and glutamatergic projection to the SNr and GPi, as well as to the SNC and GPe.
5 Animal models

5.1 The 6-hydroxydopamine animal model of Parkinson’s disease and levodopa-induced dyskinesias

The 6-OHDA rat model is one of the oldest and most widely used rodent models for PD (Ungerstedt, 1968). 6-OHDA is a synthetic neurotoxin and a structural analogue of dopamine neurotransmitter carried on by dopamine transporter. This toxin does not cross the blood brain barrier, and therefore it is stereotaxically injected locally into certain brain regions. In preclinical research 6-OHDA is most commonly injected unilaterally into the SNc, medial forebrain bundle, or striatum. When injected into the SNc it induces a fast and specific degeneration of the dopaminergic neurons. After striatal injection lesions are generated via retrograde transport of the neurotoxin to the SNc cell bodies and tend to form a more progressive partial lesion. The efficacy of the unilateral lesion can then be easily assessed by drug-induced rotation tests, usually with injection of the dopamine receptor agonist apomorphin (Jerussi and Glick, 1975; Dunnett and Lelos, 2010). An alternative are drug-free behavioural tests such as cylinder test (Schallert et al., 2000; Glajch et al., 2012). Electrophysiological studies in this model showed, in general, similar findings with regard to the firing rate (Mallet et al., 2006; Kita and Kita, 2011), burst-firing (Bergman et al., 1994; Soares et al., 2004; Tachibana et al., 2011; Wichmann and Soares, 2006; Mallet et al., 2008) and oscillatory activities (Jenkinson and Brown, 2011; Kühn et al., 2009) as compared to patient with PD and non-human primate. However, inter-species differences still need to be cautiously taken into account. Despite the fact that the 6-OHDA model cannot mimic all stages of PD (Papa et al., 1994) and that the acute nature of lesion effect is different from the insidious progression of PD observed in patients, this model has been proved a good tool for studying PD and remains popular after decades since its first introduction.

Chronic treatment of levodopa in this model of PD has been reported to induce LID-like movements, such as movements with dystonic or hyperkinetic features, which were observed in axial and orofacial muscles (Andersson et al., 1999; Cenci et al., 1998). In order to model the LIDs and evaluate its severity, a special rating scale has been developed by...
Cenci et al. in 1998 to quantify the abnormal involuntary movements (AIMs) induced by levodopa treatment. The AIMs rating scale, which is currently still in use, evaluates four aspects of the movements in rats following administration of levodopa, including locomotion, axial dyskinesia, orolingual dyskinesia and limb AIMs (Fig. 5.1). The rating is based on both amplitude and time, which give the AIMs test a large dynamic range and allows for precise evaluation.

5.2 Ethylcholine mustard aziridinium ion-induced pedunculopontine nucleus cholinergic lesion and evaluation of motor function in rats

The ethylcholine mustard aziridinium ion (AF64-A) has been used as a selective presynaptic cholinergic neurotoxin since about three decades ago (Fisher and Hanin, 1980). It acts by inhibitory irreversible alkylation of the choline uptake system and different choline-related enzymes (Fisher et al., 1982; Leventer et al., 1985a; Leventer et al., 1985b) and is considered as a potent and remarkably selective cholinergic neurotoxin for the PPN in a dose and site-dependent manner (Hanin, 1996; Kása and Hanin, 1985; Lana et al., 2000). Alterations of the motor function following PPN lesion can be evaluated via different behavioural tests. The traditional open field test can be used to quantify the general locomotor activity level of the rat by placing it in an open field arena, such as a $60 \times 60 \times 30 cm^3$ black box. The Rotarod test can be used to assay the motor coordination of the animal by placing it on a suspended rotating rod (namely rotarod) and measuring how long the rat is able to maintain its balance on the rotarod. Utilizing high speed digital camera, the motion of a rat walking on a treadmill can be captured and different gait parameters can thus be measured for further analyses.
Figure 5.1: Subtypes of levodopa-induced abnormal involuntary movements in the unilateral 6-OHDA rat model of Parkinson’s disease. After injection of levodopa, rat was affected by locomotive (A), axial (B) orolingual (C), and forelimb AIMs (D; adapted from Winkler et al., 2002)
6 Objectives

Both LIDs and gait disturbances frequently occur in patients with advanced PD. These conditions not only severely impact the quality of life and increase the financial burden for patients and social healthcare systems, but also bring major therapeutic challenges to clinics. In-depth studies are needed in order to enhance our understanding of the pathophysiology underlying these conditions.

Project one

We aimed to investigate the neuronal firing characteristics of the EPN, the rat equivalent of the human GPi and output nucleus of the BG, and its coherence with the motor cortex field potentials in the 6-OHDA rat model of PD with and without LIDs.

Project two

We aimed to investigate the effect of anterior or posterior cholinergic lesions of the PPTg (equivalent to the PPN in primates) on gait-related motor behaviour, and on neuronal network activity of the PPTg area and BG motor loop in rats.
Title:
Coherence of neuronal firing of the entopeduncular nucleus with motor cortex oscillatory activity in the 6-OHDA rat model of Parkinson’s disease with levodopa-induced dyskinesias

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Contribution:
Authors Jin, Alam and Schwabe designed the study and wrote the protocol. Experiments were performed by author Xingxing. Authors Mesbah and Xingxing undertook the statistical analysis of the data and wrote the first draft of the manuscript. All authors contributed to and have approved the final version of the manuscript. Critical revision was done by authors Schwabe and Krauss.
Coherence of neuronal firing of the entopeduncular nucleus with motor cortex oscillatory activity in the 6-OHDA rat model of Parkinson’s disease with levodopa-induced dyskinesias

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Abstract

Objective: The pathophysiological mechanisms leading to dyskinesias in Parkinson’s disease (PD) after long-term treatment with levodopa remain unclear. This study investigates the neuronal firing characteristics of the entopeduncular nucleus (EPN), the rat equivalent of the human globus pallidus internus and output nucleus of the basal ganglia, and its coherence with the motor cortex (MCx) field potentials in the unilateral 6-OHDA rat model of PD with and without levodopa-induced dyskinesias (LID).

Methods: 6-hydroxydopamine lesioned hemiparkinsonian (HP) rats, 6-OHDA lesioned HP rats with LID (HP-LID) rats, and naïve controls were used for recording of single unit activity under urethane (1.4 g/kg, i.p.) anesthesia in the EPN “on” and “off” levodopa. Over the MCx, the electrocorticogram (ECoG) was recorded.

Results: Analysis of single unit activity in the EPN showed enhanced firing rates, burst activity and irregularity compared to naïve controls, which did not differ between drug-naïve HP and HP-LID rats. Analysis of EPN spike coherence and phase locked ratio with MCx field potentials showed a shift of low (12-19Hz) and high (19-30Hz) beta oscillatory activity between HP and HP-LID groups. EPN theta phase locked ratio was only enhanced in HP-LID compared to HP rats. Overall, levodopa injection had no stronger effect in HP-LID rats than in HP rats.

Conclusions: Altered coherence and changes in the phase lock ratio of spike and local field potentials in the beta range may play a role for the development of LID.

Keywords: Entopeduncular nucleus, Motor cortex, Parkinson’s disease, Neuronal coherence, Phase locking
The degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc), which leads to the depletion of dopamine in the striatum, the entrance region of the basal ganglia (BG) motor loop, is one of the pathophysiological hallmarks of Parkinson’s disease (PD). Chronic replacement therapy with levodopa relieves symptoms, however, eventually may lead to abnormal involuntary movements, termed dyskinesias, which become treatment-limiting. It has been thought that levodopa-induced dyskinesias (LIDs) develop as a consequence of pulsatile stimulation of dopamine receptors, with consequent dysregulation in downstream neurons resulting in changes in neuronal firing patterns (Obeso et al., 2000).

In patients with PD abnormal neuronal activity has been found in the globus pallidus internus (GPI) and the subthalamic nucleus (STN), especially an increase in synchronized oscillatory beta band activity (13-30 Hz) has been noted, along with enhanced neuronal firing rates and burst activity (Brown, 2003; Obeso et al., 2006; Wichmann and Dostrovsky, 2011; Weinberger et al., 2012). Recordings from patients undergoing pallidotomy or deep brain stimulation have shown that dyskinesias after chronic levodopa treatment are accompanied by reduced oscillatory beta band activity and enhanced theta band activity (4-10 Hz), together with an extensive decrease in firing rates and abnormal firing patterns (Lozano et al., 2000; Obeso et al., 2000; Alonso-Frech et al., 2006). These studies, however, did not address differences of neuronal single units and oscillatory activity in patients with drug naïve advanced PD with or without peak-dose dyskinesias, since almost all patients undergoing neurosurgical treatment have received chronic treatment with levodopa and therefore developed levodopa-induced dyskinesia (LID) at least to some extent at the time of surgery.

Injection of 6-hydroxydopamine (6-OHDA) into the rat nigrostriatal system leads to degeneration of dopaminergic neurons in the SNc together with concomitant abnormal neuronal activity in the BG, which closely parallels the findings in PD patients. When chronically treated with levodopa, 6-OHDA lesioned rats exhibit a broad range of behavioural, physiological, and biochemical features that are similar to LIDs in human patients (Lundblad et al., 2002; Picconi et al., 2005; Marin et al., 2008; Marin et al., 2009; Alam et al., 2014). The oscillatory theta band activity recorded in different basal ganglia regions was significantly more pronounced in 6-OHDA lesioned animals with LIDs than in drug-naïve 6-OHDA lesioned rats (Alam et al., 2014; Meissner et al., 2006).

In order to better understand the neuronal mechanisms involved in the development of LIDs, we investigated the neuronal firing activity of the entopeduncular nucleus (EPN), the rat equivalent to the human GPI, and its coherence with the motor cortex (MCx) field.
potentials in 6-OHDA lesioned hemiparkinsonian (HP) rats with LIDs on/off levodopa, i.e., a model for advanced PD with peak-dose dyskinesias. Measures were compared with the neuronal activity of HP rats without dyskinesias and naïve rats, which served as controls.

Material and methods

Animals

Thirty eight adult male Sprague Dawley rats (Charles River Laboratories, Germany) were used in this study. They were housed in groups of three to four animals per cage (Macrolon Type IV) and kept under controlled environmental conditions (temperature 22°C, relative humidity 45-55%, 14/10 h light/dark cycle) and fed with laboratory rat chow and water ad libitum. All animal procedures were in accordance with the European Council Directive of November 24, 1986 (86/609/EEC) and were approved by the local animal ethic committee. All efforts were made to minimize the number of animals used and their suffering.

Thirty two rats were rendered hemiparkinsonian by unilateral injection of 6-OHDA in the medial forebrain bundle (MFB). Subsequently, these HP rats were divided into two groups. One group (n=24) were rendered dyskinetic by long-term injections of levodopa, in the following termed HP-LID rats, while the other HP group (n=8) received no levodopa injection. Another group of rats (n=6) without surgery served as naïve controls.

6-OHDA lesion

For surgery, rats were anaesthetized with 3.6% chloral hydrate (1ml/100g body weight, i.p., Sigma, Germany) and placed in a stereotaxic frame (Stoelting, Wood Dale, Illinois, USA). Two holes were drilled over the targets above the right medial forebrain bundle and the dura was exposed. 6-OHDA was dissolved in 0.02% ascorbate saline at a concentration of 3.6µg/µl and was injected (1µl/min) in two deposits (2.5µl and 3µl, respectively) at the following coordinates in mm relative to bregma and to the surface of the dura mater: anterior-posterior (AP) = 4.0; lateral (L) = ±0.8; ventral (V) = −8.0; tooth bar at +3.4 and AP = 4.4; L = ±1.2; V = −7.8; tooth bar at −2.4, respectively. Sham lesioned rats received only the vehicle (0.02% ascorbic acid in physiological saline) at the same coordinates. After infusion, the incision was closed by stitches and the animals were returned to their home cages for recovery.

The efficacy of the 6-OHDA-induced lesion was validated 3 weeks after surgery by injection of apomorphine (0.05mg/kg, s.c.; Sigma) as previously described (Alam et al., 2014).
The lesion was considered successful in those animals that made more than 80 net counter-versed rotations in 20 min. To induce dyskinesias the rats were treated for four weeks with 6 mg/kg L-DOPA methylester (Sigma-Aldrich, Germany) plus 12 mg/kg benserazid-HCl. Both drugs were dissolved in physiological saline and injected subcutaneously (s.c.) with a volume of 1 ml/kg body. Dyskinesias were scored by the Abnormal Involuntary Movements (AIMs) scale as described earlier (Alam et al., 2014). The different subtypes of AIMs: orolingual, forelimb, and axial dyskinesias were scored separately for 2 h after levodopa injection on an ordinal scale from 0 to 4, respectively, for 1 min every 10 min (i.e., 12 monitoring periods from 10 to 120 min postinjection). The mean value of these measures was used for further analysis. Only rats with total AIMs scores higher than 4 were included in HP-LID group.

Electrophysiology

Neuronal activity was recorded in the EPN in naïve controls, HP and HP-LID rat groups before and after levodopa injections. Recordings were done under urethane anesthesia (1.4 g/kg i.p. with additional 25% doses as needed) as described previously (Alam et al., 2012). The temperature of the anesthetized animals was constantly controlled with a rectal probe and maintained at 37.2 to 37.6°C with a heating pad (Harvard Apparatus). Electrocardiographic (ECG) activity was monitored constantly to ensure the animals’ wellbeing. A drop of Silicon oil was applied to all areas of the exposed cortex to prevent dehydration. Depth of anesthesia was monitored by examination of the reflex answer to a toe pinch.

The recordings of extra cellular single unit (SU) activity were performed in the EPN (coordinates relative to bregma AP: -2.3 to -2.8 mm posterior to bregma: L: -2.6 to -3.0 mm from the midline; V: 7.5 to 8.0 mm from the dura, tooth bar at -3.3 mm. Spike train recordings from the EPN were paired with simultaneous recordings of the MCx-ECoG. Extracellular SU recordings were taken by quartz coated pulled and ground platinum-tungsten alloy core (95%-5%) micro electrode with a diameter of 80 µm, and an impedance of 1-2 MΩ at 1 kHz. The electrode was advanced using a microdrive (Thomas Recording GmbH, Giessen, Germany) in the ipsilateral EPN. The SU signals were digitized at a sampling rate of 25 kHz with 0.5 kHz-5 kHz band-pass filter and amplification of signals from ×9,500 to ×19,000. Additionally, the MCx-ECoG was recorded via a 1 mm diameter jeweller’s screw, which was positioned on the dura mater above the frontal cortex ipsilateral to the lesioned or sham-lesioned hemisphere (AP, +2.7 mm; L, 2.0 mm; which corresponds to the primary motor cortex region). Two additional screws, serving as MCx-ECoG reference and ground, were placed over the parietal lobe and cerebellum and band pass filtered (0.5 Hz to 100 Hz) with a sampling rate of 1 kHz (Alam et al.,
2012). All signals were digitized with a CED 1401 (Cambridge Electronic Design (CED), Cambridge, UK). The firing of each neuron was recorded for 8 to 10 min after signal stabilization. After termination of the experiment, electrical lesions were made at the recording sites (10 µA for 10s; both negative and positive polarity) and the rat was perfused with 4% paraformaldehyde. Each brain was then cut into 20 µm sections and stained with a standard HE protocol to verify the position of each electrode.

Analysis of electrophysiological data

One epoch of 300 sec recordings was analysed and sorted on the base of a 3:1 signal to noise ratio. Neuronal firing activity arising from a single neuron was discriminated by threshold spike detection and template matching, controlled by cluster analysis with principal component analysis and final visual inspection by using the template-matching function of the spike-sorting software (Spike2; Cambridge Electronic Design, Cambridge, UK).

The firing rate was calculated with the firing rate histograms generated in NeuroExplorer version 4 (NEX Technologies, NC). The coefficient of variation (CV) of the spike inter-spike interval sequence was computed for each recording as a measure of the regularity of the spike firing. CV is a measure of spike train irregularity defined as the standard deviation divided by the mean interspike interval. Exponential distributions have a CV of 1, i.e., describe more irregular discharge patterns, whereas distributions derived from more regular ISIs have CV values below 1.

An asymmetry index was computed as the mode inter-spike interval divided by the mean inter-spike interval. It provides information on the shape of the ISI histogram and the regularity of the discharge pattern. An asymmetry index close to 1 reveals a relatively regular firing pattern, whereas the more the index differs from unity, the more irregular the spike trains. A ratio of less than 1 reflects an asymmetrical shape, indicating a larger fraction of short interspike intervals (positively skewed), as is expected when there is bursting activity.

Firing patterns of spikes events

The analysis classified discharge patterns into 1 of 3 basic categories, i.e., regular, irregular, and bursty firing. Its discharge density histogram was estimated on the base of three reference probability density functions (PDFs) as proposed by Labarre et al. (2008). This method is a comparison of the density histogram \( d(\lambda) \) to a reference density function \( p_x(\lambda) \). For the reference functions (1) a Gaussian PDF with mean 1 and variance 0.5, (2) a Poisson PDF with mean 1 and (3) a Poisson PDF with mean 0.8 were used to represent
regular, irregular and bursting activity, respectively (Lourens et al., 2013). The smallest
distance of the estimated discharge density histogram of the neuron to the three reference
PDFs determined the type of neuron.

Coherence and phase lock of EPN spikes with MCx-ECoG
The duration of 300 sec simultaneous recorded EPN neuronal spikes and MCx-ECoG
signals were used to determine coherence between a point process and a field potential
using the neurospec toolkit (version 2.0) in MATLAB, as described in Halliday et al.
(1995). ECoG signals were notch filtered to eliminate the 50 Hz noise with a finite
impulse response (FIR) notch filter prior to analysis. Autospectra of ECoG necessary for
the calculation of coherence were derived by discrete Fourier transformation with blocks of
1024 samples using a Welch periodogram. Mean coherences were calculated for the theta
(4-8 Hz) and beta (12-30 Hz) frequency ranges. Since several studies in human PD patients
have suggested that low- and high-beta activities may have a different functional signalling
(Priori et al., 2004; Marceglia et al., 2006; Marceglia et al., 2007; Lopez-Azcarate et al.,
2010), we additionally analyzed low (12-19 Hz) and high (19-30 Hz) beta band coherence
in order to determine any possible changes within the beta frequency range.
Additionally, phase relationships between spikes and MCx-ECoG field potentials were
assessed using spike-triggered waveform averages (STWA). The ECoG channels were band
pass filtered at different bands with an ideal (noncausal) filter to prevent phase distortions.
STWAs were calculated for 150 ms before and after the spike trigger over a 300 s epoch.
Spike trains of each neuron were shuffled 20 times to create a null hypothesis for a non
phase locked spike train with the same first order statistics as the original spike train.
The phase-locked ratio was obtained by dividing the peak-to-through amplitude of the
unshuffled spike trains STWA by the mean of the shuffled distribution. A comparison
of the mean ratios was analyzed for the EPN single unit firing neuron referenced to the
MCx-ECoG filtered in theta (4-8Hz) and beta (12-30Hz) frequency ranges, beta activity
was further divided into low (12-19Hz) and high beta (19-30Hz).

Statistical analysis
Two-way analysis of variance (2-way ANOVA) was used to test for significant differences
among the groups followed by post hoc Tukey Test for multiple comparisons between
groups for detection of significance (P value less than 0.05). Pearson’s chi-square (Chi^2)
test was used to determine differences in the distribution of firing patterns. All data are
expressed as the mean ± SEM.
Results

Two of the 24 animals in the HP-LID group were euthanized because of severe and continuous loss of body weight after surgery for unilateral 6-OHDA lesions. All remaining 6-OHDA lesioned rats showed more than 80 contraversive rotations during the apomorphine challenge and were thus considered suitable for the experiments. After four weeks of chronic levodopa injection, 12 of the 22 animals in the HP-LID group showed dyskinesias as determined by the AIM (mean score of 7.53 ± 0.71, range 4.33–11.5), and were thus used for the electrophysiological recordings. All rats operated for the HP group had appropriate 6-OHDA lesions, i.e., showed more than 80 contraversive rotations during the apomorphine challenge, and were used for electrophysiological recordings.

The neuronal activity of 307 single units was recorded before and of 307 single units after levodopa injection. The average units number (mean and SEM) recorded per individual rat was 21.83 ± 1.90. All recording sites were localized in the EPN (see Fig. 7.1 for examples).

Firing rate

In the EPN the firing rate was higher in 6-OHDA lesioned than in naïve rats, long-term treatment with levodopa, however, had no additional effect. Acute levodopa injection reduced firing rates in HP and HP-LID rats without difference, but increased this measure in naïve rats (Fig. 7.2 A). Statistical analysis with two-way ANOVA showed an effect for
the factor drug ($F_{2,523} = 16.12, P < 0.001$), and an interaction between the factors drug and group ($F_{2,523} = 17.72, P < 0.001$), but no effect for the factor group ($F_{2,523} = 1.68, P = 0.19$). Post-hoc testing showed that the mean firing rates were enhanced in HP and HP-LID rats as compared to naïve controls without difference (all p-values < 0.05). Injection of levodopa increased the firing rates in naïve controls, but reduced this measure in HP and HP-LID rats without difference (all p-values < 0.05).

**CV and asymmetry index**

The CV of HP and HP-LID groups was higher as compared to naïve controls. Levodopa injection, further enhanced this measure in both groups. Statistical analysis with ANOVA revealed a significant effect for the factor group ($F_{5,523} = 34.48, P < 0.001$), the factor drug ($F_{2,523} = 27.23, P < 0.001$) and the interaction between factors ($F_{2,523} = 5.21, P < 0.006$). Post-hoc analysis revealed an enhanced CV in HP and HP-LID rats ($P < 0.05$) without difference between these groups. Levodopa significantly increased CV in HP and HP-LID groups. This enhancement was less in HP-LID rats, leading to a significant lower CV in HP-LID rats after levodopa injection compared to HP rats ($P < 0.05$; Fig. 7.2 B). The asymmetry index of HP and HP-LID groups was lower as compared to naïve controls. Levodopa injection further decreased this measure in both groups. Two way ANOVA revealed significant effects on asymmetry index for the factor group ($F_{2,523} = 40.409, P < 0.001$), the factor drug ($F_{2,511} = 29.968, P < 0.001$) and interaction between factors ($F_{2,511} = 7.609, P < 0.001$). Both HP and HP-LID rats showed a lower asymmetry index compared to naïve controls ($P < 0.001$), but without difference between groups. Injection of levodopa significantly reduced the asymmetry index in both groups, but to a lesser extent in HP-LID rats, leading to a significant lower asymmetry index in HP compared to HP-LID rats ($P < 0.05$; Fig. 7.2 C).

**Firing patterns**

The percentage of bursty pattern neurons was higher and the percentage of regular pattern neurons was lower in HP and HP-LID rats as compared to naïve control rats ($P <0.01$). Administration of levodopa increased the number of bursty neurons only in HP rats (Fig. 7.2 D).

**Coherence of EPN-spikes and MCx-ECoG**

Analysis of the coherence of theta band activity between EPN spikes and MCx-ECoG showed enhanced coherences in HP and HP-LID rats compared to naïve controls, but no difference between groups. Levodopa injection reduced this measure only in HP-LID rats,
Figure 7.2: Neuronal firing rates, (A) coefficient variation of inter-spike intervals (CV; B), asymmetry index (AI; C) and the percentage of three different discharge patterns (burst, irregular, and regular; D) of the EPN neuronal activity. Significant differences in comparison with naïve control group is indicated by asterisks (*), differences within group after treatment of L-DOPA with ($) and differences between HP and HP-LID comparisons by (#; P < 0.05; two-way ANOVA and post hoc Tukey test for the neuronal firing rate; Chi-square test with Bonferroni adjustment for the distributions of discharge patterns).
without affecting HP rats. The statistical analysis with ANOVA of EPN spikes and MCx-ECoG coherence of theta band showed a significant effect for the factor group \((F_{2,523} = 7.35, P < 0.001)\) and for the interaction for the factors group and drug \((F_{2,523} = 6.14, P < 0.01)\), but no statistical difference for the factor drug \((F_{1,523} = 1.16, P = 0.28)\). Post hoc tests confirmed that compared to controls the theta frequency band coherence was higher in the HP rats \((P < 0.05)\) and in the HP-LID rats \((P < 0.001)\), without differences between groups. Injection of levodopa decreased theta band coherence only in HP-LID rats \((P < 0.001; \text{Fig. 7.3 B})\).

Analysis of the coherence of beta band activity between EPN spikes and MCx-ECoG showed that beta band activity was more enhanced in HP than in HP-LID rats. Injection of levodopa decreased beta band coherence in both groups. Statistical analysis with ANOVA showed a significant effect for the factor group \((F_{2,523} = 6.57, P < 0.002)\), for the factor drug \((F_{1,523} = 17.70, P < 0.001)\), and for the interaction between factors \((F_{2,523} = 4.82, P < 0.008)\). The beta modulating spikes coherence with the MCx was significantly higher in HP rats compared to both naïve and HP-LID rats \((P < 0.001)\). Levodopa injection decreased beta coherence in HP rats \((P < 0.001)\), while this effect it did not reach the level of significance in HP-LID rats \((P = 0.25)\).

Adspection of the coherence spectrum showed that two frequency peaks dominated the spectrum of the beta range, one in the low-beta (12-19 Hz) and another in the high-beta range (19-30 Hz), i.e., the EPN-spikes and motor cortex ECoG coherence showed a shift in the beta spectrum between HP and HP-LID rats. Statistical analysis of the low and high beta bands with ANOVA showed a significant effect for the factor group \((\text{low}: F_{2,523} = 7.05, P < 0.001; \text{high}: F_{2,523} = 7.80, P < 0.001)\), the factor drug \((\text{low}: F_{1,523} = 18.98, P < 0.001; \text{high}: F_{1,523} = 10.28, P < 0.001)\), and the interaction between factors \((\text{low}: F_{2,523} = 6.14, P < 0.01; \text{high}: F_{2,523} = 5.67, P < 0.01)\). Post hoc analysis revealed that the low beta range frequency modulating spikes coherence was higher in both HP and HP-LID groups as compared to naïve control rats \((P < 0.05; P < 0.001)\), but also higher in HP-LID rats compared to HP rats \((P < 0.05)\). Levodopa injection decreased the low beta coherence in both HP \((P < 0.05)\) and HP-LID rats \((P < 0.001; \text{Fig. 7.3 D})\). The high beta frequency (19-30Hz) spikes coherence was higher in HP rats as compared to HP-LID rats \((P < 0.001)\), which did not differ from naïve control rats. Treatment with levodopa significantly decreased the high beta coherence only in HP rats \((P < 0.001; \text{Fig. 7.3 E})\).

**EPN spikes and MCx-ECoG phase relation**

The EPN spikes and MCx phase locked ratio of theta band activity was only enhanced in HP-LID rats. Injection with levodopa had a different effect on HP and HP-LID rats.
Figure 7.3: Coherence of EPN-spikes and MCx-ECoG spectral power as shown within the frequency range of 1-40 Hz (A). The bar plot shows the mean ± SEM ratio transformed coherence of the theta (B) and beta oscillatory coherence (C), as well as for the low and high beta oscillatory coherence across experimental groups (D and E) respectively. Significant differences in comparison with naïve control group is indicated by asterisks (*), differences within group after treatment of L-DOPA with ($) and differences between HP and HP-LID comparisons by (#; \( P < 0.05 \); two-way ANOVA and post hoc Tukey test).
Figure 7.4: The bar graphs show a comparison of the mean ratios between peak to trough amplitudes of the original STWA and the mean of 20 shuffled STWAs for MCx-ECoG frequency ranges for the theta (A) and beta (B) for EPN neuronal firing activity. While it reduced the phase locked ratio in HP-LID rats, this measure was enhanced in HP rats. Statistical analysis with ANOVA showed a significant effect for the factor group ($F_{2,523} = 3.74$, $P < 0.05$) and for the interaction between factors ($F_{2,523} = 8.59$, $P < 0.001$), while the factor drug had no effect ($F_{2,523} = 0.27$, $P = 0.61$). Post hoc testing showed that the theta phase locked ratio was significantly enhanced only in the HP-LID group ($P < 0.001$; Fig. 7.4 A). Treatment with levodopa increased the theta phase locked ratio in the HP group, but decreased this measure in HP-LID rats ($P < 0.001$).

The EPN spikes and MCx phase locked ratio of beta oscillatory activity was enhanced in both HP and HP-LID rats without difference, and injection of levodopa reduced this measure in both groups. Statistical analysis with ANOVA showed a significant effect for the factor group ($F_{2,523} = 8.58$, $P < 0.001$), the factor drug ($F_{1,523} = 17.75$, $P < 0.001$) and interaction between factors ($F_{2,523} = 4.58$, $P < 0.05$). Post hoc analysis showed that the beta phase locked ratios were higher in both HP and LID groups without difference, and treatment with levodopa decreased beta phase locked ratio in both groups ($P < 0.001$;
Similar to the coherence of EPN spike and MCx-ECoG, two-way ANOVA of the low and high beta band showed a significant effect for the factor group (low: $F_{2,523} = 11.24$, $P < 0.001$; high: $F_{2,523} = 8.47$, $P < 0.001$), the factor drug (low: $F_{1,523} = 27.24$, $P < 0.001$; high: $F_{1,523} = 7.453$, $P < 0.01$), and the interaction between factors (low: $F_{2,523} = 6.53$, $P < 0.01$; high: $F_{2,523} = 4.73$, $P < 0.01$). Post hoc analysis showed a higher spike-ECoG phase locked ratio at low beta band in both HP and HP-LID groups compared to naïve controls ($P < 0.001$), but also higher activity in HP-LID rats compared to the HP group ($P < 0.01$). Levodopa injection significantly decreased the phase locked ratios in both HP and HP-LID groups ($P < 0.001$). EPN spike-ECoG phase locked ratio at high beta frequency band was significantly higher in the HP group compared to both naïve ($P < 0.001$) and HP-LID rats ($P < 0.01$), however the HP-LID rats did not differ from naïve controls. Levodopa injection significantly reduced the phase locked ratio only in the HP group ($P < 0.001$).

**Discussion**

Analysis of single unit activity in the EPN of 6-OHDA lesioned rats showed enhanced neuronal firing rates, which were reduced by levodopa. This is in line with the classical rate-coding model of PD, which predicts that the loss of nigrostriatal dopamine leads to disinhibition of the STN, which subsequently results in overactivity of the GPi. Dopamine replacement therapy normalizes GPi activity by its action through the direct and indirect striatal output pathway. Consistent with this concept, enhanced neuronal firing rates have been found in the STN and GPi of patients with PD (Rodriguez-Oroz et al., 2001; Brown, 2003; Obeso et al., 2006; Wichmann and Dostrovsky, 2011; Weinberger et al., 2012) and in experimental studies using the 6-OHDA rat model and the MPTP monkey model of PD (Hollerman and Grace, 1992; Wichmann et al., 1994; Hassani et al., 1996; Ni et al., 2001). Further, neurophysiological studies in parkinsonian monkeys (Filion and Tremblay, 1991; Boraud et al., 1998) and patients during pallidotomy (Hutchison et al., 1997) have shown a reduction of the firing rate in the GPi after application of dopamine agonists. In PD patients with LID neuronal activity of the STN and GPi also shift from increased neuronal firing in the parkinsonian state to marked hypoactivity during expression of dyskinesias (Merello et al., 1999; Lozano et al., 2000). This corroborates findings of Papa et al., (1999), showing that the firing rates in monkeys expressing dyskinesias after administration of levodopa were substantially more reduced than in monkeys without expression of dyskinesias (Papa et al., 1999). These studies used a low dose of levodopa to examine the “on” state without dyskinesias versus the “dyskinesia” state after high
dose of levodopa, but did not differentiate between drug-naïve PD and PD with LID after longterm treatment with levodopa. In contrast, in the present study, we measured neuronal activity in drug-naïve HP rats compared to HP-LID rats “off” and “on” levodopa. With this study design, in the “off” state the EPN firing rate was enhanced in both drug-naïve HP and HP-LID rats without difference. Further, the firing rate was reduced in the “on” levodopa state in both groups without difference, i.e., levodopa did not have a stronger effect on the firing rate in HP-LID rats than on drug-naïve HP rats.

The induction of LIDs by dopamine agonists has not only been associated with a mean reduction in the firing rate, but also with an increase of burst activity in the STN and GPi when patients express dyskinesias (Lozano et al., 2000; Obeso et al., 2000; Levy et al., 2001). In the present study, we found enhanced bursty pattern neurons in HP and HP-LID rats, which did not differ between groups. Treatment with levodopa, however, increased the bursty pattern neurons only in HP rats. Likewise, in both HP and HP-LID rats the irregularity of firing activity, as measured by the CV, was increased with no difference between HP and HP-LID groups, and the effect of levodopa injection was more severe in HP than in HP-LID rats.

Overall, using the 6-OHDA rat model with LID we did not find differences between HP and HP-LID rats, and acute levodopa injection did not have a stronger effect on neuronal activity in HP-LID rats. The effects seen in the human and monkey studies may therefore be more related to different dosages of dopamine agonists, than to the underlying neurophysiological changes induced by longterm application of levodopa.

Besides the classical rate model, the oscillatory activity in different spectral bands and their synchronization provide information about brain network activity. Changes in oscillatory activity are thought to be correlates of abnormal neuronal processing in movement disorders. Further, neural spiking activity is likely transiently coupled to the field potentials in a rhythmic or non-oscillatory fashion, and allow insight into how altered firing pattern in different nuclei relate to changes in oscillatory activity throughout the basal ganglia network (Fries et al., 2007; Kayser et al., 2009). With that regard, beta oscillations are enhanced in akinetic PD and possibly contribute to bradykinesia and rigidity (Brown, 2003; Kühn et al., 2006; Chen et al., 2007; Kühn et al., 2008; Ray et al., 2008). Injection of dopamine agonists have been reported to reduce beta band activity in patients with PD (Kühn et al., 2006; Weinberger et al., 2006; Ray et al., 2008) and in patients expressing dyskinesias (Alonso-Frech et al., 2006). In the present study, the coherence of EPN spike and MCx field potentials showed enhanced beta activity in both HP and HP-LID rats when using the whole range of beta frequency band (12-30Hz), however, without difference between groups. This differ from our previous studies using the 6-OHDA rat model of PD, where we observed a higher beta band activity in HP rats than in HP-LID
rats (Alam et al., 2014). Interestingly, however, we observed a shift of high (19-30Hz) to low (12-19Hz) range of beta in the EPN spikes and MCx coherence between HP and HP-LID groups, which was also observed for the phase relation analysis. High beta range oscillatory activity in the EPN may therefore be associated with drug-naïve PD, whereas enhanced low beta range activity may be a correlate of changes in neuronal activity that parallels the behavioural development of dyskinesias after chronic injection of levodopa. The role of high versus low oscillatory beta band activity is not entirely clear. In humans it has been suggested that low-beta modulations are specific for action observation, whereas high-beta modulations have been related to the action scene (Marcegglia et al., 2009). The rigid-akinetic parkinsonian state has been associated with an increase of high range beta power (20-30Hz) in M1 motor cortex or STN (Crowell et al., 2012; Shimamoto et al., 2013). In line with this, the power and coherence of high beta (22-32 Hz) oscillations in the cerebral cortex and STN of awake free moving rats was enhanced in the 6-OHDA rat model of PD (Sharott et al., 2005; Li et al., 2012). On the other hand, several clinical studies described a predominant peak within the low-beta band (12-20 Hz) in the off levodopa state, together with a smaller peak in the high-beta (20-30 Hz) band (Lopez-Azcarate et al., 2010; Rodriguez-Oroz et al., 2011; Thompson et al., 2014). The low-beta peak disappeared after levodopa application or was greatly reduced during movement, whereas the high-beta peak remained at similar power (Thompson et al., 2014).

In patients with PD it has been shown that oscillatory activity in the theta frequency band was higher after administration of levodopa, i.e., in the “On” dyskinesia condition, than in patients in the “Off” medication state (Alonso-Frech et al., 2006). In line with this, the theta oscillatory activity recorded in different basal ganglia regions was significantly more pronounced in 6-OHDA lesioned animals with LIDs than in drug-naïve 6-OHDA lesioned rats (Alam et al., 2014; Meissner et al., 2006). In our present study, however, analysis of the coherence and phase lock ratio of theta frequency spike trains of MCx-ECoG showed that although oscillatory theta band activity was somewhat higher in HP-LID rats than in drug-naïve HP rats, levodopa injection only enhanced theta band activity in HP rats, but reduced this measure in HP-LID rats.

It should be noted that all recordings in the present study were made in the urethane anaesthetized condition, therefore, the genesis of increased theta spike-MCx coherence or phase locked ratio after treatment of levodopa occurred without the sensory motor feedback of involuntary movements. Especially theta band activity has been shown to depend on the presence or absence of the abnormal dystonic posture or the phasic movements and may therefore be more pronounced in the non-anesthetized state (Brazhnik et al., 2012; Lemaire et al., 2012).

The pathophysiological mechanisms underlying the development of dyskinesias still need
further clarification. While we did not find differences of single unit activity in the EPN of drug naïve HP and HP-LID rats, the coupling of neural spiking activity to the MCx oscillatory activity differed between HP and HP-LID. Further research is needed to investigate whether the interaction between BG activity and cortical processing would indeed be relevant for the occurrence of dyskinesias.
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to the study of physiological tremor, single motor unit discharges and electromyograms”.


8 Manuscript two

Title:
Cholinergic lesion in the anterior and posterior pedunculopontine tegmental nucleus: behaviour and neuronal activity in the cuneiform and entopeduncular nuclei

Order of Authors:
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Contribution:
Authors Jin, Alam and Schwabe designed the study and wrote the protocol. Experiments were performed by author Xingxing. Authors Mesbah and Xingxing undertook the statistical analysis of the data and wrote the first draft of the manuscript. All authors contributed to and have approved the final version of the manuscript. Critical revision was done by authors Schwabe and Krauss.
Cholinergic lesion in the anterior and posterior pedunculopontine tegmental nucleus: behaviour and neuronal activity in the cuneiform and entopeduncular nuclei

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Abstract

Objective: Loss of cholinergic neurons in the mesencephalic locomotor region, comprising the pedunculopontine nucleus (PPN) and the cuneiform nucleus (CnF), are related to gait disturbances in late stage Parkinson’s disease (PD). We investigate the effect of anterior or posterior cholinergic lesions of the PPN on gait-related motor behaviour, and on neuronal network activity of the PPN area and basal ganglia (BG) motor loop in rats.

Methods: Anterior PPN lesions, posterior PPN lesions or sham lesions were induced by stereotaxic microinjection of the cholinergic toxin AF64-A or vehicle in male Sprague Dawley rats. First, locomotor activity (open field), postural disturbances (Rotarod) and gait asymmetry (treadmill test) were assessed. Thereafter, single unit and oscillatory activity were measured in the non-lesioned area of the PPN, the CnF and in the entopeduncular nucleus (EPN), the BG output region, with microelectrodes under urethane anaesthesia. Additionally, ECoG was recorded in the motor cortex.

Results: Injection of AF64-A into the anterior and posterior PPN decreased cholinergic cell counts as compared to naïve controls ($P < 0.001$). Only anterior PPN lesions decreased the front limb swing time of gait in the treadmill test, while not affecting other gait related parameters tested. Main electrophysiological findings were that anterior PPN lesions increased the firing activity in the CnF ($P < 0.001$). Further, lesions of either PPN region decreased the coherence of alpha (8-12Hz) band between CnF and MCx, and increased the beta (12-30Hz) oscillatory synchronization between EPN and the MCx.

Conclusion: Cholinergic lesions of the PPN in rats had complex effects on oscillatory neuronal activity of the CnF and the BG network, which may contribute to the understanding of the pathophysiology of gait disturbances in PD.

Keywords:
Introduction

The late stages of Parkinson’s disease (PD) and progressive supranuclear palsy are characterized by postural instability and gait disturbances, which contribute to marked disability. These may be refractory to dopaminergic medication, but also to deep brain stimulation (DBS) of the subthalamic nucleus (STN) or the globus pallidus internus (Fasano et al., 2015; Kasashima and Oda, 2003; Schrader et al., 2013). Disturbed gait has been related to degeneration of cholinergic neurons in the pedunculopontine nucleus (PPN; Hirsch et al., 1987; Jellinger, 1988; Zweig et al., 1989), which, together with the cuneiform nucleus (CnF), forms the mesencephalic locomotor region (MLR). Deep brain stimulation (DBS) of the PPN has been tested for treating these symptoms, using stimulation parameters thought to stimulate the remaining neurons, however, with variable results and with substantial controversy, where exactly the optimal site for stimulation is located (Ferraye et al., 2010; Moro et al., 2010; Nosko et al., 2015; Plaha and Gill, 2005; Schrader et al., 2013).

In primates, the PPN consists of a compact part (PPNc) with a higher density of cholinergic neurons, and a pars dissipata (PPNd), with glutamatergic, GABAergic, and cholinergic neurons (Inglis and Winn, 1995; Martinez-Gonzalez et al., 2011; Ros et al., 2010). The PPN interacts with the basal ganglia (BG) motor loop, but also relays information to the brainstem and spinal cord relevant for postural muscle tone (Alam et al., 2011). Lesions of cholinergic PPN neurons in monkeys induce akinesia, gait and postural changes resistant to dopaminergic agents, which closely parallels findings in patients with late stage PD (Karachi et al., 2010; Kojima et al., 1997; Matsumura and Kojima, 2001). In rodents, however, the pedunculopontine tegmental nucleus (PPTg, the equivalent to the PPN in primates) has primarily been related to non-motor behaviour, such as cognition and sensorimotor gating (see Winn, 2006 for review). Bilateral excitotoxic lesions of the PPTg did not affect spontaneous locomotor activity stability, speed, stride or coordination (Inglis et al., 1994; Olmstead and Franklin, 1994; Winn, 2006). Nevertheless, more recently, Alderson et al., (2008) have observed a reduction in locomotion after lesioning a restricted portion of the anterior but not of the posterior part of the PPTg. These results are consistent with the hypothesis that in rats the anterior PPTg (aPPTg), which is thought to resemble the PPNd, has functions and anatomical connections related to motor processes (Honda and Semba, 1995; Rye et al., 1987), while the posterior PPTg (pPPTg), which is regarded the PPNc because of a high density of cholinergic neurons, has stronger anatomical connections to associative-limbic structures (Mena-Segovia et al., 2008; Manaye et al., 1999; Olszewski and Baxter, 1954). Whether the effects of the aPPTg lesions are achieved through the effects on cholinergic neurons on descending motor projections, or through
effects on the basal ganglia motor loop, possibly via the CnF as suggested by Alam et al., (2012), has not been investigated.

Ethylcholine mustard aziridinium ion (AF64-A) is an irreversible inhibitor of the choline uptake system and choline-related enzymes (Fisher and Hanin, 1980) and acts as a potent and selective cholinergic neurotoxin for the PPN in a dose- and site-dependent manner (Hanin, 1996; Kás and Hanin, 1985; Lança et al., 2000). We here tested the effects of specific AF64-A-induced cholinergic lesions of either the aPPTg or the pPPTg on rodent gait-related behaviour and extracellular neuronal activity of the unlesioned part of the PPTg, as well as on the CnF and the entopeduncular nucleus (EPN), which is regarded the rat equivalent of the human GPi and the output nucleus of the basal ganglia motor loop.

Materials and Methods

Animals

Male Sprague-Dawley rats (N = 20; weighing 220-230 g; Charles River Laboratories, Germany) were randomly divided into three groups, including naïve controls (N = 8), aPPTg lesion (N = 6) and pPPTg lesion (N = 6) animals. Rats were housed in groups of four in standard Macrolon Type IV cages (Techniplast, Hohenpeissenberg, Germany) under a 14 h/10 h light-dark cycle with light on at 07:00 h at a room temperature of 22 ± 2°C, and with food and water available at all times. All animal procedures were in accordance with the European Council Directive of November 24, 1986 (86/609/EEC) and were approved by the local animal ethic committee. All efforts were made to minimize the number of animals used and their suffering.

PPTg cholinergic lesion

The cholinergic neurotoxin AF64-A solution was prepared from acetyethylcholine mustard HCl (Sigma, Germany) as described previously by Fisher et al. (1982). The solution was freshly prepared in 1mg/mL aqueous solution, adjusted with 10N NaOH to a pH of 11.5 – 11.7 and kept in room temperature for 30 min with continuous stirring. Thereafter, the pH was adjusted to 7.4 using 6N HCl and NaOH. The AF64-A solution was further diluted with saline (0.9% NaCl) for a final concentration of 13.5ng/µL and stored at 4°C until microinjection within 6h. In preliminary studies this dosage has been found to induce selective lesions of cholinergic neurons within the aPPTg or pPPTg without damaging the tissue.

The surgical procedure was adapted according to a protocol for PPTg lesion in rats
Animals were anesthetized with 3.6% chloral hydrate solution (1 mL/100 g body weight, i.p., Sigma, Germany) and placed in a rodent stereotaxic frame (Stoelting, Wood Dale, Illinois, USA). After making a small incision to expose the scalp, a bone scraper was used to clean the skull above bregma and lambda and a small craniotomy was made with a dental drill above the PPTg area of each hemisphere. The aPPTg (AP: −7.3 mm posterior to bregma; L: ±1.8 mm and V: −7.3 mm; according to the atlas of Paxinos and Watson, 1997) and the pPPTg (AP: −8.3 mm posterior to bregma; L: ±1.8 mm and V: −7.0 mm) were lesioned bilaterally by microinjection of a total volume of 0.5 µl of AF64-A (6.75 ng) that was injected with a rate of 0.1 µl/min into each PPTg region. Controls received microinjection of vehicle only. The tooth bar was set at −3.3 mm for all coordinates.

**Behaviour**

Three weeks after surgery behavioural testing started. All tests were performed after 30-60 min habituation to the testing room during the day light cycle under artificial light with a fixed intensity and acoustic exposure to a masking noise (playing radio).

*Activity box test:* To assess spontaneous locomotion, the animals were placed in a black plastic open field (60 × 60 × 30 cm³). A video of the animals was recorded for 10 minutes by a camera installed above the box and the total distance travelled was automatically calculated by a video tracking system using the same settings for all rats (TopScan 1.0, Clever Sys. Inc., Reston, VA, USA).

*Rotarod testing:* To assess the motor coordination of the animals, an accelerating Rotarod (IITC Life Science, Woodland Hills, CA) was used. The Rotarod consisted of a suspended rod, accelerating for 60 seconds from 1 round per minute (RPM) to 12 RPM, thereafter continuing at that speed for another 60 seconds. A trial was stopped when the rat fell off the Rotarod or after 120 seconds. Three consecutive trials were performed with a rest period of 5 min in between, and the mean duration of rat staying on the Rotarod was calculated. Prior to surgery, the rats were trained for five days to achieve a stable performance.

*Automated treadmill gait test:* Treadmill gait assessment was performed with the TreadScan system (CleverSys, Inc., Reston, VA, USA). Rats were placed on a motorized treadmill within a plexiglass compartment (≈ 25 cm long and ≈ 5 cm wide). Digital video images were acquired at a rate of 100 frames per second by a camera mounted underneath the treadmill to visualize paw contacts on the treadmill belt. The treadmill was set at a fixed speed of 17 cm/sec at which most animals were able to move continuously. The videos were analysed by the TreadScan software, which automatically identifies the paw footprints. Manual adjustments of the contrast of the images were made, if neces-
sary, to properly distinguish the footprints from the background. The images were then automatically processed by the software to calculate values for gait parameters, including stance time and swing time for the front and hind paw. The stance phase is the part of the gait cycle that begins as soon as the paw contacts the ground and terminates when the paw starts its forward movement. The swing phase is defined as the period following the stance phase, when the foot is off the ground.

**Electrophysiological recordings**

After behavioural testing, electrophysiological recordings were taken in the aPPTg in rats with pPPTg lesions, and in the pPPTg in rats with aPPTg lesions, i.e., in the non-lesioned PPTg area. In controls, recordings were taken both in aPPTg and pPPTg. In all groups, additional recordings were taken in the CnF and the EPN. For recording, the rats were anesthetized with urethane (1.4g/kg, i.p.; ethyl carbamate, Sigma, St. Louis, MO) and placed in a stereotaxic frame. The body temperature was maintained at 37 ± 0.5°C by a heating device (FHC, Bowdoinham, ME). Surface ECG was recorded to monitor and ensure constant physiological conditions and wellbeing during recording. The ECoG electrodes were placed in the axilla and pelvic region (Lead II, CED1902 isolated amplifier, Cambridge Electronic Design, Cambridge, UK).

Small craniotomies were made over the target coordinates for the aPPTg and/or the pPPTg, the CnF and the EPN in both hemispheres. A single microelectrode for extracellular recordings (quartz coated pulled with a ground platinum-tungsten alloy core (95%-5%), diameter 80µm, impedance 1−2MΩ) was connected to the Mini Matrix 2 channel version drives headstage (Thomas Recording, Germany) and stereotaxically guided through the skull burr holes to the target coordinates in the aPPTg (A: −7.3 to −7.8mm; L: ±1.8mm; V: −7.0 to −7.8mm), the pPPTg (A: −8.3 to −8.5mm; L: ±2.0mm; V: −6.8 to −7.2mm), the CnF (A: −8.3 to −8.5mm; L: ±2.0mm; V: −6.0 to −6.4mm) and the EPN (A: −2.3 to −2.8mm; L: ±2.6 to ±3.0mm; V: −7.5 to −8.0mm). Regions were recorded in randomized order. The recorded signals were split into two signals, which allowed single unit (SU) activity and local field potentials (LFPs) to be analysed from the same electrode: (1) SU activity was recorded with a 0.5 to 5 kHz band pass filter at a sampling rate of 25 kHz and ×9500 to ×19,000 amplification, and (2) LFPs were recorded with a 0.5 to 100 Hz band pass filter at a sampling rate of 1 kHz, as described by Alam et al. (2012). Additionally, an electrocorticogram (ECoG) was recorded via a 1 mm diameter jeweler screw, which was positioned on the dura mater above the primary motor cortex (MCx) ipsilateral to the recording site (AP: +2.7mm; L: ±2.5mm). The ECoG reference and ground was positioned in a skin pocket over the neck muscle. All signals were digitized with a CED 1401 (Cambridge Electronic Design, UK) and recorded for 10
to 12 min after signal stabilization with Spike2 analysis software (Cambridge Electronic Design, Cambridge, UK). Recordings of 300 s were analysed and sorted on the base of a 3:1 signal to noise ratio. After termination of the experiment, electrical lesions were made at the proximal and the distal site of the trajectory to allow histological verification of the recording site (10 μA for 10 seconds; both negative and positive polarities; Fig. 8.1).

8.1 for examples).

**Data analysis**

Action potentials arising from a single neuron were discriminated by the template-matching function of the spike-sorting software (Spike2; Cambridge Electronic Design, Cambridge, UK). Only well isolated single unit activities were included in the analysis, which was determined by the homogeneity of spike waveforms, the separation of the projections of spike waveforms onto principal components during spike sorting, and clear refractory periods in inter-spike interval (ISI) histograms. All analyses were performed using custom-written Matlab (Mathworks, Natick, MA) functions unless otherwise noted.

Firing rates were calculated by taking the reciprocal value of the mean ISI for the whole 300 seconds of recording. The coefficient of variation (CV) of the ISI sequence is a measure of spike train irregularity defined as the standard deviation divided by the mean ISI. An exponential distribution has a CV of 1, which describes more irregular discharge patterns; whereas distributions derived from more regular ISIs have CV values below 1.

Firing patterns of all spike trains were classified into one of the 3 basic categories (regular, irregular, and bursty firing) using the method described by Labarre et al. (2008). The discharge density histogram $d(\lambda)$ of each spike train was compared to reference density functions (PDF) $p_x(\lambda)$. For the reference functions (1) a Gaussian PDF with mean 1 and variance 0.5, (2) a Poisson PDF with mean 1 and (3) a Poisson PDF with mean 0.8 were used to represent regular, irregular and bursting activity, respectively (Lourens et al., 2013).

The bursting characteristics of neuronal activities were analysed using traditional maximum interval method with NeuroExplorer version 4 (Nex Technologies). A burst was defined as at least two spikes with an inter-spike interval equal to or less than 80 ms, with burst termination defined as a subsequent inter-spike interval more than 160 ms (Grace and Bunney, 1984). Additionally, the minimum duration of a burst was set at 10 ms, the minimum number of spikes in a burst was n=3, and the minimum interval between bursts was set at 300 ms. Following the burst detection, a set of burst parameters were determined, including the number of bursts per minute, the percentage of spikes in bursts and the mean firing frequency in bursts.

Representative epochs of 300 seconds without major artefacts were used for the fre-
Figure 8.1: Schematic drawings of the rat coronal brain slices (adapted from Paxinos and Watson, 1986) of the aPPTg (a), the pPPTg and CnF region (b), and the EPN (c). Photographs show the histological verification of the recording electrode by electric coagulation (black arrows) in hematoxylin-eosin (HE) stained coronal sections in the anterior and posterior part of the PPTg, the CnF and the EPN with ×10 and ×50 times magnification.
quency domain signal processing for LFPs and ECoGs. After eliminating the 50 Hz power line artefacts using a finite impulse response (FIR) notch filter, data was normalized by subtracting the mean amplitude and dividing the standard deviation, which allowed the frequency domain signals to be pooled and compared with less influences from individual/non-specific differences. Frequency domain transformation was applied by computing the Fast Fourier Transform (FFT) spectra from blocks of 1024 samples, which resulted in a frequency resolution of 0.9766 Hz. Hanning’s window function was applied to overcome spectral leakage phenomena. Functional relationships between the MCx and LFPs were estimated by means of coherence using traditional methods described by Halliday et al. (1995). Coherence of oscillatory signals provides a frequency-domain measure of the linear phase and amplitude relationships between signals. It is a finite measure of values from 0 to 1, where 0 indicates that there is no linear association and 1 indicates a perfect linear association.

Choline acetyltransferase staining and quantification

After electrophysiological recordings, rats were transcardially perfused with 150mL 0.1M phosphate-buffered saline (PBS), followed by 200mL 4% paraformaldehyde (PFA) in PBS. Following perfusion, the brains were collected and post fixed with PFA overnight at room temperature, thereafter transferred to a 30% sucrose solution in PBS and stored at 4°C before cutting.

The brains were cut on a freezing microtome in the coronal plane with a section width of 30µm. Every second section that contained the PPTg or the CnF was processed for free-floating choline-acetyltransferase immunohistochemistry (ChAT; primary goat polyclonal anti-ChAT antibody, 1:200 dilution; Millipore). The sections were incubated with secondary antibody solution (1:200 dilution) with biotinylated IgG donkey anti-goat (Millipore). Staining was performed using the ABC-Standard-Kit (1:1,000; 1µL Avidin-H + 1µL biotinyl-peroxidase in 1 mL PBS; ABC-Kit, Vector Laboratories, Burlingame, CA) and 3,3’-diaminobenzidin (DakoCytomation, Glostrup, Denmark). Sections were mounted on gelatine coated microscope slides, dehydrated in ascending concentrations of alcohol, cleared in xylene and cover slipped using Vecta Mount (Vector Laboratories, Inc., Burlingame, CA).

Every first section and additional coronal sections of a width of 10µm that contained the EPN were mounted on glass slides (SuperFrost, Thermo Scientific, Germany), and stained with hematoxylin-eosin (HE), dehydrated in alcohol, and cover slipped with Vitro Clud (Langenbrinck, Emmendingen, Germany), in order to visualize the electrolytic coagulations along the microelectrode recording tracks.

To evaluate the cholinergic lesion effects of the aPPTg and pPPTg, ChAT-positive (ChAT+)
cells were bilaterally counted in four sections per animal at 400 times magnification. Two sections were located approximately between $-7.2$ to $-7.4\text{mm}$ posterior to the bregma, which corresponds to the aPPTg, whereas the other two were located between $-8.2$ to $8.4\text{mm}$, which corresponds to the pPPTg. The transition from the superior colliculus to the inferior colliculus, which was located approximately at $-8.0\text{mm}$ posterior to bregma, was used as an anatomical landmark to distinguish between aPPTg and pPPTg. Cell counts were then averaged for each PPTg subregion for each animal. The mean counts of ChAT+ cells in the aPPTg and pPPTg in naïve controls were taken as 100%. Percentages of the averaged counts in the aPPTg and pPPTg were calculated over the mean counts in naïve controls.

**Statistics**

The number of ChAT positive neurons was compared between groups by t test. Behavioural data were analysed using one way analysis of variances (ANOVA) followed by Tukey post hoc comparisons (Sigma Stat 3.5, Software, Inc., USA). For the SU activity data nonparametric Kruskal-Wallis ANOVA tests were used due to the significant deviation from normality and a lack of homogeneous variances that existed in most extracellular SU spike data. Statistically significant differences between groups were assessed by using post hoc pairwise multiple comparisons with Dunn’s method if the Kruskal-Wallis ANOVA showed significant differences ($P < 0.05$). Distributions of the firing patterns were compared using chi-square test. Further, z-test was applied between percentages of patterns by adjusting Bonferroni correction to determine significant changes of P values for individual (regular, irregular and burst) observations. Statistics were performed with Sigma Stat Software. For all tests, results were considered statistically significant with a P-value $< 0.05$. All results are shown as mean ± standard error unless noted otherwise.

**Results**

One animal from the aPPTg lesion group died during urethane injection prior to the electrophysiological recording. Therefore in the electrophysiology results only five animals were included in the aPPTg lesion group.

In the aPPTg lesion group and the naïve control group a total of 211 SU activities were recorded in the pPPTg region; similarly in the pPPTg lesion group and the naïve control group a total of 143 SU activities were recorded in the aPPTg area. In all three groups of animals ( naïve controls, rats with aPPTg lesions and rats with pPPTg lesions), a total of 207 and 154 single neuronal activities were recorded in the EPN and in the CnF, respectively. The average counts of neurons recorded per animal were $15.07 \pm 1.46$ in the
aPPTg, 11.00 ± 1.72 in the pPPTg, 10.89 ± 1.53 in the EPN, and 8.11 ± 1.31 in the CnF. All recording sites marked with electrolytic lesions were verified in the different regions (see Fig. 8.1 for examples).

Lesion effect

The mean percentage of surviving neurons after AF64-A injection in the aPPTg and pPPTg area were 38.83 ± 11.76% and 38.12 ± 9.34%, which significantly differed from the cell-count of naïve controls. Injection of AF64-A into the aPPTg only marginally affected the ChAT-positive cell count in the pPPTg (88.18 ± 8.58%). Likewise, injection of AF64-A into the pPPTg only marginally affected the number of ChAT-positive neurons in the aPPTg (73.18 ± 5.94%; see Fig. 8.2).

Motor impairment

Statistical analysis with ANOVA showed that neither aPPTg lesions nor pPPTg lesions changed measures in the Rotarod test or spontaneous locomotion in the activity box (both F-values > 0.256; both P-values > 0.7; Fig. 8.3 A and B). In the treadmill gait test, the front limb swing time was reduced in aPPTg-lesioned rats (P = 0.041 after significant ANOVA: $F_{2,18} = 3.816; P = 0.04$), while the swing time of rats with pPPTg did not differ from naïve controls and rats with aPPTg lesions. No differences in the front limb stance time were observed (Fig. 8.3 C and D). All other measures did not differ between groups.

Neuronal firing rate and CV

Neither the aPPTg nor the pPPTg lesions altered the firing rate in the non-lesioned part of the PPTg (Fig. 8.4 A and B), but the CV in the aPPTg area was significantly enhanced after pPPTg lesions as compared to that of naïve controls (0.68 ± 0.06 vs. 0.49 ± 0.05; P < 0.05; Table. 8.1). After aPPTg lesions the firing rate was significantly enhanced in the CnF compared to controls (P < 0.05 after significant ANOVA: $\chi^2 = 9.043; P = 0.011$), while the CnF firing rate did not differ between naïve controls and rats with aPPTg lesions (Fig. 8.4 C). In the EPN, the firing rate did not differ between groups (see Fig. 8.4 D). Also, the CV of the CnF and the EPN did not differ between groups.
Figure 8.2: Histological micrographs showing the choline acetyl transferase immune-positive (ChAT+) neurons in the naïve (A) and lesioned (B) anterior PPTg and the naïve (C) and lesioned (D) posterior PPTg. Schematic diagrams illustrating the lesioned areas in the aPPTg and pPPTg (E; adapted from Paxinos and Watson, 1986). The bar histograms show the mean percentage (±SEM) of ChAT+ neurons in the aPPTg and the pPPTg after aPPTg lesions (F) and after pPPTg lesion (G). Significant differences between the aPPTg and the pPPTg are indicated by asterisk (*; P < 0.05; t test).
Figure 8.3: Time spent on the rotarod (A), total distance travelled in the activity box (B), average swing time (C) and stance time (D) of the front limb measured using treadmill. Bars show the mean ± SEM of naïve, aPPTg lesioned and pPPTG lesioned rats. A significant difference to naïve controls is indicated by asterisks (*; P < 0.05; one-way ANOVA and post hoc Tukey test).

Table 8.1: Coefficient variation of ISIs (CV; all values presented as median and its 25th and 75th percentile). A comparison with naïve control has been indicated by (*) asterisks (P < 0.05; one-way ANOVA and post hoc Tukey test).
Figure 8.4: The neuronal firing rates of the aPPTg (A) and pPPTg (B), the CnF (C) and EPN (D). Bars show the mean ± SEM of naïve, aPPTg lesioned and pPPTG lesioned rats. A significant difference to naïve controls is indicated by asterisks (*; \( P < 0.05; \) one-way ANOVA and post hoc Tukey test).
Burst parameters

Neither aPPTg nor pPPTg lesions affected the burst activity in the non-lesioned part of the PPTg. In the CnF, statistical analysis with ANOVA revealed significant differences in the number of bursts per minute, the percentage of spikes in burst and the mean frequency of bursts (all $\chi^2$-values > 12; all p-values < 0.002). Post hoc testing showed that the pPPTg lesions decreased the burst per minute compared to both naïve controls and the aPPTg lesioned group ($P < 0.05$). The aPPTg lesions did not alter the number of bursts per minute in the CnF nucleus but increased the percentage of spikes in burst parameter compared to both naïve control and the pPPTg lesioned group ($P < 0.05$). No significant changes were observed after pPPTg lesions in the CnF nucleus. Additionally, aPPTg lesions increased the mean frequency of bursts compared to both naïve controls and the pPPTg lesioned group in the CnF nucleus ($P < 0.05$; Table 8.2). All other parameters did not differ between groups. In the EPN, statistical analysis showed that only pPPTg lesion significantly increased the number of bursts per minute compared to naïve controls ($P < 0.05$ after significant ANOVA: $\chi^2 = 6.678$; $P = 0.035$) but no differences were determined for the percentage of spikes in bursts and mean frequency of bursts.

Firing patterns

Chi-square test showed that pPPTg lesions significantly affected the neuronal firing pattern distribution in the aPPTg ($\chi^2 = 7.413$, $df = 2$; $P = 0.025$), while aPPTg lesions had no effect on the pPPTg area ($\chi^2 = 1.691$, $df = 2$; $P = 0.429$). Post hoc test showed that after pPPTg lesions the irregular firing patterns were enhanced in the aPPTg (31.15% vs. 15.73%; $P = 0.02$) and the regular firing patterns were decreased (66.39% vs. 83.15%; $P = 0.01$) as compared with the naïve controls, but no differences in the burst patterns were detected (Fig. 8.5 A and B).

Chi-square test also showed that the percentage of bursts in the CnF nucleus were higher after aPPTg lesions than after pPPTg lesions (38.5% and 15.1%; $P < 0.01$; after $\chi^2 = 8.821$, $df = 4$; $P = 0.06$; Fig. 8.5 C). PPTg lesions had no effect on the neuronal firing pattern distribution in the EPN ($\chi^2 = 3.198$, $df = 4$; $P = 0.525$; Fig. 8.5 D).

Coherence of LFPs and MCx-ECoG

Analysis of the coherence of PPTg LFPs and MCx-ECoG in the alpha (8-12 Hz) and in the beta (12-30 Hz) band showed that both aPPTg and pPPTg lesions did not affect the non-lesioned PPTg-LFP and MCx coherence.
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Table 8.2: Burst parameters including the bursts per minute, the percentage of spikes in bursts and the mean firing frequency in bursts (all values presented as median and its 25th and 75th percentile). A comparison with naive control has been indicated by (∗) asterisks, differences between aPPTg lesion and pPPTg lesion with (#; P < 0.05; one-way ANOVA and post hoc Tukey test).
Figure 8.5: Distribution of cellular firing patterns (burst, irregular and regular) in the aPPTg (A), the pPPTg (B), the CnF (C) and the EPN (D). Significant differences to naïve controls are indicated by asterisks (*; $P < 0.05$; Chi-square test with Bonferroni adjustment for the distributions of discharge patterns).
In the CnF, one-way ANOVA showed significant effects of PPTg lesion on the LFP-ECoG coherence of the alpha \((F_{2,146} = 12.339; P < 0.001; \text{Fig. 8.6})\) and beta frequency bands \((F_{2,146} = 4.038; P = 0.020)\). CnF-LFP and MCx coherence in the alpha band was significantly decreased after aPPTg lesions \((P < 0.001)\), but to a lesser extent after pPPTg lesions \((P < 0.05)\). The difference between aPPTg lesion and pPPTg lesions was significant \((P < 0.05)\). The beta band coherence were also decreased after pPPTg lesions \((P < 0.05)\), however, tendency for a decrease was also found after aPPTg lesions \((P = 0.058)\).

In the EPN, one-way ANOVA showed significant effects of PPTg lesion on the LFP-ECoG coherence of the beta frequency band \((F_{2,200} = 5.012; P < 0.01; \text{Fig. 8.6})\) but not the alpha frequency band \((F_{2,200} = 0.289; P = 0.749)\). Post hoc test showed a significantly enhanced beta coherence after aPPTg lesions \((P < 0.01)\), however, a tendency for an increase was also observed after pPPTg lesion \((P = 0.074)\).

**Discussion**

Cholinergic lesions in the aPPTg marginally affected gait, i.e., reduced the front swing duration of the gait cycle, while all other gait related measures were not affected by lesions of either PPTg subregion. Our results are in line with the report of Alderson et al., (2008), who found a small reduction in locomotion after lesioning the aPPTg but not the pPPTg. Nevertheless, most previous studies have not seen changes in locomotion after full or partial lesions of the PPTg in rodents (Winn, 2006), while in primates PPN lesions lead to gait dysfunction and akinesia (Kojima et al., 1997; Aziz et al., 1998; Matsumura and Kojima, 2001; Karachi et al., 2010). With that regard, species dependent adaptive compensations of gait related problems must be considered, especially when comparing the quadruped locomotion in rodents with the biped locomotion in non-humane primates and humans. Interesting with that regard is that gait deficits after cholinergic PPN lesions were only found in adult monkeys (Aziz et al., 1998), while in young monkeys balance deficits and falls were only observed with the combination of nigrostriatal dopaminergic and PPN lesions (Karachi et al., 2010). Our finding about the effect of aPPTg lesions on gait is supported by the anatomic connection of this region to the basal ganglia. In rats the aPPTg projects to the SNc, while the pPPTg projects to the ventral tegmental area (Oakman et al., 1995; Alderson et al., 2008). Further, cholinergic neurons of the aPPTg preferentially innervates the motor associated dorsolateral striatum, while the pPPTg innervates the associative-limbic medial striatum and the nucleus accumbens shell (Alderson et al., 2008; Winn, 2008).

Because of the strong anatomical input to different BG regions we hypothesized that
Figure 8.6: Mean coherence of LFPs with the MCx-ECoG in different nuclei for the alpha (8-12 Hz) and beta (12-30 Hz) frequency range. Bars show the mean $\pm$ SEM of naïve, aPPTg lesioned and pPPTg lesioned rats. A significant difference to naïve controls is indicated by asterisks (*), a differences between aPPTg lesion and pPPTg lesion with (#; $P < 0.05$; one-way ANOVA and post hoc Tukey test).
cholinergic PPTg lesions would affect neuronal activity in the EPN, the output region of the BG. With that regard, both aPPTg and pPPTg cholinergic lesions lead to enhanced coherence of EPN beta band activity with MCx. Beta oscillations are enhanced in PD and have been associated with bradykinesia and rigidity since treatment with dopaminergic medication reduces beta band activity in parallel to improvement of symptoms in PD (Brown, 2003; Weinberger et al., 2006; Chen et al., 2007; Kühn et al., 2008; George et al., 2013; Connolly et al., 2015). Our findings implicate that the loss of cholinergic neurons in the PPN area may contribute to increased beta oscillatory activity, which has been observed after loss of nigrostriatal dopamine. Interesting with that regard is that bilateral lesions of the PPN in normal monkeys induce akinesia and bradykinesia that look like PD (Aziz et al., 1998). Also, unilateral excitotoxic lesions of the PPN with kainic acid injections induced mild levels of flexed posture and hypokinesia contralateral to the lesion (Kojima et al., 1997).

Nevertheless, except enhanced burst activity after pPPTg lesions, single unit activity of the EPN was not altered after cholinergic lesions of either PPTg subregion. It has been reported that rodents have less pallidal projection neurons to the PPN than monkeys (Alam et al., 2011), and some authors have even suggested that the EPN is not linked with the PPN, but rather with a brainstem region located just medial to it, which they referred to as the ‘midbrain extrapyramidal area’ (Rye et al., 1987; Lee et al., 1988; Steininger et al., 1992). Still, electrical stimulation of the rat PPTg increases the firing rates of neurons in the EPN (Scarnati et al., 1988), suggesting a strong functional connection to this nucleus. The PPN not only interacts with the BG motor loop, but also relays information to neural structures located in more caudal areas of the brainstem, which play a role in postural muscle tone (Alam et al., 2013). Cholinergic lesions of the aPPTg enhanced the firing rate and burst parameters in the CnF, whereas after pPPTg lesions only the number of bursts was reduced in the CnF. Since the majority of CnF neurons are GABAergic, enhanced CnF activity after aPPTg lesions may provide excess inhibitory output to the muscle tone via descending projections to the lower brainstem and spinal cord, resulting in subtle alteration in gait such as seen in our study (Kerr, 1975; Menetreay et al., 1982; Bjorkeland and Boivie, 1984). Interestingly, functional MRI studies during fast imagined gait in healthy humans showed activation of the region comprising the PPN and the CnF (Karachi et al., 2010). With that regard, boundaries between the posterior PPN and the CnF are indistinct — potentially confounding precise determination of the source of neuronal recordings and of the structures responsible for clinical effects. Interestingly, PPN electrodes in PD patients were most effective when located slightly posterior to the PPNc, which corresponds to the ventral part of the CnF (Ferraye et al., 2010).

Cholinergic lesions of both PPTg subregions decreased the alpha (8-12Hz) oscillatory co-
herence of the CnF and MCx-ECoG compared to naïve controls, an effect that was even stronger after aPPN lesion. Further, beta synchronization in the CnF and motor cortex was decreased after cholinergic lesions of either PPTg region. A clinical study in PD patients has demonstrated a correlation between alpha oscillations in the PPN and gait performance, which was particularly strong in the posterior PPN region, while beta oscillatory activity in the PPN area did not correlate with gait measures (Thevathasan et al., 2012). Gait freezing was associated with attenuation of alpha activity, whilst increases in PPN alpha power correlated with improved gait. Further, treatment with levodopa strongly enhanced alpha oscillatory activity in the PPN, suggesting that attenuated alpha activity could be pathologically associated with gait in PD (Androulidakis et al., 2008a; Androulidakis et al., 2008b). In their manuscript, the authors already raised the question, whether their findings would be specific for the posterior PPN, but may rather relate to the CnF, since the boundaries between the posterior PPN and the CnF are indistinct. Nevertheless, the authors discarded this thought, since in PD loss of cholinergic neurons is refined to the PPN, whereas no cellular degeneration of neurons has been found in the CnF. However, in the present study we showed that aPPTg and pPPTg cholinergic lesions led to reduced alpha power in the CnF, either by direct input or by indirect anatomical connections via the BG.

Oscillatory activity in the alpha frequency band has been shown to correlate with performance of cognitive tasks by active suppression of task irrelevant processes (Jensen and Mazaheri, 2010; Thevathasan et al., 2012). With that regard, in PD attentional deficits have been described, together with impaired automaticity of movement so that processing demands are higher in these patients (Wu and Hallett, 2005; Wu and Hallett, 2008). In line with this, PD patients with gait freezing seem to have even more attentional deficits than those without freezing of gait (Amboni et al., 2008; Yogev-Seligmann et al., 2008). Further, dual tasking, which is thought to ‘distract’ attention away from gait, can worsen gait freezing (Giladi and Hausdorff, 2006).

Within the PPTg, only cholinergic lesions in the pPPTg lesions led to enhanced irregular firing in the aPPTg, while lesions of the aPPTg had no effect. Further, the coherence of PPTg LFPs and MCx-ECoG oscillatory activity was not affected by cholinergic lesions of either region. The PPTg is formed by a cluster of intermingled cholinergic, glutamatergic and GABAergic neurons. Little is known, however, about its internal connections, but our finding may indicate that there is no strong interaction between subregion. One explanation for the enhanced irregular firing in the aPPTg may be that lesions in the pPPTg led to more than 20% reduction of aPPTg cholinergic neurons, while aPPTg lesions only marginally affected cholinergic neurons in the pPPTg (about 10%).
Conclusion

The pathophysiological mechanisms underlying gait disturbances after loss of cholinergic neurons in the PPTg still need further clarification. Using a rat model we showed that enhanced firing activity in the CnF after loss of cholinergic neurons in the aPPTg may provide excess inhibitory output to the muscle tone via descending projections to the lower brainstem and spinal cord. Additionally, reduced oscillatory alpha band activity in the CnF after cholinergic lesions of either PPTg subregion may contribute to gait disturbances via disturbed attention. Further, enhanced beta activity in the output nucleus EPN may contribute to altered gait behaviour. Together, these findings will contribute to the understanding of how the PPTg affects BG and MLR function that may be relevant for postural and gait abnormalities.
References


9 Discussion

Neuronal activities of the entopeduncular nucleus in Parkinson’s disease and dyskinesia

The major difference between 6-OHDA lesioned rats and those with LIDs in our study existed in the oscillatory activity, where we observed a remarked shift of EPN spike-MCx ECoG oscillatory coherence from high-beta (19-30 Hz) band in 6-OHDA lesioned rats to low-beta (12-19 Hz) band in those with LIDs. The EPN spike-MCx ECoG phase locked ratio, which is also a measurement of synchronization, showed similar result. Enhanced beta oscillations were consistently found in patients with PD (Brown, 2003; Kühn et al., 2006; Chen et al., 2007; Kühn et al., 2008; Ray et al., 2008) and animal models (Devergnas et al., 2014; von Wrangel et al., 2015; Nambu and Tachibana, 2014) and were considered to play an anti-kinetic role in the pathophysiology. Low- and high-beta oscillations have been reported in the PD patients undergoing DBS implantation in the STN (Priori et al., 2004; Marceglia et al., 2006; Marceglia et al., 2007; Lopez-Azcarate et al., 2010), and were thought to be differently modulated during motor tasks depending on the context (Marceglia et al., 2009). However, the role of these oscillations is still not well known.

Another difference we have observed is that levodopa injection seems to have less effect of reversing the enhanced firing irregularity in rats with LIDs as compared to those with out LIDs, as shown in the coefficient variation of inter-spike interval, the asymmetry index and distribution of firing patterns. An earlier study by Boraud et al. (2001) using a PD monkey model reported that both D1 and D2 dopamine receptor agonists induced similar enhanced firing irregularity during the onset of LIDs. However, the use of apomorphine, which is a mixed D1/D2 agonist, failed to show similar enhancement in the irregularity. This indicates that the underlying neuronal mechanisms during LIDs are more complex than simply irregular firing (Bezard et al., 2001).

In addition, we found significantly increased firing rate, higher divergence of the neuronal firing activity and more bursting neurons in 6-OHDA lesioned rats with and without LIDs, which is in line with many clinical and experimental findings (Tang et al., 2007; Starr et al., 2005; Bergman et al., 1994; Filion and Tremblay, 1991; Soares et al., 2004;
Mallet et al., 2008) and the “firing rate” model that predicts enhanced cortical inhibition from the BG output nuclei in PD.

Nevertheless, we failed to find significant differences between 6-OHDA lesioned rats and those with LIDs with regard to firing rate. As predicted by the “firing rate” model, decreased neuronal activity in the BG output nuclei is responsible for the hyperactive motor cortex that results in LIDs. Previous studies in patient with PD also showed lower firing rate during the expression of LIDs after application of different doses of levodopa to examine the “on” state without LIDs versus with LIDs (Lozano et al., 2000; Merello et al., 1999; Papa et al., 1999). One possible explanation for the inconsistency could be that these effects were more related to the doses applied, than to the underlying pathophysiology of LIDs.

Another inconsistency comes from the theta (4-8 Hz) band oscillatory activity, which is considered to be associated with the presence of abnormal dystonic posture or phasic movements in LIDs (Brazhnik et al., 2012; Lemaire et al., 2012). Although our EPN spike-MCx ECoG coherence showed somewhat higher theta (4-8 Hz) band synchronization, the theta band activity was reduced rather than increased after injection of levodopa. This does not match results from other studies (Alonso-Frech et al., 2006; Meissner et al., 2006). One previous study carried out in our lab also found enhanced theta oscillatory activity in the same 6-OHDA rat model of PD during the expression of LIDs, however the electrophysiology recordings of rat were performed in the free-moving state (Alam et al., 2014). With that regard, this inconsistent result of theta oscillatory activity, as well as the lack of differences in firing rate, could be due to the effect of anesthesia, which abolished the sensory motor feedback of involuntary movements after treatment of levodopa that may be involved in the genesis of theta spike-MCx coherence. This is also the major limitation in our study.

In the near future, techniques of electrophysiological recording in free-moving rat will be applied in the EPN and also other BG nuclei in order to eliminate the influence from anesthesia. In a previous study we found that DBS of the EPN reduced the differences of neuronal oscillatory activity in the striatum found between 6-OHDA lesioned rats with and without LIDs (Alam et al., 2014). We therefore plan to apply experimental DBS therapy and test its effect on single unit activity in different BG nuclei.
Motor function and neuronal activities following cholinergic lesions of anterior and posterior pedunculopontine tegmental nucleus

Evaluation of the motor functions following PPTg lesion only marginally affected swing duration of the front paws of the rats after cholinergic lesion in the aPPTg, whereas posture stability in the rotarod test, the spontaneous locomotor activity in the activity box, and other gait parameters were not altered. Lack of changes on spontaneous locomotor activity after lesion of PPTg has been reported in a recent study by MacLaren et al., (2014). In this study they also reported significant impairment of rat performance on the accelerating rotarod, but not on the rotarod with fixed speed. The reason why we did not find similar result in the rotarod test in the present study could be the different parameters we applied for the test (smooth accelerating 0-12 RPM over 60 s v.s. 0-40 RPM over 180 s in MacLaren’s study). Alterations in the front paw swing time aPPTg lesion are somewhat in line with the findings by Alderson et al., (2008), where a small reduction in locomotion has been observed after lesioning the aPPTg but not the pPPTg. These findings are at least to some extent supported by the anatomic connection of this region. The aPPTg projects to the SNc, which is a critical part of the BG motor loop, while the pPPTg projects to the ventral tegmental area, which is more related with limbic system (Alderson et al., 2008; Oakman et al., 1995).

One of the main findings in our study is the significantly increased firing rate and enhanced bursting in the single neuronal activities in the CnF following aPPTg lesion, whereas no significant alterations on single unit activity have been observed in the major BG output nuclei, the EPN. Studies of PD patients during an imagined gait task also showed significant associations with the subcuneiform region dorsal to the PPN (Piallat et al., 2009) and with the CnF (Karachi et al., 2010). As one of the major components of the MLR beside the PPTg, the CnF, which consists of mainly inhibitory GABAergic neurons, may exert an enhanced inhibitory influence on more caudal areas of the brainstem and mediate the suppression of muscle tone and subtle alteration in gait after aPPTg lesion (Bjorkeland and Boivie, 1984; Oakman et al., 1995; Menetrey et al., 1982). Combined with the results in the EPN, these may to some extent answer our question in the beginning about how the motor effects of the aPPTg lesions are achieved.

However, all of our findings could be epiphenomenal and need further investigation, especially with regard to interspecies differences of human and rodents. For instance, little is known regarding the interconnections between CnF and BG nuclei. Also, it is not known how well the EPN in rodent represents the human GPi. Studies have actually shown less
pallidal projection neurons in the PPTg in rodents compared to monkey (Alam et al., 2011). Some authors have even suggested that the EPN is not linked with the PPTg, but rather with the “midbrain extrapyramidal area” located just medial to it (Rye et al., 1987; Lee et al., 1988; Steininger et al., 1992). We also found decreased alpha (8-12 Hz) oscillatory coherence of the CnF-LFP and MCx-ECoG after both aPPTg and pPPTg lesion. Alpha oscillations are thought to play an important role in attention processes, particularly when these tend to occur over the lower frequency band in the alpha range (Klimesch, 1999; Palva and Palva, 2007). Studies in rodents have also reported significant impact on sustained attention as well as other cognitive functions (Cyr et al., 2015; Ivlieva and Timofeeva, 2003; Rostron et al., 2008). A clinical study in PD patients has demonstrated a correlation between alpha oscillations in the PPTg and gait performance. Gait freezing was associated with attenuation of alpha activity, whilst increases in PPTg alpha power correlated with improved gait (Thevathasan et al., 2012). Our results of the reduction of alpha synchronization in the CnF and motor cortical region suggest that the CnF area may have a function for modulating the network internal attention, which is considered to play a critical role in the pathophysiology in the gait disturbances in advanced PD.

In addition, enhanced beta (12-30 Hz) oscillatory coherence of EPN-LFP and MCx-ECoG were found after both aPPTg and pPPTg lesion. As usually considered to play an anti-kinetic role in PD (Brown et al., 2001), this enhanced beta activity by PPTg lesion may contribute to the akinesia independent of the nigrostriatal dopamine system.

In future, recordings in other BG nuclei such as the STN could help to further understand the pathophysiology underlying cholinergic lesions of the PPTg. We will also combine the 6-OHDA rodent model of PD with the cholinergic lesions of the PPTg, as the traditional 6-OHDA model does not lead to lesion of the PPN cholinergic system, which is involved in advanced PD. Further, experimental tests of DBS targeting the PPTg area will be applied in this combined model.

Overall, our data revealed remarkable changes in the oscillatory synchronization between BG network and the motor cortex, which may play a role for the development of LID. And the significant alterations in the single unit activity and oscillatory activity in the CnF following aPPTg lesion may contribute the gait disturbances in late stage of PD via its descending projections to lower motor regions and disturbed attention. However, further investigations are still necessary.
10 Summary

Experimental models of Parkinson’s disease with levodopa-induced dyskinesias and gait dysfunction: electrophysiological and behavioural measures in rats

Xingxing Jin

Levodopa-induced dyskinesias (LIDs) and gait disturbances are two troublesome conditions frequently occurring in advanced stage of Parkinson’s disease (PD), which also represents major therapeutic challenges in clinical practice. Although progressive nigral dopamine denervation and pulsatile dopamine stimulation are considered to play an important role in LIDs, the pathophysiology remains unclear. Further, the pedunculopontine nucleus (PPN), as a novel target for treatment of gait and posture instability in advanced PD, is considered critical for the pathophysiological mechanisms leading to these symptoms. However, to date the results of therapies targeting this area have been mixed, and the high heterogeneity of the neuronal discharge properties and neurochemical nature of the PPN area make it difficult to understand its exact role in the pathophysiology. Electrophysiological recording will shed light on the understanding of the pathophysiology and mechanisms underlying these conditions.

In the first project, we investigated the neuronal firing characteristics of the entopeduncular nucleus (EPN, equivalent to the internal segment of globus pallidus or GPi in human) and its coherence with the motor cortex field potentials in the 6-hydroxydopamine (6-OHDA) rat model of PD with and without LIDs. Our results showed significantly increased firing rate, higher divergence of the neuronal firing activity and more bursting neurons in the EPN in PD and LIDs with little differences in between. A shift from high (19-30 Hz) to low (12-19 Hz) beta oscillatory activity in the EPN spikes and motor cortex (MCx) coherence were found in rats with LIDs. We conclude that altered coherence and phase lock ratio of spike and local field potentials in the beta range may play a role in the pathophysiology of LIDs.

In the second project, we compared the effects of cholinergic lesions in the anterior or
posterior part of pedunculopontine tegmental nucleus (PPTg, equivalent to the PPN in primates) on gait-related motor behaviour and electrophysiological alterations of the EPN and the cuneiform nucleus (CnF), another mesencephalic motor nucleus adjacent to the PPTg in rats. Cholinergic lesions in the PPTg area showed no differences in posture on the rotarod and spontaneous movement in open field box. Only a decreased front limb swing time of gait in the treadmill test was found after anterior PPTg lesion. Changes of firing rate were only found in the CnF, which was increased after anterior PPTg lesion. The alpha (8-12 Hz) band CnF coherence with MCx field potentials were decreased after anterior or posterior PPTg lesions, especially after lesion of anterior PPTg, whereas, beta (12-30 Hz) band oscillatory coherence was decreased by posterior PPTg lesion. Anterior PPTg lesion also increased the beta (12-30 Hz) oscillatory synchronization in the EPN and the MCx field potentials coherence. Considering the results in this study, we can conclude that cholinergic lesions of the PPTg in rats had complex effects on oscillatory neuronal activity of the CnF and the basal ganglia (BG) network, and that the CnF may contribute to gait disturbances after loss of cholinergic neurons of either PPN subregion in late stage of PD.

Together, these data of the electrophysiology and behaviour alterations in rats will shed light on the understanding of the modulation of BG motor circuitry and pathophysiology in advanced PD.
Zusammenfassung

Experimentelle Modelle für die Parkinsonerkrankung mit Levodopa-induzierten Dyskinesien und Gangstörungen: elektrophysiologische Messungen und Verhaltensuntersuchungen in der Ratte

Xingxing Jin


Phasenbezugs (phase lock ratio) von Aktionspotentialen und lokalen Feldpotentialen im Beta-Spektrum eine Rolle bei der Pathophysiologie der LIDs spielen könnte.

Im zweiten Projekt verglichen wir die Effekte von cholinergen Läsionen im anterioren und posterioren Anteil des pedunkulopontinen tegmentalen Nukleus (PPTg, Äquivalent zum PPN bei Primaten) auf gangspezifische Motorik und elektrophysiologische Veränderungen des EPN und des cuneiformen Nukleus (CnF), eines anderen mesenzephalen motorischen Kernels, der bei Ratten dem PPTg benachbart liegt. Cholinerge Läsionen im PPTg Areal führten zu keinen Veränderungen in der Haltung auf dem Rotarod, sowie der spontanen Bewegung in der Open Field Box. Einzig eine verminderte Schwunggeschwindigkeit der Vorderpfoten beim Gang wurde beim Treadmill-Test nach anterierer PPTg-Läsion festgestellt. Veränderungen in der Feuerrate wurden nur im CnF gefunden, sie war nach anteriorer PPTg-Läsion erhöht. Die Alpha-Band (8-12 Hz) Kohärenzen mit den MCx Feldpotentialen waren vermindert nach anterierer und posterierer PPTg Läsionen, insbesondere nach Läsionen des anterioren PPTg, während die oszillatorische Kohärenz im beta-Band durch posterier PPTg-Läsionen vermindert war. Anteriore PPTg-Läsionen verstärkten auch die beta-oszillatorische (12-30 Hz) Synchronisation zwischen dem EPN und den MCx-Feldpotentialen. In Anbetracht der Ergebnisse dieser Studie können wir schließen, dass cholinergen Läsionen im PPTg bei Ratten komplexe Effekte auf die oszillatorische neuronale Aktivität des CnF und des Basalganglien-Netzwerkes (BG) hatten und dass der CnF möglicherweise zu den Gangstörungen nach Verlust von cholinergen Neuronen in einer der Subregionen des PPN beiträgt.

Insgesamt tragen die gezeigten elektrophysiologischen Daten und Verhaltensveränderungen bei Ratten zum Verständnis der Modulation des BG Motor-Schaltkreises und der Pathophysiologie bei fortgeschrittenem PD bei.
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