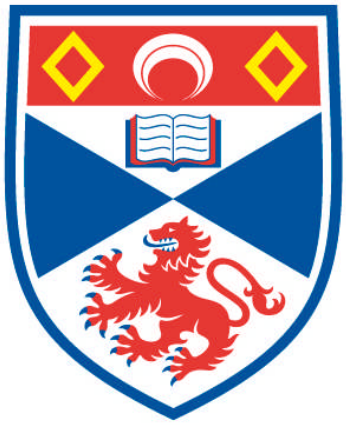
MODELLING THE EFFECTS OF DEEP BRAIN STIMULATION IN THE PEDUNCULOPONTINE TEGMENTAL NUCLEUS IN PARKINSON'S DISEASE

Nadine Katrin Gut

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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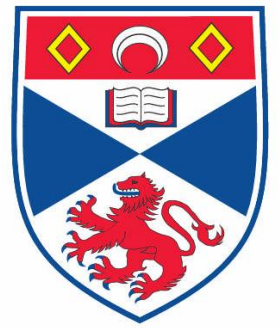

This thesis is submitted in partial fulfilment for the degree of PhD

at the University of St Andrews

March 2014

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I wish to dedicate this work to my Mum and my late Dad, who tirelessly gave all their support, understanding and patience and allowed me to follow my chosen path – wherever it took me – and to achieve my goals. I thank my brother for all his encouragement and simply for always being my big brother.

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Abstract

Based on the belief that it is a locomotor control structure, the pedunculopontine tegmental nucleus (PPTg) has been considered a potential target for deep brain stimulation (DBS) for Parkinson's disease (PD) patients with symptoms refractory to medication and/or stimulation of established target sites. To date, a number of patients have been implanted with PPTg electrodes with mostly disappointing results. Exact target site in PPTg, possible mechanisms of PPTg-DBS and likely potential benefits need to be systematically explored before consideration of further clinical application.

The research described here approaches these questions by (i) investigating the role of the PPTg in gait *per se*; (ii) developing a refined model of PD that mimics the underlying pathophysiology by including partial loss of the PPTg itself; (iii) adapting a wireless device to let rats move freely while receiving DBS; and (iv) investigating the effect of DBS at different sites in the PPTg on gait and posture in the traditional and refined model of PD.

Underlining the concern that understanding the PPTg as a locomotor control structure is inadequate, the experiments showed that neither partial nor complete lesions of PPTg caused gait deficits. The refined model showed hardly any differences compared to the standard one, but the effect of DBS in each was very different, highlighting the need to take degeneration in the PPTg into consideration when investigating it as a DBS target. The differential results of anterior and posterior PPTg-DBS show the critical importance of intra-PPTg DBS location: Anterior PPTg electrodes caused severe freezing and worsened gait while some gait parameters improved with stimulation of posterior PPTg.

The results suggest mechanisms of PPTg-DBS beyond the proposed activation of over-inhibited PPTg neurons, including aggravation of already dysfunctional inhibitory input by anterior PPTg-DBS and activation of ascending projections from posterior PPTg to the forebrain.

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List of abbreviations

2-DG	2-deoxyglucose
6-OHDA	6-hydroxydopamine
ABC	avidin-biotin complex
ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
ADS	antibody diluting solution
AIM	abnormal involuntary movement
ANOVA	analysis of variance
BG	basal ganglia
BOS	base of support
Cb	calbindinD_{28k}
ChAT	choline acetyltransferase
CM	centromedian nucleus of the thalamus
CW	CatWalk
cZi	caudal zona incerta
DA	dopamine
DAB	3,3-diaminobenzidine
DBS	deep brain stimulation
Dtx-UII	diphtheria toxin - urotensin II
FOG	freezing of gait
FR	fixed ratio
GABA	γ-aminobutyric acid
GP	globus pallidus

GPe	globus pallidus external segment
GPi	globus pallidus internal segment
HFS	high frequency stimulation
i.d.	inner diameter
i.p.	intraperitoneal
IAL	intra-aural line
IC	inferior colliculus
IgG	immunoglobulin G
LB	Lewy body
LC	locus coeruleus
L-DOPA	L-3,4-dihydroxyphenylalanine
LDTg	laterodorsal tegmental nucleus
LFP	local field potentials
LFS	low frequency stimulation
LHS	left-hand side
LID	L-DOPA induced dyskinesias
LN	Lewy neurites
MFB	medial forebrain bundle
MLR	mesencephalic locomotor region
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	multiple system atrophy
MSN	medium spiny neuron
NAcc	nucleus accumbens
NeuN	neuronal nuclei
o.d.	outer diameter

PBP	parabrachial pigmented nucleus
PBS	phosphate buffered saline
PD	Parkinson's disease
PET	positron emission tomography
Pf	parafascicular nucleus of the thalamus
PIGD	postural instability and gait disturbance
PN	paranigral nucleus
PPI	prepulse inhibition
PPN	pedunculopontine tegmental nucleus
PPTg	pedunculopontine tegmental nucleus
PR	progressive ratio
PSP	progressive supranuclear palsy
Pt/Ir	Platinum/Iridium
REM	rapid eye movement
RHS	right-hand side
s.c.	subcutaneous
SC	superior colliculus
SN	substantia nigra
SNc	substantia nigra pars compacta
SNl	substantia nigra lateralis
SNm	substantia nigra medialis
SNr	substantia nigra pars reticulate
STN	subthalamic nucleus
TH	tyrosine hydroxylase
UPDRS	Unified Parkinson's Disease Rating Scale

VA	ventral anterior nucleus of the thalamus
VIM	ventral intermediate nucleus of the thalamus
VL	ventrolateral nucleus of the thalamus
VR	variable ratio
VTA	ventral tegmental area

Chapter 1

General Introduction

Studies of post-mortem human brain, work with animal models, and the intimate connection it holds with the basal ganglia (BG), it all indicates that the pedunculopontine tegmental nucleus (PPTg) has a role in Parkinson's disease (PD). The introduction of subthalamic nucleus (STN) deep brain stimulation (DBS) to clinical practice has been a consistent success in the treatment of parkinsonian symptoms and other conditions like dystonia and essential tremor. Since 2005 DBS has also been applied to the PPTg in attempt to address symptoms of Parkinsonism with a focus on symptoms refractory to dopamine (DA) medication – gait and postural disturbances, freezing of gait (FOG) – and for patients not considered good candidates for STN-DBS. However, there is doubt about whether the PPTg will ever be considered an established target for DBS in PD: there is controversy about its actual potential, the nature of symptoms that might be addressed with it, stimulation parameters, and a major question about the optimal site for stimulation within the PPTg and the effects that can be expected by stimulating different locations within it.

1. Parkinson's disease

1.1. Clinical features

It was not until 50 years had passed after James Parkinson's publication "An essay on the shaking palsy" in 1817 (Parkinson, 2002) when this neurological disorder was first called "Parkinson's disease" by Jean-Martin Charcot, who made tremor the main symptom, correcting the understanding that the slowness of movement results from a weakness of the muscles (Lanska, 2010). Today, four symptoms are recognised as cardinal features of PD: resting tremor, rigidity, akinesia and postural instability. A flexed posture and FOG are also recognised among the classic features (Jankovic, 2008). In addition, patients can show further motor symptoms secondary to the cardinal symptoms such as hypomimia, dysarthria, dysphagia and sialorrhea, micrographia, shuffling gait and festination, dystonia and glabellar

reflexes (see Table 1.1). Non-motor symptoms include cognitive impairment such as dementia (affecting up to 80% of patients in advanced stages of PD; (Aarsland *et al.*, 2003) or deficits in executive functioning (Chaudhuri and Schapira, 2009), which can already occur in early stages of the disease. Other non-motor symptoms can be anhedonia, apathy, depression, anxiety, sleep disorders, autonomic symptoms such as bladder disturbances and orthostatic hypertension, gastrointestinal symptoms including nausea and constipation and sensory disturbances affecting vision and smell [(for a more detailed review please refer to (Chaudhuri *et al.*, 2006; Chaudhuri and Schapira, 2009)]. Individual patients experience different symptoms –or rather combinations of symptoms – with diverse degrees of severity. How much it finally affects the individual person's quality of life depends of course on the stage of the disease and the severity of the symptoms but also on their lifestyle, profession, social and familial situation.

Table 1.1. Motor and non-motor symptoms in Parkinson's disease.

Parkinson's disease symptoms	
Motor symptoms	
tremor	resting tremor (4-6Hz) in limbs with distal predominance, "pill-rolling" tremor
rigidity	increased muscular tone to palpation at rest, reduced distension to passive movement and increased resistance to stretching
hypokinesia	reduced frequency and amplitude of spontaneous movement
bradykinesia	slowness of movement, reduced speed when initiating and executing a single movement and progressive reduction of amplitude of repeated movements
postural instability	
FOG	difficulties initiating gait or interruption of ongoing gait
shuffling gait	
festination	quickening and shortening of normal strides
problems arising from chair	
decreased arm swing	
micrographia	abnormally small handwriting
slow activities of daily living	
bulbar dysfunction	
hypomimia	reduced facial expressions
dysarthria	motor speech disorder
dysphagia	swallowing difficulties
sialorrhea	excessive production of saliva
hypophonia	"weak" voice
Myerson's sign	unhabituated glabellar reflex: primitive reflex of eye blinking elicited by tapping on the forehead, which normally ceases after a few tabs
blepharospasm	increased blinking or spasms of eyelid closure
postural deformities	
striatal deformities	abnormal postures of hand and foot
scoliosis	abnormal curvature of the spine to the side
camptocormia	abnormal flexion of the trunk in standing or walking which disappears in supine position

Parkinson's disease symptoms

Non-motor symptoms	
Neuropsychiatric symptoms	
depression	
apathy	diminished motivation
anhedonia	loss of pleasure in previously enjoyable activities
anxiety and panic attacks	
cognitive dysfunction	bradyphrenia ("slowed thinking"), verbal fluency deficit, executive dysfunction
attentional deficit	
hallucinations, illusions, delusions	
dementia	
confusion	
Sleep disorders	
REM behaviour disorder	parasomnia manifested by vivid dreams associated with dream enactment behaviour
excessive daytime somnolence	
insomnia	
Autonomic symptoms	
bladder disturbances	
sweating	
orthostatic hypotension	sudden fall of blood pressure when standing up/stretching
erectile impotence	
gastrointestinal symptoms	
reflux, vomiting	
nausea	
constipation	
Sensory symptoms	
ageusia	lack or impairment of the sense of taste
anosmia	loss of the sense of smell
Pain	
Paraesthesia	"pins and needles"
visual dysfunction	i.a. impairment of spatial contrast sensitivity, visual acuity, reduced contraction amplitude (Armstrong, 2011)

1.2. Towards the understanding of the disease

About one hundred years after Parkinson's essay, cell loss in the substantia nigra (SN) could be linked to parkinsonian symptoms and 140 years after the publication Carlsson and colleagues identified DA as an independent neurotransmitter involved in the mechanisms behind the pathology. This was confirmed by Ehringer and Hornykiewicz who proved that there were decreased DA levels in the striatum of PD patients (Bjorklund and Dunnett, 2007b; Jankovic, 2008). These findings not only led to the discovery and establishment of L-3,4-dihydroxyphenylalanine (L-DOPA) as an effective treatment for parkinsonian symptoms in the late 1950s and 60s but also to the understanding of the involvement of midbrain DA neurons in motor control (and of course, later in reward signalling during learning: see for example (Redgrave *et al.*, 2008; Schultz, 2006). This ultimately led to the identification of neuropathological processes that cause movement disorders such as PD. Consequently, rapid progress was made and models of BG functioning were formulated.

1.2.1. Basal Ganglia circuitry

The BG are a group of interconnected nuclei and form an essential component of various parallel cortico-subcortical circuits in which information from cortical areas is processed in a collection of interconnected nuclei and returned to the cortical areas from which the information originated, passing via the thalamus. In addition, these processes are modulated by intimately connected midbrain and brainstem structures which in return receive information from the BG themselves. The BG are described as being comprised of the caudate nucleus and the putamen (which, together form the dorsal part of the striatum: in primate the caudate and putamen are separated by the internal capsule; in non-primate brains they form one complex, the caudate-putamen), the globus pallidus (GP; with its two separate external and internal segments GPe and GPi respectively), the STN and SN, which divides into pars

compacta (SNc) and pars reticulata (SNr). Putamen and GP as an entity are also called the lenticular nuclei (Gerfen and Bolam, 2010; Wichmann *et al.*, 2002).

It should be noted that the ventral striatum constitutes a further part of the BG. The dorsal striatum includes the caudate and putamen as noted above, and receives DA input from SNc. The portion below is the ventral striatum: the nucleus accumbens (NAcc) core and shell, as well as the olfactory tubercle, which receive DA input from the ventral tegmental [VTA; (Humphries and Prescott, 2010; MacDonald *et al.*, 2011)].

Since the early 1980s it has been acknowledged that the BG are more than a structure of "pure" motor control but that they are anatomically and topographically and also functionally more complex than that. In the Robert Wartenberg Lecture in 1982 David Marsden highlighted that the "*the striatum takes in information from virtually all areas of the nervous system to undertake some unknown transformation into instructions to the output zones of the GPi and SNr whose own output is coded in relation to motor behavior"* [(Marsden, 1982) p. 518]. Rather than causing a "more" or "less" of movement by activating individual pathways of the BG circuits ['scaling and focusing model'; see (DeLong and Wichmann, 2009)] facilitating desired movements and inhibiting unwanted movement, it is assumed that the BG have a role in action selection processes, an ability crucial for every organism and an "evolutionary ancient problem" (Redgrave *et al.*, 2011), a function that critically involves learning. Only one "final common motor path" (Redgrave *et al.*, 1999) can be executed at a time, so that the most advantageous action needs to be selected from all possible ones in any situation.

The "segregated circuit hypothesis" (Alexander *et al.*, 1986; Middleton and Strick, 2000) describes how information originating from different cortical areas enters and leaves the BG maintaining largely functional territories throughout the individual circuits and re-enters the same cortical areas it originated from. Haber and colleagues describe how the topography

results in a tight anatomical and functional organisation with interconnected thalamic and cortical afferents terminating in the same striatal area, from where the functional topography is continued to the BG output nuclei and maintained back to cortex via the thalamus (Haber, 2003). However, they also highlight the necessity to extend this concept of parallel processing by the notion of integrative circuits by which information from separate loops can influence each other (Haber, 2003; Haber *et al.*, 2000).

The resulting loop architecture enables an integration of different types of information and a functional role beyond simple motor functions. “Motor”, “cognitive”, and “limbic” loops connect different cortical, subcortical and thalamic areas in a similar manner [also often divided into “motor, associative, sensory and limbic” (Redgrave *et al.*, 2011) or including “oculomotor” and “orbitofrontal” loops (Joel and Weiner, 2000; Obeso *et al.*, 2008; Voorn *et al.*, 2004; D. I. G. Wilson *et al.*, 2009)] . Given the relevance of the motor circuit for this study the following descriptions will focus on this, but it is important to point out that those parallel loops are not always closed and information can spread between loops (Joel and Weiner, 1994; Miyachi *et al.*, 2002), allowing for a role beyond motor control by integrating various functions giving rise to behaviour, which rarely can be classified as purely “motor”.

Efferents from cortical motor areas project topographically to the putamen - the “motor portion” of the striatum (Wichmann and Dostrovsky, 2011). A minor part projects directly to the STN, which constitutes the hyperdirect pathway. It is suggested that the cortico-subthalamic pathway is separate from the corticostriatal pathway, meaning that a different type of information might be processed separately at that point (Parent and Parent, 2006). Information leaving the dorsal striatum takes two different pathways: either the direct, monosynaptic way innervating the BG output nuclei GPi (entopeduncular [EP] nucleus in rodents) and the SNr or the indirect, polysynaptic one, which involves the GPe and the STN.

These corticostriatal projections to striatal medium spiny neurons (MSNs) express D1 receptors and are involved in the activation of the direct pathway; they are distinct from those projecting to D2-expressing MSNs, which are involved in the activation of the indirect pathway (Lee *et al.*, 2005). The BG output from GPi/SNr is mainly directed toward the ventrolateral (VL) and ventral anterior (VA) nuclei of the thalamus which form reciprocal connections with the BG and also project back to cortical areas, closing the cortical-subcortical-cortical motor loop. Interestingly VA and VL also innervate the intralaminar thalamic nuclei (the centromedian nucleus [CM] and the parafascicular nucleus [Pf]) both of which receive important cholinergic input from PPTg and the laterodorsal tegmental nucleus (LDTg) and are crucial in procedural learning, providing salience information to the striatum (Kimura *et al.*, 2004). GPi/SNr make descending connections with midbrain and brainstem targets such as the superior colliculus (SC) and, relevant to this study, the PPTg, with which the BG form reciprocal connections (see Chapter 2.3.). Figure 1.1. summarises the intrinsic circuitry of the BG motor loop.

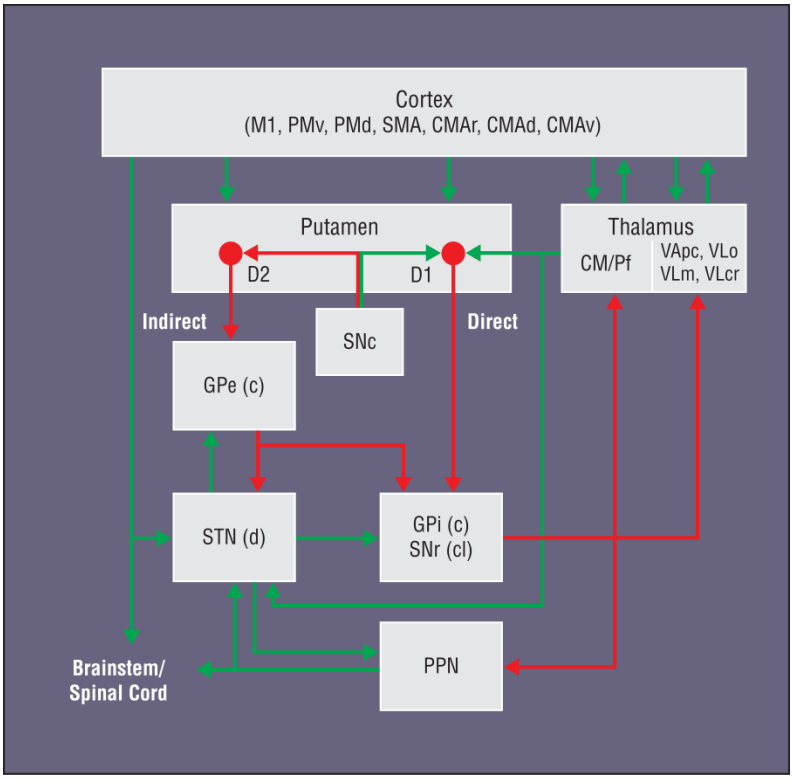

Figure 1.1. Cortico-subcortical circuit model representing the so called motor circuit. Red arrows indicate inhibitory connections, green arrows excitatory connections. This figure is lacking further connections of the PPN with BG nuclei, most notably its inhibitory afferents from the BG output nuclei GPi/SNr. For further information on PPTg connectivity please refer to Chapter 2.3.2. Abbreviations: CM, centromedian nucleus of the thalamus; CMAr, rostral portion of cingulate motor area; CMAd, dorsal portion of cingulate motor area; CMAv, ventral portion of cingulate motor area; GPe, external segment of the globus pallidus; GPi, internal segment of the globus pallidus; M1, primary motor cortex; Pf, parafascicular nucleus of the thalamus; PMd, dorsal premotor cortex; PMv, ventral premotor cortex; PPN, pedunculopontine nucleus; SMA, supplementary motor area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; VApc, ventral anterior nucleus of thalamus pars parvocellularis; VLm, ventrolateral nucleus of thalamus pars medialis; VLo, ventrolateral nucleus of thalamus pars oralis; VLcr, ventrolateral nucleus of thalamus rostral pars caudalis; c, caudal; cl, caudolateral; d, dorsal. Image reproduced from (DeLong and Wichmann, 2007).

The direct pathway results, when activated, in an inhibition of the GPi and the SNr which means a reduction of inhibitory BG output, leading to a disinhibition of targeted thalamocortical neurons and facilitation of the intended movement. The indirect pathway has an opposite effect when activated: it increases BG output by disinhibition of its activity and therefore increases the inhibition of thalamocortical projections, suppressing unintended or competing movements (DeLong and Wichmann, 2007; Galvan and Wichmann, 2008).

However, this is a very simplified model of BG functioning. Those intrinsic circuits are not that clear cut as mentioned above; many striatal projections have collaterals projecting to both segments of the GP, extending beyond functional domains (Levesque and Parent, 2005; Parent *et al.*, 2000; Wu *et al.*, 2000). Another mechanism causing an integration of circuits is through complex non-reciprocal connections between structures such as feedforward connections from the ventral striatum to the dorsal striatum via midbrain dopamine neurons (Haber, 2003; McFarland and Haber, 2002). Neither does this model acknowledge subcortical loops connecting the PPTg, SC, periaqueductal grey, cuneiform, habenula and parabrachial nuclei to the BG, offering important modulatory influence (McHaffie *et al.*, 2005; Mena-Segovia *et al.*, 2004; Redgrave and Coizet, 2007; Winn *et al.*, 2010). The lateral habenula for example provides inhibitory input to midbrain dopamine neurons via the RMTg (Balcita-Pedicino *et al.*, 2011; Hong *et al.*, 2011) responding inversely to stimuli associated to reward, omission and delivery of reward compared to DA neurons (Matsumoto and Hikosaka, 2007). It further ignores important connections of the BG circuit with cerebellar circuits (Hoshi *et al.*, 2005). Further, some authors include the VTA and NAcc into descriptions of the BG [for example (Yin *et al.*, 2006)].

The rate model of the pathophysiology of PD describes firing rate changes in the BG after striatal DA loss as a result of the disturbances in the activity of the direct and indirect pathway.

Decreased activation of D2-expressing MSNs presents a reduction of inhibition of corticostriatal transmission causing increased activity along the inhibitory indirect striatopallidal pathway, reducing the firing rate in the GPe which results in disinhibition of STN activity and facilitation of GPi firing (Breit *et al.*, 2006; Mitchell *et al.*, 1989; Vila *et al.*, 1997). In a similar manner it was suggested that a decrease of D1-expressing MSNs leads to reduced activity along the direct pathway resulting in an increased activity in the BG output nuclei, but this has not been directly demonstrated (Wichmann and Dostrovsky, 2011). The consequence of the increased inhibitory BG output would cause a reduction in thalamocortical activity and increased inhibitory output onto downstream brainstem targets (see Figure 1.2.).

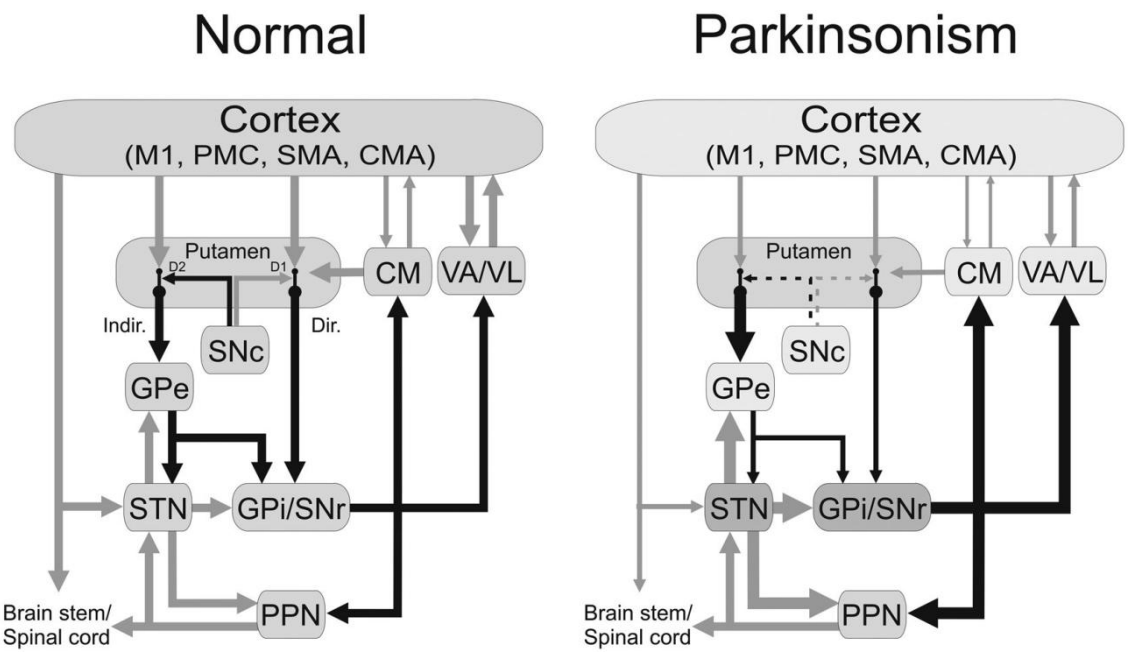

Figure 1.2. Shift of balance of BG activity towards the indirect pathway in Parkinson's disease according to the 'rate model'. Black arrows indicate inhibitory connections, gray arrows excitatory connections. The thickness of the arrows corresponds to their presumed activity. Abbreviations: CM, centromedian nucleus of thalamus; CMA, cingulate motor area; Dir., direct pathway; D1 and D2, dopamine receptor subtypes; GPe, external segment of the globus pallidus; GPi, internal segment of the globus pallidus; Indir., indirect pathway; M1, primary motor cortex; Pf, parafascicular nucleus of the thalamus; PMC, premotor cortex; PPN, pedunculopontine nucleus; SMA, supplementary motor area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; VA, ventral anterior nucleus of thalamus; VL, ventrolateral nucleus of thalamus. Image reproduced from (Galvan and Wichmann, 2008).

However, the rate model fails to explain clinical findings: contrary to what the model would suggest, lesions of the thalamus or GP did not lead to worsening of hypokinesia or bradykinesia, nor did it cause dyskinesias (Marsden and Obeso, 1994). Furthermore, the firing rates predicted by the rate model do not always correspond to findings in Parkinson's models: instead of the expected reduced firing rate in GPe and increased firing rate in GPi, several studies have found no significant changes in individual structures [GPi: (Wichmann *et al.*, 1999); motor cortex: (Doudet *et al.*, 1990; Goldberg *et al.*, 2002; Rubin *et al.*, 2012)]. GPe-lesion induced rate changes in BG nuclei similar to changes seen after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) had no behavioural effects (Soares *et al.*, 2004).

It is another distinct abnormality of BG activity that replaces the exclusive firing rate models: prominent changes of BG firing patterns, such as bursts and oscillatory patterns and abnormal synchronization have been found in parkinsonian animals but have also been recorded by DBS electrodes implanted in PD patients (for exhaustive summaries see (Eusebio and Brown, 2007; Galvan and Wichmann, 2008; Hammond *et al.*, 2007; Wichmann and Dostrovsky, 2011). Synchronized oscillatory activity was consistently shown at multiple levels of the BG-cortical loop [for example (Brown and Williams, 2005; Priori *et al.*, 2004)] and is largely understood to be the hallmark of activity in PD, which is suggested to correlate with motor deficits (Hammond *et al.*, 2007). In animal models of PD and in PD patients ('OFF' L-DOPA) increased burst activity was measured from STN, GPe and GPi forming oscillatory activity of neuronal spike trains and local field potentials (LFP). Throughout the extrastriatal BG significant oscillatory activity frequently occurred at a high alpha/ low beta frequency range of 10-35Hz [other report 8-30Hz, called 'basal ganglia beta frequency band' by (Hammond *et al.*, 2007)]. This oscillatory activity can form synchronous activity among neurons within individual nuclei and coupled between individual structures such as the STN, GPi and cortex (Gatev *et al.*, 2006); or, as reported by Heimer and colleagues, between GPe neurons, between striatal tonically

active interneurons and between those two populations (Heimer *et al.*, 2006). Beta-band activity is suppressed by treatment with dopaminergic drugs, correlating with motor performance (Brown, 2006; Heimer *et al.*, 2006; Kuhn *et al.*, 2004; Marceglia *et al.*, 2006). As a consequence of excessive synchronization it is believed that information coding through spatial and temporal patterning by individual neurons or groups of neurons is limited causing inappropriate motor performance (Wichmann and Dostrovsky, 2011). Beta band oscillations are present in non-parkinsonian patients (Sochurkova and Rektor, 2003), healthy rodents (Leventhal *et al.*, 2012) and non-human primates (Courtemanche *et al.*, 2003) and suppression of this beta band activity was related to movements. Prior to and during voluntary movement (self-paced and externally paced) beta band oscillations in LFP of STN and GPi were also shown to be reduced in PD patients (Kuhn *et al.*, 2004; Priori *et al.*, 2002) and increased during no-go tasks that require motor inhibition (Kuhn *et al.*, 2004). It is suggested that the elevated levels of synchrony might be more resistant to this movement related suppression (Hammond *et al.*, 2007). It is not yet well understood how DA loss can lead to this changed oscillatory activity but it is very likely that the profound DA degeneration is the key cause of it.

This model of PD pathophysiology has its problems as well: (i) these changes do not account for all behavioural manifestations; (ii) not all of the PD symptomatology responds to dopaminergic medication and neither can DA medication always attenuate excessive BG synchrony; (iii) neuronal changes have been reported before the manifestation of motor deficits in PD models (Bezard *et al.*, 1999); and (iv) neuronal changes have been observed after the onset of parkinsonian symptoms, making these changes unlikely to be the cause of the phenotype.

The above described models of BG circuitry and dysfunction have led to immense advances in the treatment of movement disorders. However, it shows that the understanding needs to be

extended and a better model of PD developed – discussion around the involvement of the PPTg is an example of this. Moreover, it is important to recognise that the underlying anatomical brain pathology of PD goes beyond DA degeneration in SN.

1.2.2. Underlying brain pathology of PD

The most important hallmarks of PD are considered to be Lewy neurites (LN) and Lewy bodies (LB), associated with damage to the SNc and consequential progressive loss of DA neurons. But SN is not the first and only structure to undergo PD-related degeneration (Del Tredici *et al.*, 2002). It is always accompanied by extranigral pathology which Braak and colleagues have assessed and categorised temporally, proposing a topographical development of affected structures along the time course of the disease. They describe an ascending development in six stages reporting the first lesions in stage 1 to appear in the medulla oblongata, the dorsal IX/X motor nucleus, the olfactory bulb and/or the intermediate reticular zone. In stage 2 these are extended by degeneration in the caudal raphe nuclei, reticular nuclei and the coeruleus-subcoeruleus complex. Non-motor symptoms like impaired sense of smell and autonomic dysfunction usually precede motor dysfunctions by several years (Gaenslen *et al.*, 2011; Winkler *et al.*, 2011). The characteristic SN degeneration does not start before stage 3 according to Braak, when the typical PD motor symptoms arise, around the same time that LNs start to appear in the posterior PPTg (for more details on PPTg degeneration see Section 2.1.2. of this chapter). Over the course of time these lesions augment and the structure gets affected more severely; additionally prosencephalic areas and the basal forebrain show signs of degeneration as well (stage 4). At later stages of the disease when patients show severe PD symptoms limbic structures and the neocortex are affected as well (Braak *et al.*, 2003).

1.3. Gait and freezing of gait

In this section the disturbances related to gait and in particular to FOG will be discussed in more detail, because they seem to differ to other motor symptoms in the response to dopaminergic medication, and because in the PPTg-DBS literature a particular focus is put on FOG and associated falls. FOG constitutes a very common feature of PD, but it differs in various aspects from the other cardinal symptoms. It is described as a brief, episodic absence or notable reduction of forward progression of the feet, despite the intention to walk (Nutt *et al.*, 2011). Patients experience a feeling of "being glued to the ground". Without depreciating the other cardinal symptoms of PD FOG has a significant impact on quality of life (Moore *et al.*, 2005), increases the risk of falls (Bloem *et al.*, 2001; Bloem *et al.*, 2004; Moore *et al.*, 2007; Schaafsma *et al.*, 2003) which may lead to severe injuries, the risk of nursing home admission and mortality (Gray and Hildebrand, 2000; Hely *et al.*, 1999). A further problematic characteristic of this symptom is the difficulty of assessing its frequency and severity objectively due to its paroxysmal and unpredictable nature (Fahn, 1995). Given its distinct response to L-DOPA (see below) in comparison with other symptoms it seems to be linked to a different or more complex pathophysiology than other common symptoms of PD.

PD patients with FOG can experience an inability to start walking ("start hesitation") or continue to walk ("destination hesitation"), but FOG can also present itself as "trembling in place" [at frequency of 3-8Hz; (Moore *et al.*, 2008)] and "shuffling forward" [an increase in cadence with a decreased step length (Nieuwboer *et al.*, 2001)]. It is mostly triggered when initiating walking, turning ("turning hesitation)", passing through narrow spaces, negotiating obstacles, reaching a target, in situations of stress, or during dual tasks (Hausdorff, Balash*, et al.*, 2003; Schaafsma *et al.*, 2003).

While visual stimuli like narrow doorways or obstacles can cause a freezing episode, sensory cues can also act as "releases": using external temporal or spatial stimuli (Okuma, 2006; Spildooren *et al.*, 2012; Velik *et al.*, 2012), patients can learn to facilitate movement (gait) initiation and continuation (Nieuwboer *et al.*, 2007). In clinical practice patients are taught to use external or internal cues (Snijders *et al.*, 2008), such as imagining or choosing targets for stepping, or visualising climbing a stair in order to initiate a step. By focussing on the stepping the patient might be increasing cortical control supporting impaired subcortical motor control (Rahman *et al.*, 2008), or the effect of cueing might be due to an increase of attention and distraction from an increased cognitive load caused by external stimuli or in dual task situations (Nieuwboer, 2008).

Traditionally FOG is understood as a motor symptom found in advanced PD (Macht *et al.*, 2007). FOG occurs in about 53% if patients in advances stages (Giladi, 2001). However, up to 26% of patients also experience this symptom during earlier stages (including pre L-DOPA exposure) (Giladi, McDermott*, et al.*, 2001; Giladi, Treves*, et al.*, 2001; Lamberti *et al.*, 1997). Early FOG is associated with cognitive decline and depression rather than bradykinesia, rigidity and tremor (Bartels *et al.*, 2003; Giladi, McDermott*, et al.*, 2001). Other main risk factors for the development of FOG are severe impairment of gait, balance and speech.

FOG can be differentiated into "off"-FOG and "on"-FOG. FOG that occurs in a patient's "off"-period is in general responsive to DA treatment and to DBS of STN or GPi. "On"-FOG, however is either not responsive (Krause *et al.*, 2004) or the response to DA treatment decreases over time (Bloem *et al.*, 1996). This, and the fact that many PD patients never experience FOG, suggests a different, extranigral, non-dopaminergic pathophysiology. Furthermore, FOG is also present in other hypokinetic, extrapyramidal movement disorders, such as progressive supranuclear palsy (PSP), pure akinesia syndrome (Williams *et al.*, 2005), multiple system

atrophy [MSA (Gurevich and Giladi, 2003)], corticobasal degeneration (Rinne *et al.*, 1994), higher level gait disorder (Giladi *et al.*, 2007), vascular parkinsonism (Factor *et al.*, 2002), normal pressure hydrocephalus (Giladi *et al.*, 1997), and focal frontal lesions (Nadeau, 2007). This shows that disruptions at different levels of common neural networks may still lead to similar symptoms. Regarding FOG in PD it has been suggested that the physiological mechanism responsible for it may lie in the brainstem – possibly in the PPTg and locus coeruleus (LC) (Grimbergen *et al.*, 2009).

A number of studies have investigated the relation between FOG in PD and other symptoms, and identified characteristics of FOG patients which separate them from PD patients that do not experience FOG. They describe factors that correlate with FOG occurrence and severity in order to describe predisposing risk factors or ideally formulate hypotheses about the nature and reason for FOG. (i) Abnormal gait pattern generation and sequencing of gait, also described as gait rhythmicity, can be related to FOG (Iansek *et al.*, 2006; Nieuwboer *et al.*, 2001). FOG during walking might be a "sequence effect" of gradually decreasing step length and increased stance phase, which in turn can be caused by an altered timing and premature muscle activation and termination patterns (Nieuwboer *et al.*, 2004). General stride to stride variability, and the ineffective bilateral coordination found in FOG patients might support and/or exacerbate freezing episodes (Chee *et al.*, 2009; Hausdorff, Schaafsma*, et al.*, 2003; Plotnik *et al.*, 2005). The latter is likely contributing to freezing during turning and gait initiation when coordination is more complex and independent control of both legs is required (Plotnik *et al.*, 2007, 2008). (ii) Problems with implicit learning and automatic task performance might explain problems in dual task situations (Hallett, 2008) and for patients with difficulties in executive functioning. Gait and posture can be understood as skilled and automated movements. Problems with these functions can be compensated for by additional executive control. Indeed, PD patients show an increased brain activity associated with automatic

movements compared to healthy controls indicating the need for recruitment of more executive control to compensate for inefficient brain activity in executing automatic movements (Wu and Hallett, 2005). But employing more cognitive resources to make up for dysfunctional automaticity might mean that in a situation of increased cognitive demand, such as in a dual task situation, there is a risk of cognitive overload (Heremans *et al.*, 2013). (iii) A strong association between executive dysfunction and FOG has been shown repeatedly (Giladi and Hausdorff, 2006). Implicit learning and automatic task performance was deficient in PD patients and suggested to be worse in patients with a higher incidence of falls (Hallett, 2008). Patients showing unsteady and arrhythmic gait in dual task situations also showed more deficits in executive functioning and it was suggested that the worse the deficit the higher the risk of FOG (Yogev *et al.*, 2005). FOG correlates with cognitive decline, particularly executive dysfunction such as deficient set-shifting and conflict resolution (Naismith *et al.*, 2010) and early FOG was associated with cognitive decline and depression rather than rigidity and bradykinesia (Giladi and Hausdorff, 2006; Giladi, McDermott*, et al.*, 2001). The performance in verbal fluency tasks, the frontal assessment battery, and ten-point clock test were related to "on"-state FOG (Amboni *et al.*, 2008). From all of this, it has been suggested that FOG is primarily a cognitive impairment rather than a motor disturbance (Browner and Giladi, 2010) and that "freezers" might have more difficulties in attending to this increased cognitive demand for the organisation, adaptation and generation of gait pattern. (iv) Perceptual judgement and visuospatial processing deficits have also been suggested to contribute to FOG (Johnson *et al.*, 2004). Freezing while going through narrow and confined spaces might reflect an impaired integration of vision with spatial memory – patients report judgement errors like feeling too big to fit through the door frame for example (Lee *et al.*, 1998).

The variety of symptoms and the variability in their occurrences, the findings regarding FOG and the correlation that can be drawn between certain symptoms, the anatomical and

functional complexity of the BG, and the pathological changes beyond the SN – all this leads to the hypothesis that at least some of the symptoms in PD, like gait disturbances and FOG, are the result of cognitive impairment rather than motor disturbances. How is the PPTg involved in this?

2. Pedunculopontine tegmental nucleus

The pedunculopontine tegmental nucleus has no standardised name or abbreviation. Some authors call it the pedunculopontine nucleus (PPN or PPT) or the tegmental pedunculopontine nucleus (TPP), and an older literature uses the Latin form, nucleus tegmenti pedunculopontinus (NTPP), though this is rarely used now. The human DBS literature primarily uses the abbreviation "PPN". However, some have used this abbreviation to describe the "pedunculopontine area" and a few have also distinguished between the PPTg and PPN, the PPTg being a particular structure within the pedunculopontine area, or calling the PPTg the rodent correspondent to the human PPN. This laboratory has consistently followed the convention of abbreviating pedunculopontine tegmental nucleus to "PPTg" and this is how it will be referred to in this thesis, referring to the human and animal pedunculopontine tegmental nucleus for consistency. This is the abbreviation used in Paxinos and Watson's stereotaxic atlases of the rat and mouse brain (Paxinos and Watson, 2005), the most widely used of their kind.

2.1. PPTg involvement in PD

As described above the pathological hallmark of PD is DA neuron degeneration in the SN. William Langston, however, describes this as being merely the tip of the iceberg and points out that it means neglecting the fact that the neuropathology is not confined to this part of the brain (Langston, 2006). Damage is present in a variety of structures – see the discussion of the Braak hypothesis earlier – but it is the PPTg in particular that has received increased attention

in the past few years [see for example (Pahapill and Lozano, 2000)]. The most convincing argument for moving away from the restricted focus on DA degeneration in the search for more effective treatment options is the fact that DA cell loss cannot explain all of the symptoms that PD patients may suffer from: several very debilitating symptoms are refractory to DA replacement medication, leading to the belief that other lesions in the brain may be contributing to the diverse symptomatology of PD (Hirsch *et al.*, 1988).

There are several excellent reviews on PPTg involvement in BG functioning and PD that the reader can refer to (Hamani *et al.*, 2007; Inglis and Winn, 1995; Mena-Segovia *et al.*, 2004; Pahapill and Lozano, 2000; Winn, 2006; Winn *et al.*, 1997). In the following, the focus will lie on the change of PPTg firing activity in PD and PPTg neuron degeneration. Section 3.3. of this chapter will then highlight therapeutic targeting of the PPTg with DBS and which effect this has on symptoms and neuronal activity of the circuits involved.

2.1.1. Change of firing activity

In animal models of PD, and also in PD patients, changes in PPTg neuronal activity have been observed. Increased 2-deoxyglucose (2-DG) uptake was reported by Mitchell and colleagues [Figure 1.3.; (Mitchell *et al.*, 1989)] in MPTP-treated primates and confirmed in rodents by Carlson and colleagues (Carlson *et al.*, 1999) – it is assumed that this reflects increased synaptic activity and therefore the pathologically increased GABAergic (γ-aminobutyric acid releasing), inhibitory influence from afferent structures, the pallidum and SNr, causing over-inhibition of the PPTg [see also (Palombo *et al.*, 1990)]. More recently others have reported a decrease of the metabolic marker cytochrome oxidase subunit I in MPTP-treated primates, interpreting this as a decreased activity of PPTg neurons (Gomez-Gallego *et al.*, 2007), or decreased metabolic markers in a fluoro-2-DG-positron emission tomography (PET) study in rats (Jang *et al.*, 2012). Lesioning the GPm in PD patients has been shown to alleviate

symptoms, which might be due to removal of the exaggerated inhibitory input onto the PPTg (Lang *et al.*, 1999; Lee *et al.*, 2000). However, Gubellini states that "electrophysiological support for the hypothesis of PPN hypoactivity in PD state is poor" (Gubellini *et al.*, 2009) and Carlson points out that an increase of 2-DG uptake does not necessarily have to be interpreted as a product of increased inhibitory input, but might potentially also represent hyperactivity of the PPTg itself (Carlson *et al.*, 1999). Others did indeed show increased activity by means of extracellular recording (Breit *et al.*, 2001; Breit *et al.*, 2005; Zhang *et al.*, 2008) and by measure of expression of cytochrome oxidase messenger RNA (Orieux *et al.*, 2000) (Figure 1.3.), which seems to be related to the increased activity observed in the STN, as this decreased after STN lesions (Breit *et al.*, 2001; Jeon *et al.*, 2003). Others again found no change at all (Heise and Mitrofanis, 2006).

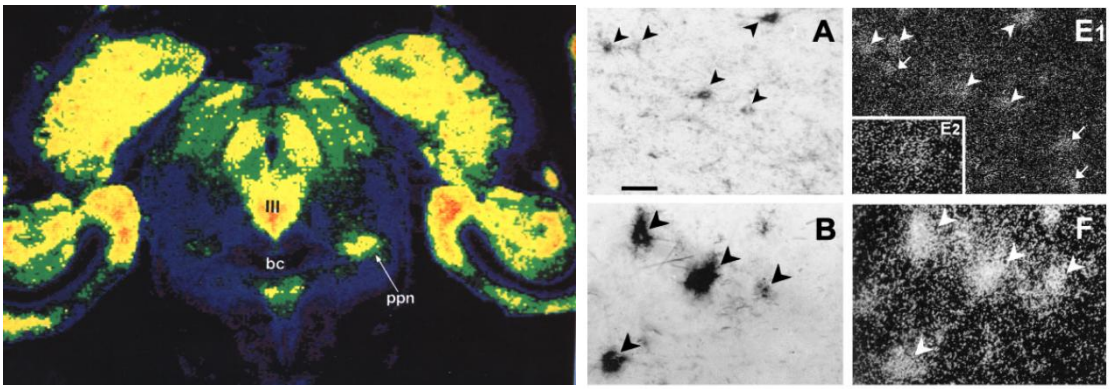

Figure 1.3. Changes of metabolic activity of the PPTg in PD models. **LEFT:** 2-DG autoradiograph of unilaterally MPTP-treated monkey, showing increased 2-DG uptake in the ipsilateral PPTg, reflecting the activity of its afferents, presumably of hyperactive pallidal, nigral and subthalamic inputs. Abbreviations: III, oculomotor complex; bc, brachium conjunctivum; ppn, pedunculopontine nucleus. Image reproduced from Mitchell et al., (1989). **RIGHT:** Measurement of metabolic activity of STN-projecting PPTg neurons showing significantly higher COI mRNA expression in 6-OHDA lesioned rats compared to sham lesioned rats. Photomicrographs show PPTg neurons labeled by WGA–HRP revelation (A, B) and COI in situ hybridization (E1, E2, F). Image reproduced from (Orieux *et al.*, 2000).

2.1.2. Loss of neurons

As pointed out above progressive cell loss of dopaminergic neurons in the SN is not the only pathophysiological characteristic of PD. Neuronal loss is present in various brainstem nuclei including subnuclei of the reticular formation, the raphe system and the coeruleus-subcoeruleus complex (Braak *et al.*, 2003). In the PPTg, cell loss of 40 – 57% of large PPTg neurons was identified, averaged across various levels along the rostrocaudal axis of the PPTg (Hirsch *et al.*, 1987; Zweig *et al.*, 1989). Loss of cholinergic and non-cholinergic neurons, including substance P, was noted in idiopathic PD and also in post-encephalitic PD, PSP and MSA (neurological disorders that can present themselves in atypical parkinsonian forms where severe impairment of gait and balance is a prominent feature as well) (Halliday, Blumbergs*, et al.*, 1990; Halliday, Gai*, et al.*, 1990; Halliday, Li*, et al.*, 1990; Hirsch *et al.*, 1987; Jellinger, 1988; Schmeichel *et al.*, 2008). In the study by Rinne and colleagues (Rinne *et al.*, 2008), the average loss of cholinergic neurons was similar to that reported by Zweig and colleagues (36%), but non-cholinergic neurons were also reduced in number by 23% and the overall reduction of all neurons in the PPTg across the rostrocaudal axis was reported as 27%. Cell degeneration and atrophy was found in surviving neurons showing a 14% (all neurons) to 26% (cholinergic neurons only) decrease in cell size (Figure 1.4.). According to Braak and colleagues the degree of cholinergic cell loss correlated with disease severity according to the Hoehn and Yahr stages (Braak *et al.*, 2003; Goetz *et al.*, 2004; Rinne *et al.*, 2008). *In vivo* cholinergic PET studies in PD patients and Parkinson's disease associated dementia patient confirmed this cholinergic deficit (Hilker *et al.*, 2005; Shinotoh *et al.*, 1999) and PD fallers had significantly decreased thalamic cholinergic innervation compared to non-fallers (Bohnen *et al.*, 2009; Bohnen *et al.*, 2012). Significant loss of brainstem cholinergic innervation from PPTg was also found in patients with PSP (Jellinger, 1988). This is of interest since PPTg and in particular cholinergic involvement has been predominately related to gait and postural deficits and cholinergic cell loss was shown to

be greater (Karachi *et al.*, 2010) and thalamic cholinergic innervations fewer in fallers than in non-fallers (Bohnen *et al.*, 2009; Bohnen *et al.*, 2012).

2.1.3. Relationship of PPTg degeneration to loss of dopamine neurons in SN

The simultaneous appearance of cholinergic and dopaminergic neuron degeneration in PD suggests the possibility of common aetiologic factors causing degeneration or cell loss in the SN and PPTg, which directly projects to the SNc and receives input from the SNr (Hirsch *et al.*, 1987). According to Braak and colleagues (Braak *et al.*, 2003) both nuclei, SN and PPTg, initially show degeneration through development of LN and inclusion of LB in stage 3, leading to substantial damage and cell loss in the SN and PPTg in stage 4. However, in early Parkinsonism, PPTg neurons are intact, which could either mean that they might contribute to the maintenance of DA functioning or that their increased activity leads to the degeneration of DA neurons. The nature of this relationship – protective, destructive or indifferent? – remains unknown.

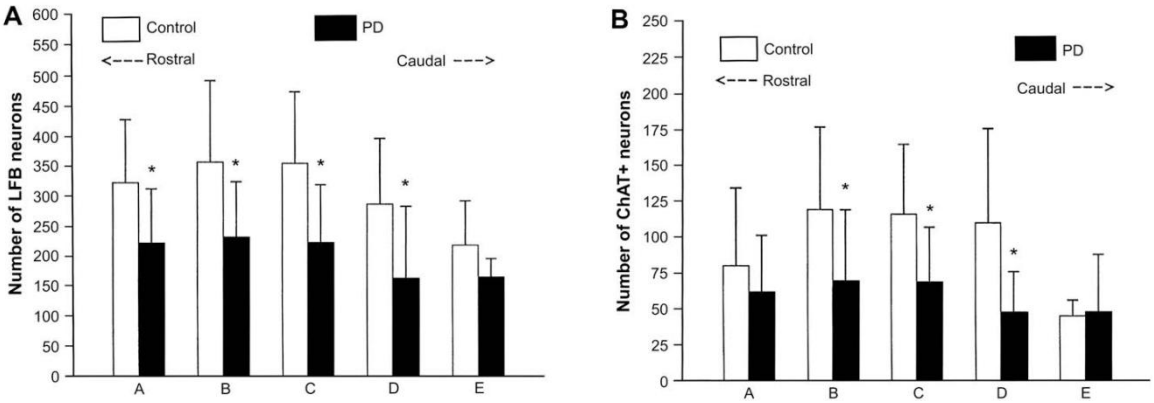

Figure 1.4. (A) Distribution of all large neurons in the PPTg (stained with Luxol fast blue) and **(B)** of cholinergic neurons (stained for an antibody against ChAT) in different levels on the rostro-caudal axis of the PPTg in controls and PD patients. Image reproduced from (Rinne *et al.*, 2008).

The literature allows the support of all three possibilities: In 1984 McGeer and McGeer showed DA cell death after kainic acid lesions of the rostral PPTg in rats, hypothesising the PPTg lesion was causing a pathological stimulation of the nigral DA cells (McGeer and McGeer, 1984). Having shown PPTg hyperactivity in 6-hydroxydopamine (6-OHDA) rat models of PD Breit and colleagues also suggest that this hyperactivity might cause excitotoxic excitatory drive on DA neurons which allows the speculation that reversing this destructive input by stimulation of STN or the PPTg itself might be neuroprotective (Breit *et al.*, 2006; Takada *et al.*, 2000). Preservation of cholinergic cells in the PPTg in the two most common PD models – MPTP-treated monkey and 6-OHDA-lesioned rat could also be shown (Heise *et al.*, 2005; Herrero *et al.*, 1993). While it is apparent that the mechanisms leading to the pathophysiology and symptomatology are different in idiopathic PD and PD models [although the mechanisms of the toxins come close - causing oxidative stress and mitochondrial dysfunction (Blandini *et al.*, 2000)] it does not only show the limitations of the models but also suggests that the abnormal circuits – overactive glutamatergic excitatory input from the STN – do not cause PPTg degeneration in these models. A different group however has found significant reduction of cholinergic PPTg neurons in aged MPTP-treated monkeys, but not young ones (Grabli *et al.*, 2013; Karachi *et al.*, 2010). The age-related atrophic changes in PPTg cells (George *et al.*, 2006; Zhang *et al.*, 2005) might contribute to the susceptibility to MPTP toxicity or to the lack of functioning DA target neurons and/or afferent input. Confirming the results of the study by Heise and colleagues but contrasting Karachi's findings, by means of design-based stereology, it has been shown that also in rats 6-OHDA induced DA cell loss does not cause a decrease in cholinergic cells, however, Pienaar and colleagues (Pienaar and van de Berg, 2013) revealed a significant loss of non-cholinergic neurons.

2.2. Interspecies differences

It is argued that there are significant differences of anatomical organization and connectivity of the PPTg between rats and primates. For example, one argument is that the evolution from quadrupedal to bipedal gait caused anatomical differences between species which are therefore reflected in the structure and organisation of various nuclei in monkeys and rats (Alam *et al.*, 2011),. Given the remarkable preservation of the PPTg through evolution with very similar structure and pattern of connections in all studied species from teleost fish to human [(Brantley and Bass, 1988), teleost fish; (Medina and Reiner, 1994), pigeon; (Manaye *et al.*, 1999), human; (Mesulam *et al.*, 1989), human; (Marin *et al.*, 1997), amphibians; (Rye *et al.*, 1987), rat); for reviews see (Martinez-Gonzalez *et al.*, 2011), (Winn, 2008)] this argument does not appear strong enough to explain fundamental differences in the results of behavioural experiments found in the literature. There are differences of strength of connections with particular structures or subregions (Alam *et al.*, 2011) but it remains unknown whether these differences can account for the apparently conflicting results found in lesion studies in rats and monkeys.

2.3. PPTg anatomy and physiology

2.3.1. Neurochemical diversity and heterogeneous structure of the PPTg

The PPTg is a highly heterogeneous structure at cellular, molecular and electrophysiological level, composed not only of a collection of neurons of different neurochemical phenotypes but also of neurons with distinct connectivity, with functionally distinct neuronal systems reaching to forebrain structures as well as to the spinal cord.

Situated in the mesopontine tegmentum of the rostral brainstem, the PPTg has strong reciprocal connections with the BG and many other ascending and descending connections. It reaches rostrally from the caudal pole of SN, just below the red nucleus, to the lateral tip of

the superior cerebellar peduncle caudally. Dorsally it lies just beneath the cuneiform nucleus and its more caudal part is medially adjacent to the LDTg (Inglis and Winn, 1995; Winn, 2006).These borders are conventionally defined by the column of cholinergic neurons of that structure because experimental work has for a long time focused on this cell group (Mesulam *et al.*, 1983). However, these borders seem to extend dorsally and medially from the cholinergic population given the actual neurochemical complexity of the PPTg (Mena-Segovia *et al.*, 2004).

Apart from cholinergic neurons, which constitute the cholinergic cell group Ch5, the PPTg consists of glutamatergic, GABAergic and peptidergic neurons [i.a. substance P and atrial natriuretic peptide; (Ryan and Gundlach, 1995; Vincent *et al.*, 1986)] which are interdigitated but not homogenously distributed throughout the structure (Lavoie and Parent, 1994; Mesulam *et al.*, 1983; Wang and Morales, 2009; Winn, 2006). Small amounts of serotonergic and tyrosine hydroxylase (TH) containing neurons (dopamine or noradrenaline containing) have been found in the PPTg of the guinea pig (Leonard *et al.*, 1995) but have not been confirmed in the rat (Winn, 2006). Populations of cholinergic neurons have been reported to co-express glutamate, GABA and NADPH-diaphorase (the synthetic enzyme for nitric oxide [NO]) and neuropeptides such as substance P (Charara *et al.*, 1996; Jia *et al.*, 2003). More recent studies using *in-situ* hybridization in combination with immunohistochemistry revealed that less than 5% of cholinergic neurons co-express GABA or glutamate (Wang and Morales, 2009).

The distribution of neurons and their connectivity support the idea of at least two functionally distinct regions within the PPTg. This consideration of functional dichotomy is important for the present study because of the possibility that PPTg-DBS has different effects dependent upon the exact location of the stimulation in PPTg.

The focus on the cholinergic population of the PPTg led to the assumption that this is its largest population of neurons. However, GABAergic and glutamatergic neurons outnumber cholinergic neurons – in anterior PPTg there are twice as many GABAergic neurons than cholinergic neurons, and in posterior PPTg the concentration of glutamatergic neurons is 1.5 times higher (Wang and Morales, 2009). The number of cholinergic neurons increases toward the caudal end - this is where cholinergic neurons are packed densely. In the anterior portion they are distributed more sparsely (Mena-Segovia *et al.*, 2009). Therefore, the neurochemical distribution has been shown to be as follows: in the anterior part 40% of the neurons are GABAergic, 37% glutamatergic and 23% cholinergic; in the posterior part the glutamatergic neurons are more densely concentrated with 50%, 31% are cholinergic and 19% GABAergic (Wang and Morales, 2009).

The segregation into anterior and posterior [by others also named rostral and caudal; or pars dissipata and pars compacta; for example (Martinez-Gonzalez *et al.*, 2012) (Pienaar and van de Berg, 2013) respectively] is possible and commonly made according to differences in number and arrangement of cholinergic neurons, but there is no definite hallmark for such a division. Mena-Segovia and colleagues suggest a separation according to the concentration of GABAergic neurons which they found to lead to a split in the middle of the structure on the rostro-caudal axis. This leaves the anterior half as a predominantly GABAergic part and the posterior half more evenly mixed (Mena-Segovia *et al.*, 2009). This segregation seems to coincide with and likely contribute to the functional topography of the PPTg [see (Alderson *et al.*, 2006, 2008; Andero *et al.*, 2007; Inglis *et al.*, 2001); see chapter 2.4.5.)].

Electrophysiologically, neuronal subtypes of differing discharge properties have been identified: There are two types of cholinergic neurons: type I are slow firing neurons coupled to phasic increases in cortical fast frequency oscillations during sleep and type II, fast firing and

coupled to downstate of cortical slow oscillations (Mena-Segovia, Sims*, et al.*, 2008). Two types of non-cholinergic neurons have also been described: type I are fast firing neurons that are associated with cortical slow oscillations and type II, neurons that are very slow (even quiescent) and independent of cortical activity (Ros *et al.*, 2010).

2.3.2. Connectivity of the PPTg

The subdivision into anterior and posterior is further supported by a neurochemically diverse connectivity of the PPTg with most parts of the brain. Many detailed and exhaustive reviews have been dedicated to PPTg connectivity (Martinez-Gonzalez *et al.*, 2011; Winn, 2006). In the following the relevant connections are summarised with a focus on a differentiation of efferent and afferent projects to the anterior and posterior part of the structure where this is applicable and known. Figure 1.5. illustrates the most important PPTg connections.

The PPTg has extensive connections with a variety of structures above and below. It has (1) connections to the musculature via the spinal cord and connections with other brainstem nuclei. Furthermore, it is strongly connected with (2) the thalamus and also holds connections with (3) frontal areas. However, most prominent is its influence on (4) the BG and the input it receives from here, supporting the proposal that the PPTg should be understood as a part of the BG family (Mena-Segovia *et al.*, 2004).

(1) PPTg neurons project to the inferior and superior colliculi (IC, SC). These projections arise from cholinergic neurons predominantly from posterior PPTg and non-cholinergic ones from anterior PPTg (Beninato and Spencer, 1986; Motts and Schofield, 2009). The PPTg also sends efferents from a mix of cholinergic and non-cholinergic neurons of the posterior part to other brainstem structures such as the pontine and medial reticular formation (Nakamura *et al.*, 1989; Semba *et al.*, 1990; Takakusaki *et al.*, 1996), nucleus pontis oralis (Garcia-Rill *et al.*, 2001), the motor trigeminal (Fay and Norgren, 1997a, 1997b, 1997c), the medulla (Skinner,

Kinjo, Ishikawa*, et al.*, 1990), the medulla oblongata (Nakamura *et al.*, 1989) and spinal cord (Rye *et al.*, 1988; Skinner, Kinjo, Henderson*, et al.*, 1990). PPTg afferents from the brainstem target preferentially the posterior part and arise from the IC and SC (Redgrave *et al.*, 1987; Semba and Fibiger, 1992), the dorsal raphe (Steininger *et al.*, 1997), the LC (Jones and Yang, 1985) and spinal cord (Semba and Fibiger, 1992). Further, the PPTg is intimately connected with the LTDg (Semba and Fibiger, 1992; Winn *et al.*, 2010).

(2) The thalamus is heavily innervated by the PPTg (Hallanger *et al.*, 1987; Hallanger and Wainer, 1988) and has an impact on several thalamic structures [i.a. mediodorsal, ventral anterior, ventro-lateral and parafascicular, centromedian and centrolateral thalamic nuclei; (Erro *et al.*, 1999; Kobayashi *et al.*, 2007). The input is predominantly cholinergic from the posterior PPTg. Essentially all thalamic cholinergic innervation comes from the PPTg, LDTg and parabigeminal nucleus; the VL thalamic nuclei for example receive over 50% of their cholinergic afferents from the PPTg (Capozzo *et al.*, 2003; Holmstrand and Sesack, 2011; Kolmac and Mitrofanis, 1998). In the cat (Steriade *et al.*, 1988) and monkey (Lavoie and Parent, 1994) the input was shown to be topographically organized, though the exact patterns are yet to be described. Given the important impact on the thalamic burst firing and its output to neocortex and striatum such an organization is likely – Pf neurons for example that receive solely input from the PPTg project directly to the striatum (Kobayashi *et al.*, 2007).

(3) The cortical influence of the PPTg arises mainly via mono- or polysynaptic connections via the thalamus, but there is some evidence for direct projections to medial and sulcal frontal cortical areas (Saper and Loewy, 1982). The PPTg further projects to sites of non-specific cortical input, such as from anterior and posterior PPTg to the lateral hypothalamus (Ford *et al.*, 1995; Semba and Fibiger, 1992), from the anterior portion to the nucleus basalis magnocellularis (Semba and Fibiger, 1992), which sends a bulk of cholinergic input to cortex.

Auditory (Schofield, 2010) and motor (Matsumura *et al.*, 2000) cortex send projections to the PPTg and so does the extended amygdala (Zahm *et al.*, 2001).

4) Through direct and indirect connections essentially all BG nuclei receive input from the PPTg. Anterior PPTg projects directly to the GPi (EP in rodents) and SNc (Charara and Parent, 1994; Lavoie and Parent, 1994; Semba and Fibiger, 1992) through glutamatergic, GABAergic and cholinergic input. Posterior PPTg innervates VTA (Charara *et al.*, 1996) and STN (Kita and Kita, 2011). These afferent projections are also through glutamatergic, GABAergic and cholinergic. Non-cholinergic input is nine times higher than cholinergic efferents from the PPTg, though the latter form denser innervations by creating much larger terminal fields (Kita and Kita, 2011). Via the STN PPTg can influence the activity of the GPe and the striatum via SNc, VTA and the GPe-STN pathway, and there are also direct projections to the striatum (Saper and Loewy, 1982). Afferent innervation – and therefore forming reciprocal connections – comes from the STN and GPi (glutamatergic) and further BG output arises from SNr, innervating anterior PPTg (GABAergic) (Parent *et al.*, 2001; Semba and Fibiger, 1992).

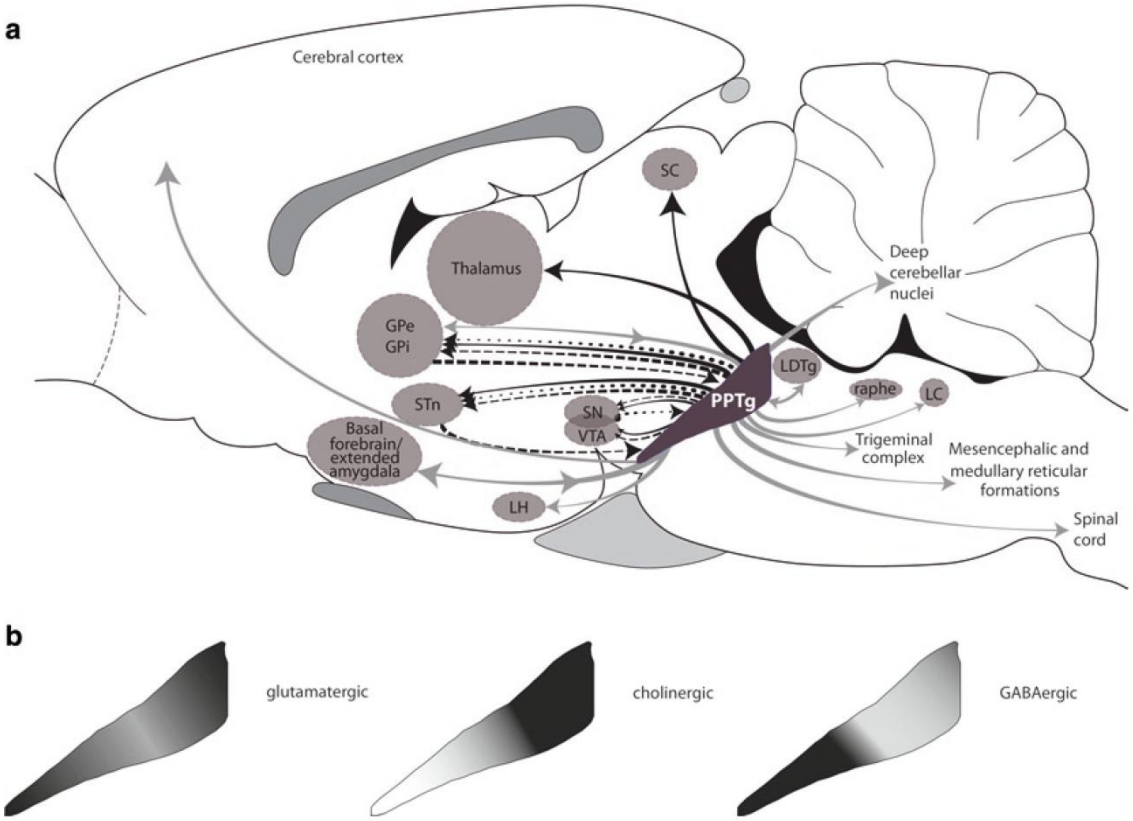

Figure 1.5. Illustration of the location, connections and composition of the PPTg.
Panel A. Sagittal view on a rat brain section (taken from the atlas of Paxinos and Watson). Schematic representation of the principal afferent and efferent connections of the PPTg with forebrain and brainstem nuclei. Black arrows represent cholinergic connections, dotted arrows GABAergic, and dashed arrows glutamatergic connections. Grey arrows represent unclassified connections. **Panel B.** Schematic graded distributions of glutamatergic, cholinergic and GABAergic neurons within the PPTg. The gradient represents the rostro-caudal distribution of each neuron type based on the cell identification of Wang and Morales 2009 and Mena-Segovia et al., 2009. Abbreviations: GPe globus pallidus externa, GPi globus pallidus interna, LC locus coeruleus, LH lateral hyphothalamus, LDTg laterodorsal tegmental nucleus, SC superior colliculus, SN substantia nigra, STn subthalamic nucleus, VTA ventral tegmental area. Image reproduced from Gut and Winn, 2012.

2.4. PPTg functions

2.4.1. Motor

In the early literature the PPTg was understood and commonly portrayed as a motor structure. This idea has carried forward to the recent literature and been related to movement disorders including the search for potential treatment options in PD. Predominantly early work published on PPTg-DBS in PD patients is based on the assumption that "The pedunculopontine nucleus (PPN) is an integral component of the midbrain locomotor region and plays an important role in the initiation and maintenance of walking behaviour" (Mazzone *et al.*, 2005); "The pedunculopontine nucleus (PPN) is a key part of the reticular activating system and mesencephalic locomotor region..." (Androulidakis *et al.*, 2008); or that "The pedunculopontine tegmental nucleus (PPTg) is a part of the upper brainstem locomotor region that has an important role in the control of gait and muscle tone..." (Mazzone *et al.*, 2009); and that "...gait failure is induced by PPN lesion or dysfunction..." (Karachi *et al.*, 2010). This supposed PPTg-MLR association, however, is a doubtful basis for PPTg-DBS.

The mesencephalic locomotor region (MLR) is an area in the mesencephalon described as a brainstem command centre, which, together with the diencephalic locomotor region, was thought to regulate the activity level of the spinal central pattern generators. It is defined functionally (rather than anatomically; the precise targets for electrical or chemical activation for the studies mentioned bellow have been a point of debate) as an area from which it is possible to elicit locomotor activity on a treadmill in decorticate animals (Skinner, Kinjo, Henderson*, et al.*, 1990; Skinner, Kinjo, Ishikawa*, et al.*, 1990) the speed of the activity depending on stimulation strength. Some describe it as being located at the mesopontine border at the caudal pole of the cholinergic pedunculopontine and the cuneiform nuclei others include the cholinergic PPTg as crucial/important part of or even synonymous with the MLR

(Garcia-Rill, 1991). Induction of controlled locomotion was possible on a treadmill in the decerebrate rat (and cat) applying low amplitude current pulses to (presumed) cholinergic PPTg neurons (Garcia-Rill *et al.*, 1987; Garcia-Rill *et al.*, 1990). Stimulation induced locomotion could be blocked by injections of the muscarinic receptor antagonist atropine sulphate into the medioventral medulla, where efferent cholinergic fibres from PPTg terminate (Garcia-Rill *et al.*, 1987).

However, this locomotion inducing effect is also possible after stimulation of other structures within the so called MLR area, including the cuneiform nucleus, the SC, central gray, lateral lemniscus, the pontomedullary locomotor strip, Probst's tract, the parabrachial nucleus and periaqueductal gray (Coles *et al.*, 1989; Garcia-Rill *et al.*, 1983; Steeves and Jordan, 1984). With the brainstem being disconnected from descending control from higher systems it is not surprising that stimulation of structures with descending projections to the spinal cord can drive locomotion (not leaving much opportunity for much more than this), especially given that treadmill locomotion was the only behaviour presented to engage in. Further, electrical and chemical (L-sodium glutamate/NMDA, kainic acid, picrotoxin and bicuculline) stimulation of the region of the PPTg in rats (non-decorticate) could manipulate locomotor activity (Milner and Mogenson, 1988; Mogenson and Wu, 1988); injecting L-sodium glutamate and the GABA antagonist picrotoxin for example was reported to increase locomotor activity (Brudzynski and Wang, 1996). Interestingly, when the primate PPTg was inhibited by local injection of the GABA agonist muscimol, a significant decrease of motor activity was observed (Nandi *et al.*, 2002). Conversely, stimulating the PPTg with the GABA antagonist bicuculline, after the same primate had been made Parkinsonian by MPTP, has been reported to alleviate akinesia (Nandi *et al.*, 2002).

This input onto other brainstem structures and the spinal cord however does not prove that the main role of the PPTg is locomotor control or its production. On the contrary: decorticate animals even seem to be able to select appropriate simple movements in the homecage, retaining the ability to eat, groom, climb, and also to swim, albeit being a little awkward conducting those tasks, and are able to improve on some tasks with practise (Grill and Norgren, 1978; Humphries *et al.*, 2007). Furthermore, the PPTg has been proven not to be critical for the production of locomotion. A series of experiments in this laboratory and by others could not show that bilateral PPTg lesions caused by electrolytic lesions (Homs-Ormo *et al.*, 2003) or – as in most cases – fibre-sparing excitotoxins reduce spontaneous locomotor activity in locomotor boxes (photocell cages), locomotor behaviour in the home cage (Inglis, Allen*, et al.*, 1994), circular corridor, or exploratory behaviour in the open field (Alderson *et al.*, 2003; Dellu *et al.*, 1991; Inglis, Allen*, et al.*, 1994; Inglis, Dunbar*, et al.*, 1994; Olmstead and Franklin, 1994; Steiniger and Kretschmer, 2004; Swerdlow and Koob, 1987; D. I. G. Wilson *et al.*, 2009), nor have an effect on amphetamine-induced locomotion (Alderson *et al.*, 2003; Inglis, Allen*, et al.*, 1994; Inglis, Dunbar*, et al.*, 1994; Olmstead and Franklin, 1994; Steiniger and Kretschmer, 2004) [for an extensive review see (Winn, 2006)]. Further, a comprehensive examination of the performance of rats bearing bilateral lesions on the radial arm maze confirms the lack of a locomotor deficit. Despite changes in the overall performance these do not reflect deficits of their motor behaviour. Keating and colleagues (Keating and Winn, 2002) for example showed that the measures of speed of the rats moving along the arms are not decreased but that the rats fail to select the correct arms. In a similar manner it is possible to suggest that learning deficits after PPTg lesions could have been confounded for locomotor deficits in a study conducted by Bechara and colleagues (Bechara and van der Kooy, 1992) where the task required the acquisition of conditioned locomotion. The presented locomotor deficit might therefore rather represent difficulties to make an association between the

reinforcing event and the motor behaviour. Likewise Matsumura and colleagues (Matsumura *et al.*, 1997) measured neuronal activity in PPTg of non-human primates while they were conducting a simple operant task and found changes in activity in response to arm movements that were preceding movement onset. They interpreted these changes as related to movement initiation and execution; however it is interesting to point out that these movements were conducted during an operant task where movements were made in the context of a learned stimulus-reward association. Locomotor activity did not differ comparing lesioned and sham operated rats after systemic administration of d-amphetamine or its direct infusion into the NAcc (Inglis, Dunbar*, et al.*, 1994). Alderson and colleagues showed that while the levels of spontaneous locomotion were not affected in lesioned rats over all sessions when they were treated with saline, the locomotor response to the first injection of d-amphetamine did not differ from shams either (Alderson *et al.*, 2003). Locomotor sensitisation to the drug, however, was attenuated by PPTg lesions (which was not caused by locomotor-incompatible stereotyped behaviour including/or rearing). This confirms previous findings of rats with bilateral excitotoxin PPTg lesions which did not show any impairment either in gross regulatory mechanisms such as eating and drinking and maintaining their body weight or in spontaneous or d-amphetamine induced locomotor activities (Inglis, Allen*, et al.*, 1994; Inglis, Dunbar*, et al.*, 1994). However, the same lesions caused significant interruption of the development of a conditioned place preference to morphine or amphetamine (Olmstead and Franklin, 1994). Taking into consideration the heterogeneity of the PPTg's anatomy and functional dissociation regarding the development of sensitization of nicotine in an IV self-administration paradigm (Alderson *et al.*, 2006), Alderson and colleagues compared bilateral lesions to the anterior part of the PPTg to lesions of the posterior part regarding spontaneous locomotion and in response to repeated nicotine administrations (Alderson *et al.*, 2008). They showed for the first time that bilateral lesions of the anterior PPTg but not posterior PPTg generate a deficit in baseline

activity. This was not replicated in a later study published by Wilson and colleagues where neither anterior nor posterior PPTg lesions caused deficits in free locomotor testing (D. I. Wilson *et al.*, 2009). Despite the rats not showing any impairment immediately after surgery during operant testing the locomotor tests were conducted with a delay of 2 months after surgery, which might present a plausible explanation for the conflicting findings.

These consistent findings are contrasted by PPTg lesion studies in non-human primates where excitotoxic lesions – kainate – of the PPTg in monkeys were reported to induce akinesia and other parkinsonian symptoms (Aziz *et al.*, 1998; Kojima *et al.*, 1997; Munro-Davies *et al.*, 1999). Kojima and colleagues described hemiparkinsonian symptoms, flexed posture, hypokinesia in the contralateral upper limb and muscular rigidity after unilateral PPTg lesions, also induced by kainic acid in macaque monkeys (Kojima *et al.*, 1997). Munro-Davies and colleagues reported transient hypokinesia when only one side of the brain was lesioned, which developed further to include profound paucity and slowness of movement after a subsequent lesion in the contralateral side (Munro-Davies *et al.*, 1999). Kainic acid is a very potent and difficult excitotoxin that (in the rat at least) induces strong seizures and lesions remote from the site of injections. Further, motor assessment in these studies was conducted immediately after recovery from surgery and was reported to abate a few days later or after one to two weeks in monkeys with larger lesions caused by a higher dose of kainic acid. Given the seizure promoting activity, the possibility of lesions comprising neighbouring structures of the PPTg and the general malaise during the first days after surgery which may confound behavioural data, these studies have to be interpreted with caution [for more details please refer to (Winn, 2006)]. Thermal lesions of the PPN region were also described to cause a transient hemi-parkinsonian state in a normal macaque monkey (Aziz *et al.*, 1998). The deficits declined after a week, more permanent deficits were caused with bilateral lesions. Radiofrequency lesions damage fibres of passage and the possibility of this damage being the cause for motor

problems needs to be included in the interpretation of these results. Finally, in an inactivation study Nandi and colleagues reported reduced activity in normal primates after administration of GABA agonist muscimol (Nandi *et al.*, 2002). The dose of the agonist used was 40x higher than the dose used in this lab for successful inactivation of PPTg that caused learning deficits (Maclaren *et al.*, 2013). Evidently, a monkey brain is bigger and there is not necessarily a linear correlation between concentration and effect, but this enormous difference allows the rise of concern.

In a more recent paper, Karachi and colleagues presented data from PPTg lesioned macaques (without dopaminergic depletion) which displayed gait deficits in form of decreased step length and speed, postural changes, as well as rigidity in the extremities without affecting the global motor behaviour as observed in the home cage (Karachi *et al.*, 2010). These monkeys did not develop akinetic features as the previous PPTg lesion studies in monkeys which can be attributed to more concise lesions – however gait deficits only occurred in monkeys that received a very high dose of the fusion toxin Dtx-UII (urotensin II-conjugated diphtheria toxin). This toxin is selective for cholinergic neurons at the appropriate concentrations in rat (Clark *et al.*, 2007) but the concentrations used here led to a loss of 70-80% of non-cholinergic neurons, making the lesions non-selective, as the authors report themselves. Lesioning the PPTg of MPTP-treated macaques – to our knowledge the only other study that examines the behavioural effect of a combination of PPTg lesions and DA degeneration in the SN – led to an increase of global activity and step speed, as well as an increase – not statistically significant – of step length. The authors further reported a slight increase in balance deficits and adverse postural changes, which however were not significant either (Grabli *et al.*, 2013).

Clinical lesion reports of PPTg lesions are rare but occasionally drawn upon to support the locomotor hypothesis. In those cases of lesions caused by stroke and haemorrhage in the

caudal midbrain and at the pontomesencephalic junction causing gait initiation problems and ataxia the involvement of neighbouring structures cannot be excluded – in both cases it is suggested that the lesions must have been extensive (Hathout and Bhidayasiri, 2005; Masdeu *et al.*, 1994). More confined lesions of the pontine region in a single case study caused FOG, leg stiffness and intermittent rest tremor (Kuo *et al.*, 2008). Other reports, in contrast, draw a strong association of pontine stroke lesions with executive dysfunction (Omar *et al.*, 2007; Vataja *et al.*, 2003).

Is the notion of a MLR necessary at all (Allen *et al.*, 1996) or does it rather tempt to assign discrete/ discriminable functions to individual brain structures, neglecting their often diverse connections with other structures and their integrated roles? Evidence that, while involved, the PPTg is not necessary for locomotion clearly challenges the notion of the PPTg being synonymous with the MLR. After studying the stepping-inducing sites in the brainstem Garcia-Rill himself now states that "as far as the PPN is concerned,..., we concluded that this was not a locomotion-specific region" (Garcia-Rill *et al.*, 2011). Many years of dedicated study of the PPTg – of anatomy and function – have shown that for this structure it is true: the role is an integrative one. Ros and colleagues describe the large variability of projections from PPTg neurons, which gives an idea of how the PPTg could be involved in diverse behavioural processes including the control of movement, but also sensorimotor coordination, sleep-wake mechanisms, attention and autonomic functions (Ros *et al.*, 2010) as well as reinforcement processes and learning (Alderson *et al.*, 2005). In the following sub-chapters I will describe those functions that are possibly relevant to the understanding of the PPTg within the context of movement disorders. This does not only apply to rodent studies or work on non-human primates. An increasing number of studies of PPTg-DBS in PD patients do not limit their observations to motor functions. A few studies show for example increased activity of most cortical (bar occipital and parietal areas) and many subcortical structures induced by PPTg-DBS,

proving a much more far-reaching role than simply the activation of movement patterns in the spinal cord. Further, the notion of an integrative structure that is not limited to a locomotor function is supported by the interest of neurologists in the effect on PPTg stimulation on cognitive and autonomic functions (grammar, executive functions). The lack of clear results of PPTg-DBS on motor deficits and the search of effects on, and hence involvement in, a much broader spectrum of functions proves my initial statement that the association of the PPTg with the MLR is a doubtful basis for PPTg-DBS and a wider perspective on the functions of the PPTg is required to understand its role in movement disorders.

For an extensive review of the role in PPTg in sleep, anxiety, reward perception and motivation, reinforcement and learning, and attention please refer to the exciting exhaustive reviews (Inglis and Winn, 1995; Mena-Segovia *et al.*, 2004; Pahapill and Lozano, 2000; Winn, 2006).

2.4.2. Ascending reticular activation system and sleep

Cholinergic neurons located in the upper brainstem forming the Ch5 and Ch6 cell groups are considered to form wake promoting connections with higher brain regions. They provide major innervations to the thalamic relay nuclei, the intralaminar and reticular thalamic nuclei, as well as the lateral hypothalamus, basal forebrain and the prefrontal cortex (Hallanger *et al.*, 1987; Satoh and Fibiger, 1986) and change their firing patterns from rapid firing during wakefulness and REM (rapid eye movement) sleep to slow firing during non-REM sleep (Steriade *et al.*, 1993). The PPTg has therefore been considered part of the ascending reticular activation system, playing a role in behavioural state switching, and to have a key part in sleep – and to be precise, in the generation of REM sleep (Deurveilher and Hennevin, 2001). However, it is now clear that these firing pattern changes are not simple (Mena-Segovia and Bolam, 2011) and, moreover, many other sources have also an arousal influence and/or change their firing

patterns along different wakefulness and sleep states. These include projections from the parabrachial nucleus, the LC and raphe nuclei (Lu *et al.*, 2006) as well as several forebrain neuronal systems [see (Saper *et al.*, 2010)]. Moreover, an involvement in modulating states as suggested by changing firing patterns does not automatically mean a participation in the switching process itself. In fact, lesions of the PPTg showed only minimal effects (Lu *et al.*, 2006) or no effect at all (Deurveilher and Hennevin, 2001) on the amount of wakefulness and REM sleep. A recent study, however, suggests that, despite an equal distribution of sleep and wake states in PPTg lesioned rats and controls, the number of transitions between states, mainly wake and REM states (Petrovic *et al.*, 2013), differs. While the PPTg seems to be involved in behavioural state control, it clearly cannot be considered a master switch for sleep-wake transition, not changing the amount of sleep and even REM sleep.

2.4.3. Reward perception and motivation

Do rats have an altered reward perception and/or changes in motivation after PPTg lesions? Most likely not. Conditioned place preference using different sucrose solutions is not impaired after PPTg lesions (Alderson *et al.*, 2001): rats seem to be able to appreciate the rewarding nature of the stimulus independently of the deprivation state. Furthermore, they adjust their net energy intake accordingly to accommodate increased sucrose solution consumption (Keating *et al.*, 2002). Even though the breaking point drops when undergoing a progressive ratio (PR) schedule, this does not show a deficit of reward perception - or motivation for that matter – since the responding of the non-reinforced lever is significantly increased (Alderson *et al.*, 2002). In tasks that involve collecting rewards in radial mazes the motivation to find a reward is the same in PPTg lesioned rats as in control rats: they might make errors when selecting the arms, but the speed in which they are moving around the maze to find food pellets remains the same (Keating and Winn, 2002; Taylor *et al.*, 2004) and changes in the

reward value (food pellets changed to chocolate drops) are perceived as well (Taylor *et al.*, 2004).

2.4.4. Attention

Testing attention separately from other psychological functions is notoriously difficult. It is conceivable that the PPTg is well positioned to serve this function: the ability to decide which aspects of the environment are relevant and which are not is indispensable for successful adaptive behaviour so it is likely an evolutionary early function – quick processing of incoming sensory data when considering so called bottom-up attentional processes is a vital survival skill. In a test of sustained attention PPTg lesioned rats performed less successfully than controls, failing to respond timely to a light cue, which improved with lengthening of the visual signal (Kozak *et al.*, 2005). This might point towards a deficit in attentional processing but cannot rule out deficits in other processes involved in this task, like movement and learning. When replicating the experiment a detailed analysis of the reason for the increased number of omissions showed that the animals did indeed notice and respond to the light, but not with the correct behaviour (Rostron *et al.*, 2008), which would suggest an action selection problem rather than an attentional problem. It therefore cannot be ruled out that the increased time to respond only increased the chance to conduct the correct behaviour, rather than providing more time to notice and react to the cue. Increasing the attentional load, instead of decreasing as described by Kozak and colleagues, by introducing distracting noises and decreasing the salience of the cue caused a worsening in the performance of the PPTg lesioned rats (Inglis *et al.*, 2001), which could support the hypothesis of an attentional impairment caused by PPTg lesions. But again, an action selection process problem cannot be excluded because the introduced distractions might not only distract attentional processes, but also other processes such as selection or execution of actions.

2.4.5. Learning

It can be shown that the PPTg is involved in complex associative processing. Lesions of the PPTg affect responding for conditioned reinforcement under d-amphetamine. PPTg lesioned rats show an increased rate of response on non-reinforced levers, as opposed to control rats that learn to discriminate between the levers and focus almost exclusively on the reinforced lever. This indiscriminate lever pressing reflects a disruption in the process of reward-related behaviour but not motor deficit *per se* (Inglis, Dunbar*, et al.*, 1994). The same inability to learn the association between a conditioned stimulus and a reward has been shown in experiments using different experimental paradigms after bilateral excitotoxic lesions of the whole PPTg: in a visual autoshaping task (Pavlovian conditioning based on a spatial discrimination task) and conditioned reinforcement (instead of having to discriminate between two stimuli rats learn a stimulus reward association and need to transfer this in the testing session to a new response) rats with PPTg lesions were unable to learn the required associations (Inglis *et al.*, 2000). Complex cognitive processing as required to complete the delayed spatial win-shift radial maze task has also shown to be affected by whole bilateral PPTg lesions in the absence of any locomotor deficits, an effect that cannot be attributed to disrupted motivation, because measures of speed were not decreased. Here it made no difference whether or not the rats received the lesions before or after being trained on the task. They made significantly more errors than sham operated rats and showed therefore impairment in making the appropriate choice of the arm to enter in the maze (Keating *et al.*, 2002; Keating and Winn, 2002). Given the strong connections to midbrain DA neurons and their importance for establishing associations between action and reinforcement (Schultz, 2002), an involvement of the PPTg in drug reward was expected. Responding for intravenous self-administration of amphetamine (Alderson *et al.*, 2004), heroin (Olmstead *et al.*, 1998), cocaine (Corrigall *et al.*, 2002) and nicotine (Corrigall *et al.*, 1994; Corrigall *et al.*, 2001) is affected by lesions or inactivation of the

whole PPTg; they appear to abolish the ability to learn a specific contingency between an action and its outcome, unless the association between action (lever pressing) and reward (outcome) has been made prior to the lesion. However, when the requirements for obtaining a reward change, rats with PPTg lesions that previously performed well show impairments (Alderson *et al.*, 2002; Alderson *et al.*, 2004). PPTg lesioned rats increased inappropriate responses to the non-reinforced lever on a (PR) schedule when schedule requirements increased, showing difficulties to update the learned action outcome associations and making therefore the wrong choices of actions. These data strongly support the contention that the PPTg is involved in learning about the selection of appropriate actions rather than movement *per se*. Another method of studying the behavioural functions of the PPTg is inactivation of the structure by direct pharmacological manipulations by for example infusion of the GABA agonist muscimol into the PPTg (Samson and Chappell, 2001). PPTg inactivation was used to test its effect on the sensitivity of contingency degradation between actions (lever pressing) and outcomes (food reward): when the action outcome association was degraded saline treated rats reduced rates of lever pressing whereas muscimol treated rats maintained previous lever pressing rates, showing a deficit in updating of the action outcome association (Maclaren *et al.*, 2013). Kobayashi and colleagues showed how the PPTg could transform a sensory cue into a behavioural action by providing DA neurons with information about sensory input: in a reward-based performance task different populations of PPTg neurons responded not only to the reward itself, but also to the cue indicating the reward. The magnitude of the predicted reward correlated with the PPTg firing rate (Kobayashi and Okada, 2007; Okada *et al.*, 2009). Short latency responses to sensory input had been shown before (Dormont *et al.*, 1998; Reese *et al.*, 1995). Further evidence showed that PPTg neurons respond not only to sensory information *per se*, but also to the value of that input. VTA DA firing responses in rats developed in response to learned cues that were signalling reward delivery. This change in

firing pattern from tonic to burst firing in midbrain DA neurons is implicated in reward related learning (Mena-Segovia, Winn*, et al.*, 2008). Uwe Maskos explains how the cholinergic neurons of the mesopontine tegmentum, including the PPTg, may be essential for this change: restoration of the high-affinity nicotinic–cholinergic receptor subunit β2 in the VTA restores burst firing, which is completely absent in the mouse knockout of the same receptor subunit (Maskos, 2007, 2008). This phasic DA response was suppressed by unilateral PPTg inactivation (Pan and Hyland, 2005). In monkeys Kobayashi and colleagues found that the PPTg responds to reward and reward related cues even before DA neurons respond (Kobayashi and Okada, 2007; Okada *et al.*, 2009) (Figure 1.6.): In a primate visually guided reward task, electrophysiological recordings proved PPTg activity in response to stimuli predicting reward and to actual reward delivery itself, depending on reward magnitude. PPTg may therefore forward sensory information to the BG that has already been analysed regarding its salient aspects (reward predicting stimuli, reward itself, magnitude of reward). This role in sensorimotor decision-making has very recently been confirmed in mice that were performing a spatial choice task. Electrophysiological recording of PPTg activity showed neuron activity related to movement direction and outcome (Thompson and Felsen, 2013).

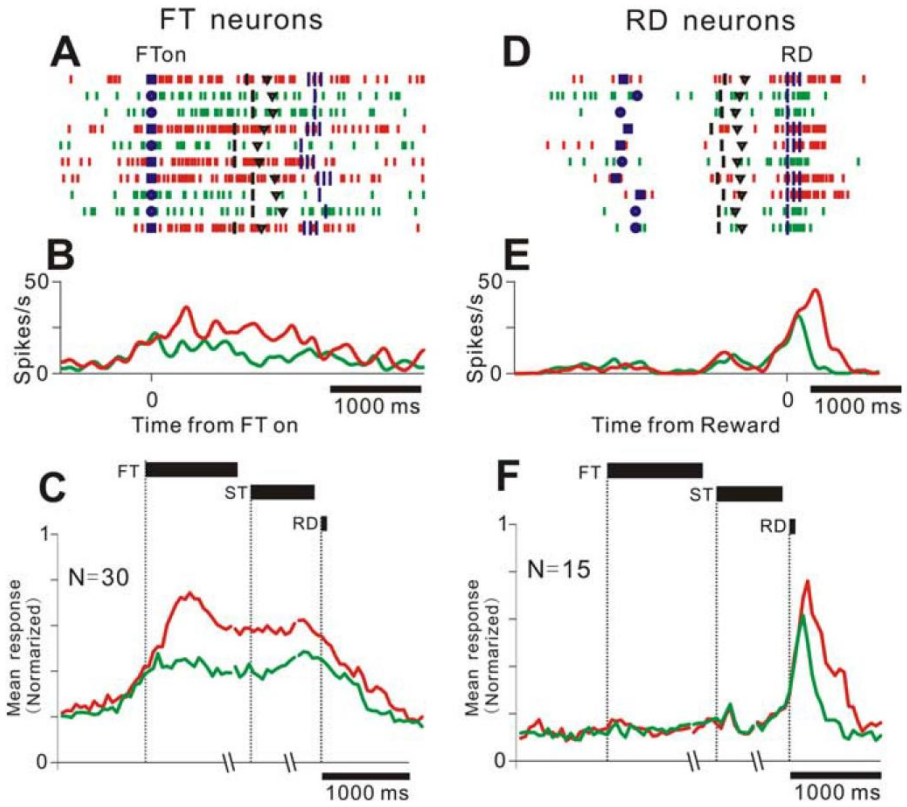

Figure 1.6. Single-neuron activity recorded from the PPTg of monkeys performing a visually guided saccade task in which the predicted reward value corresponded to the shape of the fixation target. Two groups of neurons were identified: FT = fixation target neurons; neurons responding to FT onset and RD = reward delivery neurons; neurons significantly more active after reward delivery. The level of activity varied according to the magnitude of expected reward and actual reward, respectively. **Panels A –F** show responses of FT and RD neurons to task events. Red represents large reward trials and green small reward trials. **A,D,** Rastergrams of FT and RD neuron activity; blue squares and circles = cue onset; black bars = saccade target onset; black triangles = saccade onset; blue bars = reward delivery. **B,E,** Spike-density functions of activity presented in **A and D**. **C,F,** Spike-density functions (averaged data); black bars indicate the periods of FTon, STon, and RD as denoted. Responses are aligned to the FT onset, ST onset, and RD throughout. Image reproduced from (Okada *et al.*, 2009).

Based on the anatomical and behavioural findings that allow making a dissociation between the anterior and posterior part of the PPTg this laboratory tested anterior and posterior PPTg lesioned rats on different reinforcement schedules that differed in the number of actions required to gain food reinforcement (fixed ratio; FR) and the probability of reinforcement on a given action (variable ratio; VR) and extinction, in which they learned to inhibit responding in the absence of reinforcement (D. I. Wilson *et al.*, 2009). Rats with posterior PPTg lesions made fewer correct bar presses than sham lesioned rats on all reinforcement schedules. Anterior PPTg lesions did not interrupt learning to bar press in low FR and higher VR schedules. However, anterior lesioned rats showed a behaviour that can be described as disorganized response control: in some schedules rats made more checks for food prior to its signalled delivery, more inconsequential bar presses between food delivery and food consumption, and more bar presses throughout extinction. The data confirm previous findings: anterior, but not posterior, PPTg lesions influenced locomotion (Alderson *et al.*, 2008), whereas posterior, but not anterior, PPTg lesions altered self-administration of nicotine (Alderson *et al.*, 2006).

3. Treatment for PD

3.1. Medical treatment

Only four years after the identification of DA as an independent neurotransmitter in 1957 Walther Birkmayer and Oleh Hornykiewicz in Vienna and Andre Barbeau, Ted Sourkes and Gerald Murphy in Montreal initiated the first trials of L-DOPA in PD patients (Bjorklund and Dunnett, 2007a). Today, L-DOPA in combination with a peripheral dopa-decarboxylase inhibitor (benserazide or carbidopa) is considered the gold standard of PD therapy. The response to L-DOPA, however, is not predictable and depends on the clinical phenotype and stage of progression of the disease. The initial motor improvement is said to lie between 20% and 70% (Fahn *et al.*, 2004). Tremor is often difficult to treat and an improvement is sometimes only seen after a long duration of treatment. Axial symptoms are little responsive all together. Early adverse events to L-DOPA administration are nausea, anorexia, faintness, hypomania, depression and delirium (Isaacson and Hauser, 2009). A slow and gradual optimization of the drug therapy can often avoid those problems.

Long term difficulties with L-DOPA are however more problematic. Motor fluctuations and involuntary movements (chorea, athetosis, and dystonia) are common features after long term use of L-DOPA. Initially they can be controlled by adjustments of dose and inter-dose interval. Additionally, DA agonists (pramipexole, ropinirole, rotigotine, piribedil) can, at least partially, substitute L-DOPA to reduce dyskinesias. Administration of the glutamate antagonist amantadine can also reduce dyskinesias and anticholinergic drugs (benztropine, biperiden) can reduce painful dystonia. Catechol-O-methyltransferase inhibitors (COMTIs; entacapone and tolcapone) can prolong the effects of L-DOPA.

Alternatives to L-DOPA are DA agonists – though these are rarely sufficient and L-DOPA will be necessary with time. Selective type B monoamine oxidase inhibitors (selegiline, rasagiline) are

well tolerated, but are also less efficacious. Apomorphine, a strong non-selective DA agonist, is water soluble and lipophilic and therefore suitable for intravenous, subcutaneous and transdermal routes. As a rapid rescue from severe off periods it can be injected subcutaneously but it is also offered as a pump for programmed infusion in order to more effectively control off-periods. Those injections can however, apart from other possible side effects of apomorphine (Pietz *et al.*, 1998) cause skin problems and the formation of subcutaneous nodules. A further advanced treatment is the delivery of L-DOPA in form of an intestinal gel by an intra-jejunal pump. Tube blockages and dislocations are adverse events to be considered (Olanow *et al.*, 2014).

Despite many different alternatives, which all bring some kind of side effects (most of which are tolerable) the main difficulties of fluctuating medication response, troublesome dyskinesias and/or L-DOPA unresponsive symptoms remain. Further available options are different surgical approaches.

3.2. Deep brain stimulation

The pioneering work on DBS for movement disorders was conducted and published by the French neurosurgeon Benabid and colleagues from Grenoble in 1987 in a paper called "Combined (thalamotomy and stimulation) stereotactic surgery of the ventral intermediate (VIM) thalamic nucleus for bilateral Parkinson disease" (Blomstedt and Hariz, 2010). It presents an alternative to ablative stereotaxic surgery, also pioneered by the group from Grenoble. The STN as a target was introduced in 1993 (Pollak *et al.*, 1993): from this point forward not only tremor could be treated with DBS but also other cardinal symptoms of PD and levodopa-induced motor complications.

The advantages of DBS compared to surgical destruction of tissue are obvious: it does not involve making a lesion, is theoretically reversible (in that it can be switched off) and

stimulation parameters can be adjusted post-operatively: Stimulation frequency, intensity and pulse width can be changed until maximal efficacy with minimal adverse effects can be achieved; also adapting to the course of the disease. The stimulation leads have several contacts which can be activated, deactivated and combined to produce a stimulation field that has the best effect. Today, DBS of STN, thalamus and GP are considered evidence-based routine treatments (Volkmann, 2007), which are nevertheless invasives procedures and therefore to be considered after thorough evaluation of alternatives. PPTg-DBS remains an experimental intervention.

3.3. Deep brain stimulation of the pedunculopontine tegmental nucleus

In recent years a considerable amount of articles have been published on PPTg-DBS in PD patients. Table 1.2. summarises the majority of those publications. The table, however, cannot be considered exhaustive, because articles on special single case studies describing for example rare side effects during PPTg-DBS [oscillopsia (Jenkinson *et al.*, 2012); urinary incontinence (Aviles-Olmos *et al.*, 2011)] or conditions other than PD [patient with multiple system atrophy with PD symptoms (Acar *et al.*, 2011); primary progressive freezing gait disorder patient (Ostrem *et al.*, 2010; Wilcox *et al.*, 2011); PSP patient (Brusa *et al.*, 2009)] were excluded. Pure anatomical reviews without direct references to surgical procedures were not included either. A more detailed analysis of these publication reveals that the actual amount of studies that offer information about the clinical benefit of PPTg-DBS is limited, especially considering that several publications report on the same patient cohort. (Where apparent this is remarked in the table.) Between 2008 and 2013 a total of 23 reviews on PPTg-DBS were published (13 of which exclusively focusing on the PPTg, the rest on DBS targets with reference to the PPTg). Not included in this list are numerous publications on the potential of the PPTg as a target for PD, reviewing the anatomy and animal studies [for example (Hamani *et al.*, 2007)] few papers discuss different surgical techniques that aim to improve PPTg targeting

and other focus on electrophysiological and metabolic changes in the brain during PPTg-DBS. The remaining studies assessed different clinical dimensions, motor deficits, cognitive abilities, sleep disturbances:

The first pioneering studies appeared encouraging (Mazzone *et al.*, 2005; Plaha and Gill, 2005; Stefani *et al.*, 2007). The authors reported safe surgical implants of DBS leads in the PPTg and presented subjective reports of improved feeling of 'well-being' and of improved motor symptoms. Subsequent studies, however, did not deliver the same promising results and early improvements could possibly be attributed to placebo effects; follow-ups reported a decline of the transient gait amelioration (Stefani *et al.*, 2013). The first double-blind trials proved the importance of careful assessment of the potential results of the stimulation: in blind conditions these studies did not show motor improvements with PPTg-DBS as measured by the Unified Parkinson's Disease Rating Scale (UPDRS) – a widely used tool to assess the impact of the disease and the degree of disability it causes, covering motor and non-motor features affected in the daily life and as assessed in the clinical setting (Ferraye *et al.*, 2010; Goetz *et al.*, 2007; Moro *et al.*, 2010) – and by objective freezing measures (Ferraye *et al.*, 2010). The main focus was on postural instability and gait difficulties (PIGD); when assessed by either corresponding items of the UPDRS or by objective gait measures (Thevathasan, Cole*, et al.*, 2012) these symptoms showed improvement with PPTg-DBS – but only FOG and/or falls were reported to improve with PPTg-DBS, subjectively (Ferraye *et al.*, 2010) and objectively (Thevathasan, Cole*, et al.*, 2012), not gait parameters such as stride length. 'StartReact' – an acceleration of a response after a loud auditory stimulus – was shown to be deficient in patients with freezing problems and could be restored by PPTg-DBS (Thevathasan, Pogosyan*, et al.*, 2011). Reaction time in various attentional tasks (Thevathasan *et al.*, 2010) and working memory tasks (Alessandro *et al.*, 2010; Costa *et al.*, 2010) improved, but not the accuracy of the responses; improvement was also found in verbal fluency, executive functioning and delayed recall

(Alessandro *et al.*, 2010; Ceravolo *et al.*, 2011). Further, it was reported that an increase of REM duration could be achieved with PPTg-DBS (Lim *et al.*, 2009; Romigi *et al.*, 2008).

Thus, in terms of motor outcome of PPTg-DBS, the results of the clinical trials published so far offer heterogeneous and moderate results and an overall lack of consistent improvement. Surgical and experimental protocols differed substantially across the research groups. PPTg electrode implants were unilateral, bilateral or combined with STN-DBS or caudal zona incerta (cZi)-DBS; and surgical techniques for accurate targeting differed, the importance of which was highlighted by Mazzone and colleagues when showing considerable individual anatomical variability from patient to patient (Mazzone *et al.*, 2013). Specific targeting within the PPTg varied as well – the Italian group implanted electrodes in caudal parts of the PPTg (Insola *et al.*, 2012; Mazzone *et al.*, 2008) but others were placed rather rostrally (Alessandro *et al.*, 2010; Moreau *et al.*, 2009). Thevathasan and colleagues have pointed out the importance of considering the placement within the PPTg itself (Thevathasan, Pogosyan*, et al.*, 2012)– a suggestion strongly supported by the heterogeneous anatomy, physiology and connectivity of the PPTg – and a fact clearly supported by data from this lab. Patient selection was not consistent either across the trials; disease duration, symptomatology and diagnosis and response to medication differed, very likely having an impact on the underlying pathophysiology – possibly and importantly the degree of degeneration in the PPTg itself – and therefore differing responses to the treatment.

Table 1.2. Summary of studies published up to date on PPTg-DBS in PD patients (pages 53-55)

Authors	no of patients	Type of assessment	Key points
Mazzone et al. 2005	2	surgical; intraoperative clinical score	safe surgery
Plaha and Gill 2005	2	clinical (motor)	slight improvement of UPDRS part III, today it is suggested that this was rather a placebo effect
Stefani et al. 2007	6	clinical (motor)	PPN not as good as PPN+STN
Pierantozzi et al. 2008	6	spinal reflex excitability	spinal cord excitability: increased Hoffman reflex threshold normalized with PPN DBS
Androulidakis et al. 2008	6	electrophysiological (EEG, microelectrode recording)	alpha oscillatory synchronization in PPN area and coupling with cortical EEG, promoted by levodopa
Weinberger et al. 2008	5	electrophysiological (microelectrode recording)	different electrophysiological characteristics
Strafella et al. 2008	1	functional imaging (PET rCBF)	increased activity in subcortical areas, especially thalamus
Romigi et al. 2008	1	polysomnography	increase of REM episodes with LFS, STN DBS and PPN DBS improve sleep architecture disruptions (nocturnal awakenings, increased stage I and wakefulness after sleep onset and decrease REM), but only PPN DBS increases REM duration
Aravamuthan et al. 2008	healthy Ps	imaging (DTI); surgical	connections for surgical targeting
Mazzone et al. 2008	13	surgical	ventriculography vs CT scan
Zanini et al. 2009	5	clinical (motor, cognitive)	overall cognitive profile did not change, grammar improved (but also with STN DBS)
Ballanger et al. 2009	3	functional imaging (PET rCBF)	increased blood flow in subcortical and cortical areas
Costa et al. 2010	5	clinical (motor, cognitive)	RT improved in working memory tasks but not accuracy; facilitation of speed of processing possibly through modulation of attentional resources
Lim et al. 2009	5	polysomnography	REM duration increased, probably by lowering the REM sleep threshold
Arnulf et al. 2010	2	polysomnography	increased alertness with LFS, increased propensity to sleep with 60-80Hz
Alessandro et al. 2010	6	clinical (cognitive, sleep scales)	improvements, RT decreased in WM task, patients from previous study
Ferraye et al. 2010	6	clinical (motor)	previously implanted with STN DBS; STN DBS ON throughout study, double blind, some better, some no improvement, some worse, PIGD did not respond to stimulation, bilateral better than unilateral; lack of strict correlation between lead position and performance
Shimamoto et al. 2010	4	electrophysiological (microelectrode recording); surgical	electrophysiological identification of PPTg for implantation surgery
Moro et al. 2010	6	clinical (motor)	double blinded, no benefits, only subjective report of reduced falls, 50-70Hz
Khan et al. 2010	13	surgical	use of implanted guide tube

Authors	no of patients	Type of assessment	Key points
Stefani et al. 2010	6	functional imaging (18-FDG PET); clinical (cognitive)	improvement of attentive and executive domains, increased metabolic activity in cortical and limbic areas, patients from previous study
Thevathasan et al. 2010	11	clinical (cognitive, motor)	improved RT
Yeh et al. 2010	8	electrophysiological (microelectrode recording)	suggesting direct somatosensory inputs to PPTg
Tsang et al. 2010	7	electrophysiological (microelectrode recording, EEG)	PPTg activity during movement preparation and execution, modulated by DA medication
Peppe et al. 2010	5	clinical (motor)	uncertainties, patients from previous study
Schweder et al. 2010	1	imaging (DTI)	suggesting neuroplasticity after LFS
Ceravolo et al. 2011	6	functional imaging (18-FDG PET); clinical (cognitive, motor)	PPN ON better than PPN OFF (verbal long term memory; executive functions), increased cortical metabolism, but only minor and variable motor benefit, patients from previous study
Khan et al. 2011	7	clinical (motor)	PPTg + cZi, improvement of axial subscales UPDRS when stimulated in combination
Thevathasan et al. 2011	8	clinical (motor); RT and startle reflex tasks	'StartReact' absent in FOG patients, PPTg-DBS improved this; "Optimization of motor systems releasing pre-programmed movement may not be the only mechanism by which PPTg-DBS may improve gait"
Thevathasan et al. 2011	5	clinical (motor)	improvement of gait freezing (gait and falls questionnaire), UPDRS not sensitive to this treatment
Caliandro et al. 2011	3	electromyography (Tibialis anterior)	cases individually presented
Thevathasan et al. 2012	7	electrophysiological (microelectrode recording)	correlation of alpha oscillation and gait performance, case for topographical dissociation of PPTg: posterior PPTg relevance in gait
Thevathasan 2012	7	clinical (motor)	FOG, double blinded, freezing improved but not objective gait measures, patients from previous study
Khan et al. 2012	4	functional imaging (15O-H20 PET); clinical (motor)	PPN + cZi increased blood flow, improved control of symptoms when medicated
Khan et al. 2012	5	clinical (motor)	PPN + cZi, on-medication; improvements only when stimulated in combination
Mazzone et al. 2012	14	oromotor movements	jaw movements may be restored, patients from previous studies
Insola et al. 2012	10	surgical	obtaining Obex-to-brainstem distance by P16 and P14 latency measurements
Peppe et al. 2012	5	clinical (sleep scales)	1 year follow up, improvement of night time sleep and daytime sleepiness
Schrader et al. 2013	1	clinical (motor)	PPTg, GPi and combined, individually: mild effect on gait ignition and FOG, combined: marked effect
Mazzone et al. 2013	28	imaging (MRI); clinical (motor)	no correlation between lead position, contact setup and clinical outcome, patients from previous studies
Neagu et al. 2013	2	electrophysiological (microelectrode recording)	bilateral STN DBS, recording from unilateral PPN electrodes, demonstrating functional connection

REVIEWS

Authors	Focus	
Pereira et al. 2011	PPTg	non-human primate and patients in Oxford
Garcia-Rill et al. 2011	PPTg	from slice to human
Scarnati et al. 2011	PPTg and spinal cord projections	
Hamani et al. 2011	PPTg	
Mazzone et al. 2011	PPTg	very brief overview
Amara et al. 2011	PPTg and sleep	
Profice et al. 2011	PPTg neurophysiology	
Ferraye et al. 2011	STN vs PPN	
Bohnen et al. 2011	options for gait and balance	
Tykocki et al. 2011	PPTg	
Follett and Torres-Russotto 2012	DBS	no single target is superior, PPTg needs further investigation
Benabid and Torres 2012	DBS	new targets
Fasano et al. 2012	DBS	treatment of motor and non-motor symptoms with DBS
Benarroch 2013	PPTg	functional organization and clinical implications
Kim et al. 2013	postural instability	PPTg as new surgical target, but remains experimental
Blanco et al. 2013	PPTg	anatomical review with focus on Barrington nucleus
Stefani et al. 2013	PPTg	no gait, but sleep and cognitive improvement
Duker and Espay 2013	Surgical treatment of PD	PPTg as new target
Fournier-Gosselin et al. 2013	PPTg	anatomical review and guideline for accurate targeting
Poetter-Nerger and Volkmann 2013	DBS for gait and postural symptoms	PPTg not yet considered established target
Castrioto and Moro 2013	new targets for DBS	PPTg has initially appeared encouraging, however enthusiasm now mitigated
Lukins et al. 2014	DBS	latest evidence on target selection – PPTg has shown promising improvement in axial symptoms

4. Parkinson's disease models

Traditional PD models have provided a great amount of information about PD pathophysiology and treatment options. However, when trying to understand the involvement of the PPTg in this condition and the possibility of targeting this structure with DBS the actual physiological condition of the PPTg in PD needs to be mimicked in more detail. As discussed above DA depletion alone is not enough to account for all symptoms seen in PD: the PPTg is affected by neurodegeneration as well (as are other brainstem structures). Stimulating a fully intact structure will clearly have different effects compared to stimulating a structure that shows partial cell loss which therefore needs to be taken into account.

4.1. 6-hydroxydopamine

Very early models of PD were created by means of reserpine or haloperidol injections in rodents and rabbits. This does not cause nigral dopaminergic cell degeneration but only mimics symptomatic characteristics of the disease. In order to also mimic the pathophysiological state of PD the toxin 6-OHDA is widely used in rats (Duty and Jenner, 2011; Emborg, 2004). It has to be stereotaxically injected either into the SN itself, the medial forebrain bundle (MFB) or the striatum because it does not cross the blood-brain-barrier. 6-OHDA causes selective catecholaminergic cell death through a combination of oxidative stress and mitochondrial respiratory dysfunction once inside the cell. The many different effects it has leading to the cell death resemble processes also apparent in PD [for more details see (Duty and Jenner, 2011)]. Infusion site and the amount of toxin determine the extent and severity of DA depletion. Medial forebrain bundle and SNc injection sites are most often used to induce very extensive lesions, but the DA depletion can spread to include the A10 neurons of the VTA. Infusion of the toxin into the striatum can cause very severe lesions as well – depending on the number of infusions and toxin concentration – but can be more controlled,

spares (however not completely) VTA cell loss and induces cell death retrogradely. Kirik and colleagues tested different doses and injection sites comparing the extent of DA depletion and the behavioural effect (Kirik *et al.*, 1998). They showed that rats with extensive DA depletion caused by striatal injections over four injection sites showed strongest behavioural effects measured as deficit in skilled paw use. This observation is in line with results from a comparison of two bilateral 6-OHDA models, which both received striatal injections into the same sites but using different concentrations: the higher concentration caused a more reliable and more persistent behavioural effect (Gut, 2009). For many purposes unilateral lesions can be sufficient, but when both sides of the body need to be tested, such as in gait, bilateral DA depletions are required. This means that post-operatively the animals will suffer from a period of marked aphagia, making them reliant on being hand fed. The choice of model clearly depends on the scientific question at hand but it needs to be pointed out that this traditional 6-OHDA model is limited as the degeneration in PD is not restricted to DA neurons (Hirsch *et al.*, 1987; Rinne *et al.*, 2008; Zweig *et al.*, 1989). Especially in the light of the development of new therapeutic methods targeting affected brainstem nuclei this limitation needs to be addressed as the restriction to DA neurons is not only untrue to the actual underlying pathophysiology but is also not enough for the symptoms of interest (Grabli *et al.*, 2013). Therefore, when targeting the PPTg with DBS electrodes or when recording from the PPTg it is questionable if those findings are comparable to the human condition because those traditional models do not take into account the degeneration in the PPTg (Alam *et al.*, 2011) making a refined model necessary (Pereira *et al.*, 2011).

4.2. Combination of dopamine depletion in SN and lesions of the PPTg

Only one study has been published that examines the behavioural effect of a combination of PPTg lesions and DA degeneration in the SN in Parkinson's models [in non-human primates (Grabli *et al.*, 2013)] despite the increasing number of PPTg-DBS studies in patients and the few

experimental (and mainly electrophysiological) approaches in rodents (Alam *et al.*, 2012; Capozzo *et al.*, 2009; Rauch *et al.*, 2010; Saryyeva *et al.*, 2011). A few studies have been conducted combining DA depletion and PPTg lesions, not to test the effect on behaviour, but their effect on SNr and STN firing and thalamic firing [(Breit *et al.*, 2006) and (Yan *et al.*, 2008) respectively]. Matsumura and colleagues (Matsumura and Kojima, 2001) administered MPTP to a monkey that was already bearing kainic acid lesions of the PPTg and report that these developed only mild – if any – parkinsonism compared to MPTP models without PPTg lesions. Given the marked degeneration in the PPTg in PD patients it is important to develop a model that considers these changes, especially when this very structure is the one to be targeted for DBS.

4.3. Deep brain stimulation of the PPTg in rodent models of Parkinson's disease

The effect of PPTg lesions on STN activity in parkinsonian rats was assessed before (Breit *et al.*, 2005; Breit *et al.*, 2006) and the only other PPTg stimulation studies in PD rats focused on its modulatory effect on electrophysiological activity of other structures, mainly the STN, in anaesthetised hemi-parkinsonian rats (Alam *et al.*, 2012; Capozzo *et al.*, 2009; Saryyeva *et al.*, 2011). Despite the interest in PPTg-DBS for PD and the conflicting results in the clinical literature there is to date only one published study in which the PPTg was targeted with stimulation electrodes in a rodent PD model to assess the potential of DBS in this structure as a therapeutic target (Rauch *et al.*, 2010). However, those animals had an intact PPTg on both sides. Rauch and colleagues found that stimulation with different frequencies of the intact PPTg in a 6-OHDA lesioned rat had complex effects on different parameters of motor activity and postural stability: whereas the stimulation with a high frequency or low frequency but high intensity seemed to improve the postural instability, limb use and motor activity

worsened, which in return could be improved by low frequency stimulation with a low intensity.

5. Aims

There is a need for a detailed and controlled investigation of the potential role of the PPTg in the treatment of PD symptoms. This Introduction has highlighted many reasons for this:

- The underlying brain pathology of PD includes extranigral degeneration.
- The PPTg is highly interconnected with the BG and other structures such as the thalamus and brainstem structures.
- Electrophysiological changes in the PPTg (and in BG structures that are closely connected to the PPTg) have been shown in PD models and patients.
- The evidence regarding the functions of the PPTg, meticulously accumulated over decades, provide a consistent picture of the role of the PPTg beyond motor control.
- Particular PD symptoms do not respond to dopaminergic medication: the relationship of these symptoms to PPTg cell loss and further functional disturbances match the functional role of the PPTg.
- PPTg-DBS in Parkinsonian patients has very inconsistent results.
- There is an uncertainty in the experimental literature about the functions of different parts of the PPTg.

On this basis a series of experiments was conducted to define more accurately what the potential for PPTg-DBS might be, in order to make predictions about what effects might be expected from which parts of the PPTg following DBS.

In order to confirm and expand on the literature showing that the lack of a fully functioning PPTg does not cause motor deficits, the effect of both full and partial PPTg lesions on gait and posture was assessed; the results are presented in Chapters 3 and 4.

By adding a partial lesion of the PPTg to bilateral destruction of SNc DA neurons, the traditional PD rodent model was refined to mimic better the pathological state in PD. Chapter 4 reports on the comparison of this novel model with the traditional 6-OHDA model.

Doubting the effect of PPTg-DBS as a corrective means of gait deficits on the basis that the PPTg is more highly interconnected and possess a more complex functionality than hypothesised by the majority of the PPTg-DBS literature, the effect of PPTg-DBS on gait was examined. The clear functional and structural differences between anterior and posterior PPTg raise the question whether the consequences of PPTg-DBS are dependent on location within the PPTg, as well as the question whether the effects might differ. Therefore different sites within the PPTg were targeted with DBS and their effect on gait and also L-DOPA induced dyskinesias were compared (Chapter 5). Clarity about this is an essential precursor to decisions about which patients might be considered best candidates for PPTg-DBS and to which PPTg sites DBS should be targeted.

Chapter 2

General Methods

1. Subjects

A total of 134 adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used for this project; 26 for pilot work and 108 for experiments. The rats were housed in a temperature and humidity controlled environment under a 12h light/dark cycle (lights on at 6am [7am during UK daylight saving time]). All animals were single housed as soon as the behavioural training started: they had to be on food control because of the sensitivity of the gait analysis device to weight changes. Once the target weight for surgery and testing was reached animals were weighed daily and the food was adjusted accordingly, but never below 15 g/rat/day, in order to maintain a stable weight. Water was available *ad libitum* in the homecages. The cages consisted of a solid plastic base with a wire mesh top, measuring 36 cm x 56 cm x 23 cm or 25 cm x 45 cm x 15 cm in the case of the experiment described in Chapter 3. Whenever possible the cages were environmentally enriched with plastic tubes or perspex huts. For the DBS studies access to the food hoppers was blocked off and the wire mesh tops were covered with semi-transparent plastic sheets to avoid loss of the headcaps. For the experiments described in Chapter 3 and 4 the target weight for surgery was 330g, which was increased to 380g for the DBS studies (Chapter 5) in order to increase headstage stability. Actual weights and group sizes are stated in the corresponding chapters. Compliance with the Animals (Scientific Procedures) Act 1986 and European Communities Council Directive of 24/11/86 (86/609/EEC) was maintained throughout the project.

All behavioural testing was conducted during the light phase. All care was taken not to disrupt the animals during the dark phase. However, in the case of high maintenance PD models some interaction with the rats during the dark phase was inevitable in order to provide them with the care they needed.

2. Surgical procedures

Depending on the experimental design and group affiliation rats underwent 1, 2 or 3 surgeries, allowing a 7-day recovery period between every 2 surgeries. Surgeries comprised either stereotaxically guided infusion of neurotoxic agents and/or stereotaxically guided implantation of stimulating electrodes. All surgeries were performed on “Model 900” Kopf stereotaxic frame (David Kopf, Tujunga, CA, USA) in a clean surgical environment. The following rodent models underwent a single or combinations of several surgical procedures:

Rats with bilateral PPTg lesions:	Two surgeries each comprising unilateral infusions of ibotenic acid into anterior and posterior PPTg.
Rats with partial PPTg lesions:	The same procedure using a lower concentration of the toxin.
Rats with bilateral DA depletion of the SNc:	Bilateral infusion of 6-OHDA into the striatum distributed over 4 different sites along the rostro-caudal axis in 1 surgery.
Rats with a combination of DA depletion and partial bilateral PPTg lesions:	3 surgeries starting with unilateral infusions of ibotenic acid into the PPTg followed by DA depletion in the third surgery.
Rats with either bilateral DA depletion or rats with combined lesions with bilateral implantations of stimulating electrodes into either anterior or posterior PPTg:	The corresponding procedures as described above, during the DA depleting surgery the electrodes were implanted subsequently.

2.1. Anaesthesia

Rats were anaesthetised using isoflurane (Abbot Laboratories Ltd, Maidenhead, UK) in an induction box, starting at a concentration of 0% isoflurane increasing this to 5% isoflurane at an approximate rate of 1 %/min in O_2 delivered at 4 L/min (care was taken that reflexes were lost before the concentration was increased from 3% to 4%) before being placed into the stereotaxic frame where anaesthesia was maintained through a facemask (specifically designed for the Kopf stereotaxic frame) at 2-2.5% isoflurane in 1.2-1.6 L/min O_2.

2.2. Bilateral PPTg lesions

The non-steroidal anti-inflammatory analgesic Rimadyl (carprofen 0.05 ml/rat, 5% w/v; s.c., Pfizer Ltd, Kent, UK) was administered subcutaneously, the head shaved, the skin cleaned and disinfected with povidone-iodine (Henry Schein Animal Health, Dumfries, UK). A midline incision was made and the skull exposed by scraping the periosteum from the skull, removing all soft tissue, holding the incision open with artery clamps. The incisor bar of the stereotaxic frame was then elevated. The height was determined by multiplying the distance between the interaural line and the back of the incisor bar by the sine of 8°29' (0.147) as described by Whishaw and colleagues (Whishaw *et al.*, 1977). Craniotomies were made using a hand held dental drill guided under a surgical microscope after measurements were taken for the burr holes at the specific locations (see the relevant experimental chapters for coordinates). The infusion of 0.09 – 0.12M ibotenic acid (solution in phosphate buffer; final pH adjusted to pH 7.0 using 2m NaOH; Sigma-Aldrich, Gillingham, Dorset, UK](please see the details in Chapter 3) was performed by pressure injection from a drawn glass micropipette (tip diameter 30-40µm) made from borosilicate capillary tubes (1.16 – 1.19 mm o.d.; 0.49 mm i.d.) pulled into pipettes on a pipette puller (Model 720 Needle Pipette Puller, David Kopf, Tujunga, CA, USA) with the tip broken off to the appropriate diameter under a light microscope. With the pipette filled with the toxin and held by an electrode holder on the stereotaxic frame, the measurements

were re-taken, dura cut with a sterile 29 ga needle and the pipette carefully lowered into the brain. Air pressure was applied manually by a syringe connected to the pipette via polythene tubing and released once the target volume has been in infused, as indicated by a scale on the pipette. The pipette was then left *in situ* for 5 min after infusion and then slowly lifted. This procedure was repeated for a second infusion. Subsequently the skull and wound edges were cleaned with saline and the wound closed with Michel clips (Henry Schein Animal Health, Dumfries, UK). Before being placed into a heated recovery cage rats were treated with 1-1.5 ml of Hartmann's solution (approximately 1 ml/h of surgery injected intraperitoneally; Baxter Healthcare Ltd, Norfolk, UK) for fluid maintenance accounting for the time that the rat was not able to drink, as well as with 0.02 ml of benzodiazepine (Diazepam, 5 mg/ml; Henry Schein Animal Health, Dumfries, UK) to reduce the risk of convulsive activity, also injected intraperitoneally. Rats were closely monitored during the entire post-operative recovery period because convulsive activity and barrel-rolling have to be expected after this procedure (for more details see Chapter 3). In order to create bilateral lesions surgeries were performed in 2 separate unilateral procedures separated by 7 days to reduce the risk of a fatal outcome from complications during the post-surgery recovery phase.

2.3. Partial PPTg lesions

Exactly the same procedure was performed as described above, but with a concentration of 0.06M of ibotenic acid.

2.4. Bilateral DA depletions of the SNc (6-OHDA lesions)

The non-steroidal anti-inflammatory analgesic Rimadyl (carprofen 0.05 ml/rat, 5% w/v; Pfizer Ltd, Kent, UK) was administered subcutaneously, the head shaved, the skin cleaned and disinfected with povidone-iodine. A midline incision was made and the skull exposed by scraping the periosteum from the skull, removing all soft tissue, holding the incision open with

artery clamps. The nose bar was then set to 0 mm [following (Kirik *et al.*, 1998)]. Craniotomies were made using a hand held dental drill guided under a surgical microscope view after measurements were taken for the burr holes bilaterally above the striatum in order to deliver the toxin at 4 sites on each side at the following coordinates (in mm): (1) AP: + 1.3 (measured from Bregma), ML: ± 2.6 (measured from midline at the skull surface), DV: - 5.0 (measured from Dura); (2) AP: + 0.4, ML: ± 3.0, DV: - 5.0; (3) AP: - 0.4, ML: ± 4.2, DV: - 5.0; (4) AP: - 1.3, ML: ± 4.5, DV: - 5.0. Care was taken to avoid damage to dura during the drilling process. Toxin infusions were made via a 30 ga cannula connected to a 10 µl SGE syringe (Speck & Burke, Alva, UK) (containing 70% alcohol) via polythene tubing (containing sterile distilled water) mounted in a Harvard microdrive pump (PHD 22/2000; Harvard Apparatus, Inc., Holliston, MA, USA). After filling the cannula with toxin (leaving an air bubble between water in the tubing and the toxin in order to check the flow of the infusion) and re-measuring the coordinates with the cannula (secured by an electrode holder on the stereotaxic frame) dura was cut with a bent tip of a 29 ga needle and the cannula lowered into position. At a rate of 1 µl/min 7 µg (free base weight) of 6-OHDA-hydrochloride (Sigma-Aldrich, Dorset, UK) dissolved in 2 µl of 0.1% L-ascorbic acid /0.9% saline was infused into each of the 4 sites of both sides of the brain. The cannula was left *in situ* for 2 min after the infusion before carefully retracting it. The toxin was weighed out before the surgical procedure, kept on ice and only dissolved just before the infusion to avoid oxidation. Subsequently the skull and wound edges were cleaned with saline and the wound closed with Michel clips (Henry Schein Animal Health, Dumfries, UK). Before being placed into a heated recovery cage rats were treated with 2-2.5 ml of Hartmann's solution (approximately 1 ml/h of surgery; Baxter Healthcare Ltd, Norfolk, UK).

2.5. Combination of DA depletions and partial bilateral PPTg lesions (Combined lesions)

Rats that received what in the following will be called 'combined lesions' underwent 3 separate surgeries. During the first 2 bilateral partial PPTg lesions were created as described above. Following a further 7 days of recovery the DA depleting procedure was performed. The burr holes for microinfusions of ibotenic acid into the PPTg of rats used in the DBS experiments were filled and covered with Gelfoam (Pfizer Ltd., New York, USA) before closing up in the attempt to keep the holes as clean as possible for an accurate implantation of the electrodes through the same holes (see below).

2.6. Electrode implantation

For DBS experiments rats of the DA depletion group and rats with combined lesions received implantations of concentric bipolar electrodes into either the anterior or posterior PPTg. These procedures were conducted during the same surgery as the infusion of 6-OHDA for DA depletion. After the last infusion of 6-OHDA into the striatum the skull was cleaned and dried and bone wax (Henry Schein Animal Health, Dumfries, UK) was used to close the burr holes above the striatum. The nose bar was then elevated to create a horizontal angle of 8°29' between the incisor bar and the interaural line (see above). In the case of a rat with solely a DA depletion at this point burr holes were drilled above the anterior or posterior PPTg on both sides using the same coordinates as stated for the PPTg lesion procedure. In case of a model with combined lesions the Gelfoam was removed from the burr holes above the PPTg and the hole was cleared as much as possible to allow for measurement from dura or to allow for a close enough approximate. If necessary the burr hole was slightly enlarged to allow the view onto a clean area of dura. Five to 6 stainless steel round-head machine screws with sharply cut threads (0-80 x 1/8 and 0-80 x 3/32); Plastics One, Roanoke, VA, USA) were screwed into the skull, 1 or 2 at the front, 2 between midline and the striatal burr holes and 2 behind lambda,

placing them in a slight angle to the skull surface to increase headcap stability. At this point the skull was cleaned and dried again to increase adhesion of the dental cement (see below). With an electrode (for details about the electrode design and material please see Chapter 5) secured into the electrode holder on the stereotaxic frame measurements were taken and the electrode was then lowered carefully into place (anterior or posterior PPTg) after widening the dura incision. Covering temporarily the other PPTg burr hole with bone wax the electrode was fixed onto the skull and neighbouring screw using a methyl methacrylate based dental acrylic (Simplex Rapid, Kemdent Works, Wiltshire, UK). Once the dental cement had dried the electrode was detached from the holder and the guiding cannula attached to the electrode by means of dental acrylic to aid implantation was carefully removed. This procedure was repeated for the implantation of the second electrode. Anode and cathode of the electrodes were then carefully connected to the headstage (see details about the headstage in Chapter 5; right-hand side electrode was always connected to the first row of connectors, left-hand side electrode to the second row, the cathode of each electrode was always connected to the left connector, anode to the right one). The headstage was then fixed with more dental cement in a layering manner to increase stability taking care it is level with the skull surface. Finally the wound edges were washed with saline solution. Once removed from the stereotaxic frame rats were given an i.p. injection of 2.5 ml Hartmann's solution (Baxter Healthcare Ltd, Norfolk, UK).

3. Post surgery animal care

PPTg lesioned animals did not require any special care following the immediate post-surgery recovery. After the immediate post-operative recovery period they did not show any apparent deficits that would compromise their well-being.

Bilaterally DA depleted rats (including the combined group) show severe aphagia. The animals' weight was strictly monitored after surgery and animals were hand fed with Liquid Rat Diet

(LD'82, Shake and Pour, control, BioServ, Frenchtown, NJ, USA) twice a day to secure a stable weight. Additionally they received 2 ml Hartmann's solution on the first day after surgery.

Rats carrying headstages received 0.05 ml subcutaneous injections of the non-steroidal anti-inflammatory analgesic Rimadyl for 2 days post surgery.

4. Behavioural procedures

4.1. Food restriction

All rats used in this project were kept on food control before (at the latest starting with the pre-surgical baseline testing) and after surgery to maintain a constant weight avoiding interference of the rats' body weight with the behavioural data from the CatWalk. The rats' weight was taken daily at about the same time and the food was adjusted accordingly.

4.2. CatWalk training and testing

Gait testing was conducted on the CatWalk (CW) 7.1 (Noldus, Wageningen, Netherlands). The principle working mechanisms of the CW are well explained by Hamers and colleagues (Hamers *et al.*, 2006). In brief, rats traverse a 100 cm x 12 cm x 0.6 cm glass walkway, motivated by their own homecage being present at the other end of the CW, for them to return to directly after testing. Green LEDs arranged alongside the glass floor project into the glass. This light gets scattered once the rats' paws touch the surface, illuminating the contact area; a high-speed camera sitting underneath the walkway capture these reflections and transmit the captured video clips to the connected computer. These video clips can then be analysed with the corresponding CW software. Prior to pre-surgery testing the rats were trained for 10-12 days on the CW to assure that they were able to complete at least 3 consecutive runs fulfilling the criteria of runs free from any interruptions such as stopping, rearing or jumping off the CW. During testing, runs that did not meet these criteria were

repeated (see details regarding slowing and freezing in PD models in Chapter 5). The mean of 3 runs was calculated for each testing session.

4.3. CatWalk settings

The camera's lens aperture was set at 1.4 - 1.6. For the full PPTg lesion experiment the software was set to 3990 for contrast and brightness was set to - 290 with a pixel intensity threshold of 12 and pixel number threshold of 7. For the combined lesion experiment the brightness was set to - 330 and maintained for the DBS studies.

4.4. Gait parameters

Base of support (BOS)	This parameter measures the distance (in mm) between the two forepaws or the two hind paws. It serves as an indicator for gait stability. A large BOS can compensate for an unstable gait (Hamers *et al.*, 2006).
Duty Cycle	The stance phase is the duration of contact of a paw with the walkway in a step cycle. But since the stance duration depends on the overall walking speed this parameter is expressed as a percentage of the duration of the step cycle [stance / (stance + swing) x 100%]. This parameter is an indicator for the stability of the animal. Shorter swing duration and longer stance duration can compensate for an unstable gait (Hamers *et al.*, 2006).
Support	The support formula describes the number of paws that are on the glass plate at any time during a run expressed as the relative duration (in percent) of contact with the ground of all combinations of numbers of paws. The combinations analysed here are diagonal pair of paws and three paws.
Swing Speed	This describes the velocity measure in m/s of the paws during the phase when it has no contact with the walkway (= swing). It is calculated as stride

length / swing duration.

Stride Length — This is the distance (measured in mm) between two successive placements of the same paw.

Print Position — Print position is the distance (expressed pixels) between the position of the hind paw and the position of the previously placed front paw on the ipsilateral side within the same step cycle. This also belongs to the group of parameters assessing the regularity of the animal's gait patterns. Most rodents place their hind paw at the previous position of the forepaw (best chance for a safe placement) and with increased step length the hind paw tends to be placed in front of the front paw.

4.5. Freezing of Gait

Some rats showed moderate to severe freezing during testing. In the combined lesion study (Chapter 4) rats showed mostly gait hesitation in the middle of a run (slowing down and coming to a stop) which most likely reflects destination-hesitation according to the classification of Fahn (Fahn, 1995; Schaafsma *et al.*, 2003) (rather than hesitation in tight quarters, given that the rat had already been in and had been walking through the narrow space of the CW before the freezing started). This type of freezing was not recorded separately – usually the repetition of the run led to a run that fulfilled the required criteria. In the DBS studies however some rats were suffering from severe start hesitation. If this did not change after several minutes the rat was placed back into the homecage for a short rest and the test was then repeated. Should the rat still not cross the walkway it was carefully encouraged by gentle tapping or pushing. If no run at all or not the required 3 runs could be recorded the performance was recorded as such (freezing leading to <3 runs or freezing leading to complete lack of data for the day).

4.6. Stimulation protocol (intensity setting, battery changes and regular checks)

According to the experimental design (please see the Chapter 5 for details) chronic stimulation was carried out by attaching and securing the DBS device (for full details see Chapter 5) to the headstage on the rats' skull. But first, stimulation frequency and pulse width were programmed in C on a Sony Vaio laptop using the software IAR Embedded Workbench IDE (IAR Systems Ltd., Chalgrove, UK) and uploaded onto the DBS device connecting it to the computer using a debug-interface module (MSP-FETU430IF, Texas Instruments, Dallas, USA) and a custom-built reprogrammer circuit. The stimulation intensity was then manually set to the lowest point possible (~30 μA) by tightening the appropriate trimmer on the outside of the device, reading the emitted intensity off by means of a connected oscilloscope (TDS 3032, Tektronix, Beaverton, USA). Then, the threshold for stimulation-induced behavioural side-effects was determined for each rat individually in a step-wise procedure. Care was taken to make each stimulation epoch no longer than approximately 20 sec with at least 1 min in between. The device was attached to the headstage and only loosely secured to reduce the time spent manipulating the headstage. The rat was then sat into an empty cage bottom, then the device was switched on and the rat closely observed. Typical side effects were slowing or an arrest of exploratory behaviour up to "freezing" and "staring", erected whiskers and/or ears. If no side effect was seen, the device was detached, the intensity increased by 20 μA and re-attached to the headstage and the test repeated. Once one or several of the above described side effects were detected the intensity was reduced by 20% of this side effect inducing intensity and re-tested on the animal. In the case of continued side effects the intensity was further reduced. Once satisfactory stimulation intensity was determined the device was wrapped in parafilm to protect it from dirt and water, securely fixed onto the headstage and switched on. The rat was then returned into its homecage.

Red LEDs on the device corresponding to the connected electrodes indicated functioning of the device. The devices were checked at least twice a day. Every other day the battery of the device was changed because preceding tests showed that at similar stimulation settings a stable energy supply could be guaranteed for 48h. Battery changes were quick and offered a chance to check the stimulation settings when the device was briefly detached from the rat's head. This procedure took no longer than 2-3 min.

4.7. L-DOPA injections and scoring of abnormal involuntary movements

Before the start of DBS and after completion of CW testing under the condition of chronic stimulation the rats received injections of L-DOPA (see Chapter 5 for full details). Immediately after the injection they were returned to their homecage but kept under close observation (with the top of the cage removed) to classify and quantify L-DOPA induced abnormal involuntary movements (AIMs). Ratings of behaviour were made using a modified version of the Creese-Iversen scale (Creese and Iversen, 1973). Behaviour was rated as follows: 1: normal (absence of any AIMs); 2: active with bursts of stereotyped sniffing and rearing; 3: continuous stereotyped sniffing and rearing over the entire area of the cage; 4: stereotyped behaviour in one place (for example up and down head movements, crossing and lifting of the forelegs); 5: additional bursts of stereotyped licking or gnawing of paws, legs or sides (self-mutilation) and/or gnawing of the cage; 6: continuous licking and/or gnawing. For each animal the time was noted for which they displayed these 6 different stages of AIMs. Care was taken to check for behavioural changes every 5 min at minimum. In case of self-mutilation rats were offered an alternative to gnaw on (wooden chewing stick or a piece of cloth) and in very severe cases rats were removed from the cage and held by the observer to prevent further biting. No rats came to any permanent harm and were all able to move freely following instances of self-biting.

Thirty min after the L-DOPA injection the rats were supposed to be tested on the CW. Sham operated rats had no difficulties performing acceptable runs and were tested as described above. DA depleted animals had difficulties of different degrees to cross the walkway because of the AIMs. After 3 unsuccessful attempts or crossings that were obviously influenced by AIMs (like stopping for licking and gnawing or sideway walking while attempting to turn around) the rat was returned to its homecage for further timing and rating of the stereotypies until this AIM inducing effect of the L-DOPA had worn off. Typically animals turned to their food bowls then and started eating undisturbedly.

5. Histological procedures

5.1. Perfusions

At the end of behavioural testing the rats were given a lethal intra-peritoneal injection of 200 mg/ml sodium pentobarbitone (0.6 – 0.8 ml/rat Euthatal, Merial Animal Health Ltd., Harlow, UK). Once deeply anaesthetised they were perfused transcardially with 0.1% phosphate buffered saline followed by a fixative (4% paraformaldehyde in 0.1M phosphate buffer) until fixed (approximately 350ml). Brains were left to post-fix *in situ* for at least 60 min before removal and subsequent storage in 20% sucrose solution in 0.1 M PB) at approximately 4^{o} C.

5.2. Sectioning

Coronal 30 µm sections were cut in a series of 1:6 on a freezing microtome and separated into striatum, SN and PPTg before being transferred into a cryoprotectant (ethylene glycol and sucrose solution) for storage at - 20 C.

5.3. Immunohistochemical staining

5.3.1. Stains performed

Three different stains were performed in order to assess the regions of interest: (1) Choline Acetyltransferase (ChAT): Sections taken from the upper brainstem containing the PPTg were stained for ChAT, an enzyme expressed in nuclei and terminals of cholinergic neurons responsible for the synthesis of acetylcholine (ACh), to assess the number of surviving cholinergic neurons in this structure in case of PPTg lesioned animals and to aid the determination of electrode placement in brains of rats from the DBS studies. (2) Neuronal nuclei (NeuN): PPTg sections were furthermore stained with NeuN, a nuclear antigen that binds to neuron-specific nuclear protein in neuronal nuclei and to a lesser extent the cytoplasm of neuronal cells and is therefore commonly used to non-selectively mark neurons. Here, NeuN stains were used to assess the extent of ibotenic acid lesions in the PPTg and placement and track lesion sizes of implanted electrodes where applicable. (3) Tyrosine hydroxylase/ CalbindinD_{28k} (TH/Cb): On midbrain sections containing the SN a double stain was performed staining for TH, an enzyme responsible for the synthesis of the DA precursor L-DOPA, in order to mark DA containing neurons and the calcium binding protein Cb. Within the midbrain dopaminergic cell groups Cb is heterogeneously distributed and is not expressed in DA containing neurons in the SNc. However, neurons in the SNm, SNl, and most importantly the VTA, including the parabrachial pigmented nucleus (PBP) and paranigral nucleus (PN), and the neuropil of the SNr stain positively for anti-calbindinD_{28k} (McRitchie *et al.*, 1996). This differentiated staining helps to distinguish between DA neurons of the SNc and VTA/ PBP/ PN.

5.3.2. Protocols

Chat

Immunohistochemistry was performed on free floating sections at room temperature. Rinsing and incubation was aided by careful agitation on a horizontal shaker. The sections were removed from the cryoprotectant and washed 3 times for 3 min in phosphate buffered saline solution (PBS). They were then incubated for 45 min in blocking solution (79.9 % PBS; 20 % rabbit serum; 0.1% of 10% Trition X-100 [diluted in distilled water]) and subsequently rinsed in PBS for 3 min before being incubated with the primary antibody overnight (approximately 16h). The goat derived polyclonal anti-ChAT (Merck Millipore, Billerica, MA, USA) was diluted in an antibody diluting solution (ADS) consisting of 98.9% PBS, 1% rabbit serum and 0.1% Triton x-100 at a concentration of 1:5000. For incubation with the secondary antibody sections were first rinsed (5 times for 3 min) and then transferred to a biotinylated secondary antibody solution (anti-goat IgG, 1:200 in ADS) and incubated for 90 min. After further washing (as before) they were incubated in avidin-biotin complex (ABC solution; each, avidin and biotinylated horseradish peroxidase reagent, diluted 1:50 in ADS) for 45 min. IgG, avidin and biotinylated horseradish peroxidase reagent derived from the Vector Labs Elite ABC kit (Vector Laboratories, Peterborough, UK). Finally, after washing, the sections were stained with 3,3 diaminobenzidine tetrahydrochloride (Sigma Fast DAB; Sigma-Aldrich, Gillingham, UK) for approximately 12 min and then washed thoroughly. ChAT stained sections were then mounted on glass microscope slides, cleared in Xylene and coverslipped using DPX mounting medium (Sigma-Aldrich, Gillingham, UK).

NeuN/Cresyl protocol

The protocol for NeuN stains is consistent with the protocol described above. The serum used for blocking solution and antibody diluent was normal goat serum and the monoclonal

antibody was mouse derived (Merck Millipore, Billerica, MA, USA) and diluted in ADS to a concentration of 1:20000. The secondary antibody anti-mouse IgG was taken from the corresponding Vector Labs Elite ABC kit (Vector Laboratories, Peterborough, UK). Sections were mounted on gelatine coated glass slides and subsequently counterstained with cresyl-violet. Before conducting this procedure care was taken to completely dry out the sections by leaving the slides in a paraformaldehyde gas bath in a dessicator overnight. The slides were then cleared in xylene for 3 min and rehydrated through two changes of graded alcohol (100% and 50% in distilled water) to water. They were then placed in cresyl violet stain for 30-60 sec (depending on the strength of the stain) and then thoroughly rinsed in running tab water for 5 min before getting dehydrated through graded alcohol and cleared in xylene. They were then coverslipped as described above.

TH/Cb protocol

For the TH/Cb double stain the protocol was slightly modified. The Cb stain was performed first, following the same protocol as described above for ChAT. The monoclonal anti-calbindinD_{28k} was mouse derived (Sigma-Aldrich, Gillingham, UK) and diluted to a final concentration of 1:800. Blocking solution and ADS contained normal goat serum. Anti-mouse IgG and the avidin-biotin complex were made from the Vector Labs Elite ABC kit and the sections were finally stained using DAB. After a thorough wash the sections were pre-treated with avidin (using a concentration of 1:5 in a blocking solution made with goat serum) for 15 min followed by biotin (1:5 in ADS made with goat serum) to block remaining biotin sites on the avidin. The sections were then ready for the TH stain. Anti-TH was a monoclonal mouse derived antibody (Merck Millipore, Billerica, MA, USA) used at a concentration of 1:25000 diluted in goat serum based ADS with which the sections were incubated overnight. The secondary antibody and avidin-biotin complex were made up with the mouse version of the Vector Labs Elite ABC kit. Here the incubation times were shorter than before: 30 min for IgG

and 30 min for ABC. Finally the sections were stained with a slate gray peroxidise substrate (ImmPACT SG, Vector Laboratories, Peterborough, UK) to reveal the TH + cells. The double stained sections were then mounted, dried, cleared and coverslipped as described above.

6. Histological analysis

6.1. Lesion assessment

6.1.1. NeuN+

The extent of non-selective damage caused by ibotenic acid in and around the PPTg was assessed on the NeuN/Cresyl stained sections judged by the lack of stained neuronal nuclei. To this purpose sections were examined under a light microscope (Leica DM LB2) connected to a desktop computer by a Leica camera (type DF320: Leica Microsystems, Milton Keynes, UK) at a magnification of x 1.6. All available sections were inspected under the microscope. The lesion outline was then drawn on selected schematics from the Paxinos and Watson rat brain atlas (Paxinos and Watson, 2005) representing sections just rostrally to the PPTg (including the SN), anterior PPTg, posterior PPTg and sections caudally to the PPTg. Ten schematics were chosen taking care to pick a relatively even distribution of sections on the rostro-caudal axis covering the area 3.12 mm from IAL to – 0.72 mm from IAL. Those sections that corresponded best to the chosen schematics were photographed and the outline of the lesion shown by the stain transferred onto the schematic (section choice was based on the shape of the section, anatomical landmarks and the distribution of cholinergic neurons on corresponding sections on ChAT stained sections). Lesions were rated on a scale from 0 to 4 (0 = no lesion of the PPTg, 1 = small/ partial lesion of the PPTg, 2 = partial to full lesion of the PPTg, 3 = fully lesioned PPTg, 4 = extensive lesion covering the PPTg and surrounding tissue). Animals that had either a lack of lesion in the PPTg on at least one side (0; PPTg not hit) or lesions that were so extensive, that they damaged SN (4 on both sides) were excluded.

6.1.2. Chat+ counting

Additionally to the lesion extent the number of surviving cholinergic neurons was also quantified. Cholinergic neurons have proven to be surprisingly resistant to the excitotoxin ibotenic acid so for the study conducted to assess the behavioural effect of full lesions of the PPTg it was of interest to assess the number of possibly remaining ChAT+ neurons. In the case of the PD models that included partial PPTg lesions the number of remaining and presumably functioning cholinergic neurons was required to compare these to the degree of PPTg degeneration reported for PD, which in the human neurological literature is usually quantified in terms of degeneration of cholinergic cells. For this purpose each ChAT+ stained section was photographed with the camera-microscope set up as described above at a total magnification of x10. These pictures were then opened in Image-J (U.S. National Institutes of Health, Bethesda, Maryland, USA), an analysis programme that assisted the manual counting of ChAT+ PPTg neurons. The total number of surviving cholinergic neurons of the anterior, the posterior and the PPTg as a whole were expressed as a percentage of the average total cell count taken from sham lesioned animals. Here it needs to be pointed out that this cell count cannot be considered an exact count of present neurons but rather a representative estimate. In the case of brains with track lesions from the implanted electrodes the presented cell count reflects the number of surviving cells of the non-targeted part of the PPTg (anterior or posterior respectively).

6.1.3. TH+ counting

All TH+ neurons within the dorsal and ventral tier of substantia nigra part compacta (SNc; SNcd and SNcv respectively), substantia nigra pars medialis (SNm) and substantia nigra pars lateralis (SNl) were included into the count. The additional Cb stain was performed in order to distinguish DA containing neurons of the SN from VTA (including PBP) neurons. In the case of

SNc this technique makes separation relatively easy since the SNc does not contain Cb. When in doubt – where neurons are densely packed or interdigitated – the triangular shape of the SNc neurons with ventrally directed processes distinguished them from the rather elongated neurons of the PBP which have a mediolateral orientation. SNl can also contain Cb, here however the distribution and orientation of the neurons at the dorsolateral pole of the SN allows a confident classification. SNm also contains Cb which makes a separation from VTA/PN neurons more difficult. Here the shape of the SNm cluster, SNm neurons (larger than PN) and position had to be determining factors for classification. Identification of these subsections was aided by the chemoarchitectonic atlas of the rat brain (Paxinos *et al.*, 2009). Counting was performed at a 20 x magnification using a handheld cell counter starting with the first appearance of TH+ cells caudally from the STN in control groups and non-DA-depleted animals and with corresponding sections in the PD models [approximately at - 4.56 mm from Bregma (Paxinos and Watson, 2005)], counting through to the last sections showing DA neurons at around - 6.60 mm from Bregma. Cell counts of all sections were added and the total expressed as a percentage of the average total cell count of sham operated rats.

Please note: Due to time constraints no TH staining was conducted to assess the density of striatal DA, nor NeuN staining to check for non-dopaminergic damage. This had been done previously (Gut, 2009) and it had been shown that the track damage of the injecting cannulae was negligible or non-existant, that there was no non-dopaminergic damage in the striatum and that profound loss DA terminal density was focused on but not restricted to the dorsolateral striatum.

6.2. Electrode placement

Electrode tip location in the DBS experiments was determined by examining electrode track evidence on ChAT+ stained and counterchecked on NeuN/Cresyl stained sections where there were not enough ChAT+ neurons visible to make a judgement.

7. Behavioural data analysis

All statistical analysis was performed using SPSS version 21 (IBM Corp., Armon, NY, USA). Details of specific tests performed on the according data are given in each chapter. All effects were considered statistically significant when $p \leq 0.05$.

7.1. CW data labelling

Using the CatWalk 7.1 all paw prints had to be labelled manually. For this purpose the first 3 runs that fulfilled the above criteria of all runs recorded of any particular day of each animal were chosen for further processing. Under normal conditions the video clips start with the animal already in full motion (start of recording triggered by the rat passing a pre-determined "start box" on the walkway) and gets stopped automatically at the end of the runway (ideally before the animal slows down the run). However, occasionally those clear start and endpoints were not given – for example when the red LEDs of the stimulation device triggered the recording before the rat started to cross the walkway or when the rats (mostly parkinsonian rats) stopped a few centimetres before the end of the glass plate (i.e. having crossed the "finish line" but not being far enough out of the pre-determined "finish box" to trigger the automated stop of the recording). In those cases the video clips were cut manually. Some DA depleted rats showed occasional to severe slowing down or freezing. Runs were included when movement, however slow, was still detectable on the video clip. When rats froze completely during the run the data from the run was discarded and the trial repeated.

7.2. CW data analysis

Please refer to the individual chapters for details on the statistical tests used. In general the data were explored by performing mixed, univariate and/or repeated measures ANOVAs with Tukey HSD corrected post hoc tests where appropriate. Where the data were heterogeneous the Huynh-Feldt correction was applied when epsilon > 0.75 and Greenhouse-Geisser correction when epsilon < 0.75.

7.3. FOG

The occurrence of FOG was expressed as a frequency count and presented as the percentage of the number of tests run in each experimental group per condition (number of rats x days of testing). To test for a significant association between group affiliation and occurrence of freezing chi-squared tests were performed for each experimental condition.

7.4. Dyskinesias/Stereotypies

The severity of L-DOPA induced dyskinesias was assessed by determining the time for which the rats displayed AIMs in form of L-DOPA induced stereotypies of each level according to the modified Creese-Iversen scale as described above and presented in minutes. Mixed ANOVAs were performed on these data and further investigated by testing the simple main effects for groups (univariate ANOVA) and mixed ANOVAs for individual scores with condition (L-DOPA administration with or without DBS) as within and group as between factor.

Chapter 3

The effect of full lesions of the PPTg on gait

1. Introduction

As discussed in Chapter 1 the question regarding the role of the PPTg in the generation of locomotion is an important one in light of the continued attempts to target this structure with DBS in order to alleviate parkinsonian symptoms [see, among others, (Ballanger *et al.*, 2009; Mazzone *et al.*, 2005; Pahapill and Lozano, 2000; Plaha and Gill, 2005; Stefani *et al.*, 2007). The literature offers differing views on this question stretching from the finding that an intact PPTg is not necessary to generate normal locomotor behaviour (Alderson *et al.*, 2003; Homs-Ormo *et al.*, 2003; Inglis, Allen*, et al.*, 1994; Inglis, Dunbar*, et al.*, 1994; Steiniger and Kretschmer, 2004; Winn, 2006) to the notion that the PPTg can be considered a movement generator (Garcia-Rill, 1991; Pahapill and Lozano, 2000). Anatomically the PPTg is in a strategic position for an involvement in aspects of motor control. It is reciprocally connected with the BG and has descending projections that mainly target lower brainstem structures and targets the medulla and spinal cord (Martinez-Gonzalez *et al.*, 2011; Mena-Segovia *et al.*, 2004). Early experiments in decerebrate cats and rats showed that stimulation of the PPTg induced motor responses in form of stepping on a treadmill, consistent with descending influence/control over motor regions (Garcia-Rill *et al.*, 1987; Skinner, Kinjo, Henderson*, et al.*, 1990). However, stimulating other structures in the brainstem produces the same effect (Garcia-Rill, 1991). A further argument for questioning an essential role in motor production is the fact that rats with PPTg lesions do not show deficits in spontaneous locomotor behaviour. When tested in photocell cages or in the open field they do not behave differently to control rats; in operant chambers they make just as fast or frequent lever presses, however incorrectly; and in mazes they do not show reduced speed when moving about (Alderson *et al.*, 2003; Dellu *et al.*, 1991; Inglis, Allen*, et al.*, 1994; Inglis, Dunbar*, et al.*, 1994; Keating and Winn, 2002; Olmstead and Franklin, 1994; Steiniger and Kretschmer, 2004; Swerdlow and Koob, 1987; Taylor *et al.*, 2004;

D. I. Wilson *et al.*, 2009). It is unquestionable: the PPTg is not necessary for spontaneous co-ordinated locomotion. There are changes in the locomotor response to psychomotor stimulants after PPTg lesions (Alderson *et al.*, 2003) but this reflects its relation to corticostriatal circuitry. For example, repeated administration of d-amphetamine results in a progressive increase in locomotor activity. Lesions of the PPTg did not change the effect of the drug on the first day, but attenuated sensitisation, probably as a result of loss of ascending projections of the PPTg to midbrain DA neurons in VTA and SN. By contrast, experimental work on non-human primates has shown that bilateral PPTg lesions caused akinesia and hypertonia (Kojima *et al.*, 1997; Munro-Davies *et al.*, 1999). However, the use of kainic acid in those studies most likely caused very big lesions comprising many more structures than just the PPTg (Winn, 2008). Furthermore behavioural testing took place during the immediate post-surgical recovery period so that surgery related deficits cannot be excluded (Peterson and Moore, 1980; Winn, 2006).

To date the effect of PPTg lesions on motor activity in rodents has focused primarily on spontaneous and general locomotor behaviour measured as beam breaks and rearing activity in locomotor boxes, latencies to run down an arm in a maze or to collect pellets in an operant box. If the PPTg is to be a target for DBS in patients it is necessary to take a more refined approach and to examine individual gait parameters in animals.

To this purpose the gait of rats with bilateral lesions of anterior and posterior PPTg were tested the same way as the gait of PD rodents. The same gait parameters that have shown deficits in PD models (Gut, 2009) were examined for similar deficits in rats bearing lesions of the whole PPTg.

2. Methods

2.1. Subjects

Twenty adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used. At the start of CW training their mean weight was 333g (range 312 – 349g). All animals were put on food control at that point as described in the General Methods. All animals reached the target weight of 330g for the first surgery (mean weight 336g; range 331 – 350g). The mean weight during pre-surgical baseline testing was 336g (range 325 – 342g) and a stable mean weight of 335g with a small range of 320 – 341g could be maintained over the entire period of the experiment. For further details on housing please refer to the General Methods.

2.2. Experimental protocol

The experiment followed the timeline presented in Figure 3.1. In brief, the rats were trained on the CW for 10 days. All rats were proficient at that point to deliver acceptable runs on the CW. Over the following 4 days their pre-surgical performance was recorded. They then underwent 2 consecutive surgeries, separated by a week, during which they received unilateral PPTg lesions. After a week of recovery the rats were then re-exposed to the CW on the first day and then tested daily for 7 days.

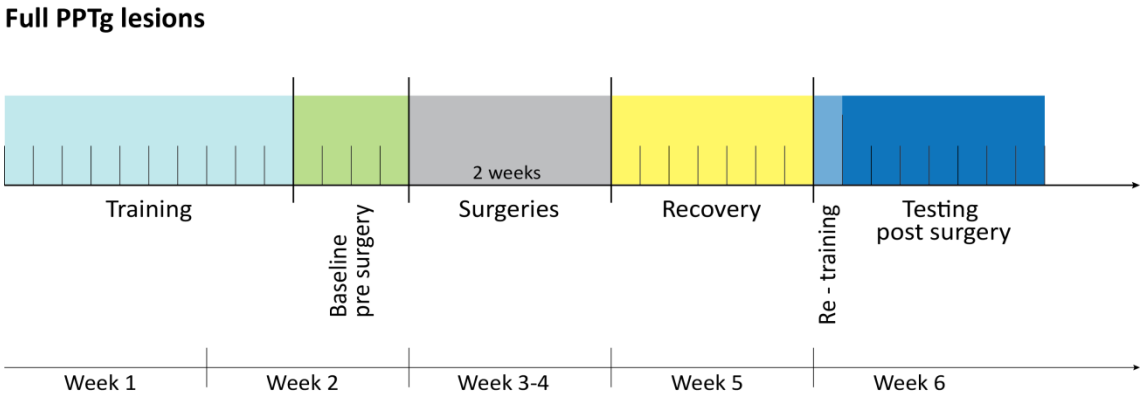

Figure 3.1. Experimental design. At the start of the experiment all animals were trained for 10 days on the Noldus CatWalk. They were then tested for 4 days to obtain a pre-surgical baseline. Rats then underwent PPTg lesion surgeries. Seven days after the last surgery they were then re-tested on the CW. For more details please refer to the text.

2.3. Bilateral lesions of anterior and posterior PPTg

Surgery followed the protocol as described in the General Methods. In 2 separate surgeries the PPTg was bilaterally lesioned by microinfusions of ibotenic acid into the anterior and posterior PPTg at the following coordinates (in mm): anterior PPTg: + 0.6 from IAL, ± 2.0 from midline and - 7.0 from dura; posterior PPTg: - 0.8 from IAL, ± 1.9 from midline and - 6.5 from dura. Infusions were always made anterior first but the order of infusions regarding the choice of side was counterbalanced. Initially rats were infused with 180 nl of 0.12M ibotenic acid per infusion site. Since five out of the first six rats receiving this concentration of the toxin did not survive the recovery period the concentration was then dropped to 0.1M (at 180 nl) – 1 out of 2 rats survived – and then to 0.09M maintaining the same volume, which proved to be a concentration with a better survival rate. Control rats received infusions of vehicle only (here: PB) into the same sites. At the end of surgery rats were treated with 1-1.5 ml of Hartmann's solution (i.p.) to account for the lack of fluid intake during the time of surgery. Furthermore, they also received an i.p. injection of 0.2 ml Diazepam to reduce the risk of injury due to the immediate effect of the excitotoxin acting on the targeted brain stem sites.

2.4. Immediate post-surgery recovery period

Ibotenic acid has agonist activity at NMDA receptors (Zinkand *et al.*, 1992) causing excitotoxicity by increasing the entry of calcium ions into neurons which will ultimately lead to the neuronal death. Initially, however, it causes hyperactivity resulting in a problematic post-surgery recovery that may lead to injuries of the animals or fatalities. If rats survive the initial 6 to 12h (mainly depending on the concentration of the toxin) they show no signs of a compromised long-term recovery such as pain or discomfort and appear rather normal-behaving. In the short term recovering rats show in general fit-like tremors that last several seconds followed by whole body rotation ("barrel rolling"). The lesions affect respiratory control in the brainstem. During those tremors the animals stop breathing which is then

followed by continuous hyperventilation. This seems to cause a build-up of fluids in the lungs which ultimately is often the reason for death during the subsequent stage of recovery, during which the fits cease and the animal's breathing becomes very flat. Several measures have been taken to reduce injuries and fatalities: The recovery cages were padded with "bubble wrap paper". This not only prevented injuries but also appeared to decrease the number of fits. Also the rats were held with the head elevated to prevent significant fluid build-up in the lungs. When the breathing rate became alarmingly low breathing was tried to be stimulated by giving short whiffs of CO_2 to stimulate the breathing reflex. If this did not help and breathing did not improve the rat was given an intramuscular injection of doxapram hydrochloride (Dopram, 0.5ml/1 kg), a respiratory stimulant. All these measures were approved or instructed by the named vet. Ultimately, the reduction of toxin concentration was the most effective measure to increase the likelihood of survival.

2.5. Training and testing on the CW

Rats were trained for 10 days prior to baseline testing and surgery and tested as described in the General Methods. The order of rats trained and tested was randomised.

2.6. Gait parameters

The following gait parameters were examined: BOS, swing speed, stride length and duty cycle of both front and hind legs, print position of ipsilateral paws and the support formula of diagonal pairs of paws and three paws. For details on the gait parameters please see the General Methods.

2.7. Lesion assessment

The area of the PPTg was histologically assessed for overall neuronal damage and cholinergic cell loss following the methods as described in the General Methods. In brief, at the end of behavioural testing rats were transcardially perfused with a fixative, the brains removed and

stored in sucrose solution until they sank. A series of 1:6 coronal 30 µm sections was cut on a freezing microtome and immunohistochemically stained for NeuN and ChAT, mounted on glass slides and examined under a light microscope. Overall neuronal damage was assessed visually on the NeuN/Cresyl stained sections and rated on a scale from 0 (no lesion) to 4 (extensive lesion). Lesions were accepted and rats included into the analysis when at least one side was rated a 3 (PPTg fully lesioned) and the other side not less than a 2 (partially to fully lesioned). Cholinergic cell survival was assessed by software assisted counts of ChAT+ neurons.

2.8. CW data analysis

In order to test the behavioural data for an effect of PPTg lesions on normal gait, differences between the lesion and sham group were explored performing mixed ANOVAs across condition (pre and post-surgery condition, within subject factor) and between groups (PPTg lesion and sham, between subjects factor) and further examined for a simple main effect on the post-surgery data performing an univariate ANOVA between groups. Preceding this, the data were assessed for pre-existing differences (univariate ANOVA between groups) and for differences between all 7 post-surgery testing days (repeated measures ANOVA across days) in order to rule out a time effect and pool the data. Normality of the data was identified by the Shapiro-Wilk test and homogeneity of variance by the Leven's test. The handling of violations of these assumptions is reported in the results section when they occurred. All tests were conducted using SPSS version 21 (IBM Corp., Armon, NY, USA) and results were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Recovery

In the past the same concentration of toxin purchased from the same supplier, prepared following the same protocol and injected using the same or very similar coordinates produced a mortality rate that was substantial but within an acceptable margin. Here, the toxin was associated with a considerably worse survival rate. As described above during the immediate recovery period the animals showed fits, barrel rolling, hyperventilation which was presumably causing a build-up of fluid which lead to death in 8 cases. Of the several measures taken to reduce the fatality rate the most effective (apart from lowering the concentration of the toxin) was to keep the animals as quiet as possible, which included holding them during the critical period where the fitting occurred (starting approximately 60 min after surgery lasting for up to 2.5h, but this can be very idiosyncratic). This reduced injuries and also seemed (subjectively) to decrease fitting. Close monitoring of the breathing rate and holding them upright to avoid the lungs filling up with fluids seemed to help as well. Injections of doxapram hydrochloride were not effective, neither was CO_2 to stimulate breathing.

3.2. Histological analysis

The presented results are obtained from a total of 20 rats, of which 16 underwent surgery for bilateral PPTg lesions and 4 received control infusions of vehicle. Due to a suboptimal perfusion and a consequently faint stain one rat of the control group was not included in the histological analysis; however the behavioural data was considered for the other analyses.

As noted above the first used concentration of ibotenic acid proved to be too high to guarantee an acceptable survival rate. Accordingly, a total of 7 rats did not survive the first unilateral infusion of ibotenate (n = 5 died after a concentration of 0.12 M, n = 1 after 0.1 M ibotenate and n = 1 after 0.09M ibotenate) and a further animal died after the second

unilateral infusion of 0.09M ibotenate. Hence, n = 8 rats constituted the final group of bilaterally PPTg lesioned rats (n = 1: 0.12M [first side] + 0.09M [second side]; n = 1: 0.1M [first side] + 0.09M [second side] and n = 6: 0.09M both sides) and n = 4 rats were in the sham group.

After histological analysis 2 rats were excluded from all analysis due to very extensive lesions not only covering the PPTg area but also the surrounding tissue, most importantly damaging the SN (n = 1; rat that survived the high concentration of 0.12M ibotenate) and due to an infusion having been placed too laterally and therefore missing the PPTg (n = 1). The remaining 6 brains therefore were all (bar one side) lesioned with a concentration of 0.09M and showed at least on one side a lesion that covered the PPTg fully along the rostro-caudal axis and the second side was at a minimum partially to fully lesioned Figure 3.2.). The single side lesioned with 0.10M ibotenate was not distinguishable from the others.

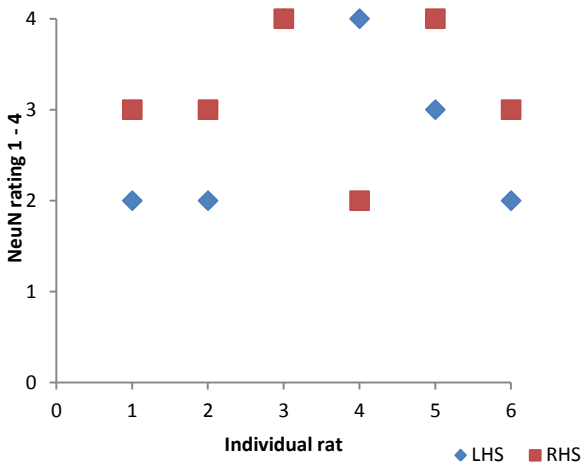

Figure 3.2. Lesion size for each rat of the PPTg lesion group rated based on NeuN+/Cresyl stained brain sections. Lesions were rated on a scale from 0 to 4 (0 = no lesion of the PPTg, 1 = small/ partial lesion of the PPTg, 2 = partial to full lesion of the PPTg, 3 = fully lesioned PPTg, 4 = extensive lesion covering the PPTg and surrounding tissue). For rat 3 LHS and RHS both scored a 4 (square covering diamond).

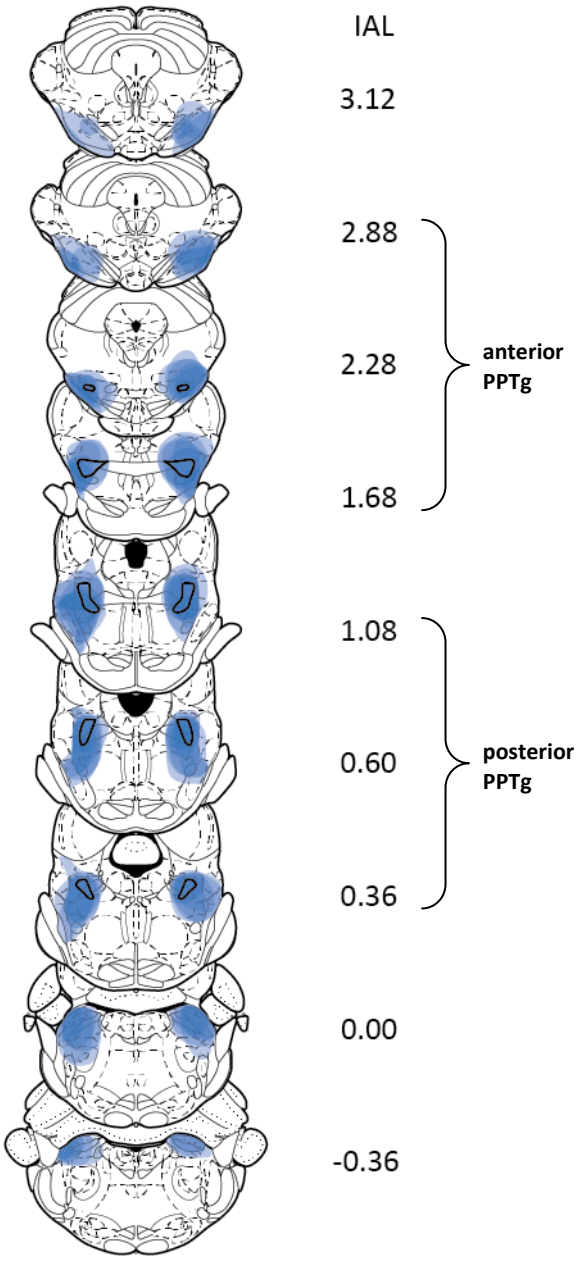

Figure 3.3. Representation of lesion extension along the rostro-caudal axis after ibotenic acid infusion on a selection of NeuN+/Cresyl stained sections. The position of the schematics (adapted from Paxinos and Watson, 2005) on the rostro-caudal axis is represented as the distance from the interaural line (IAL; mm). Sections IAL + 2.88 mm through to IAL + 1.68 mm were identified as sections containing anterior PPTg neurons (identified by PPTg cholinergic neurons in shams and partially lesioned rats) and those from IAL + 1.08 mm to IAL + 0.36 mm represent sections containing posterior PPTg.

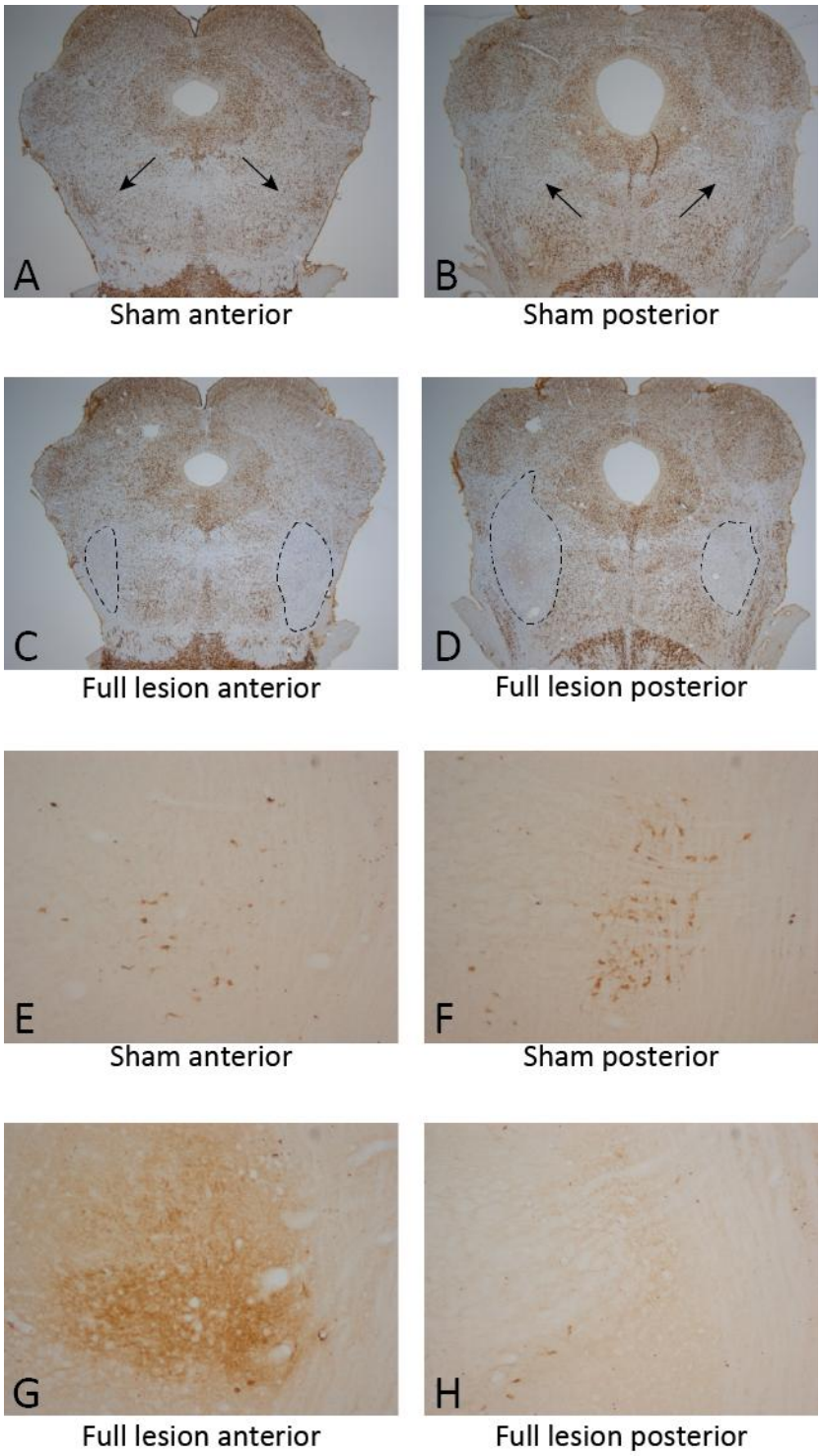

Figure 3.4. Representative histological photomicrographs of lesions in anterior and posterior PPTg of rats bearing full lesions **(C,D,G,H)** and sham controls **(A,B,E,F)**. **A-D**: NeuN/Cresyl stained; **E-H**: ChAT stained.

Of the remaining group of n = 6 rats with bilateral PPTg lesions a mean of 69% of ChAT+ PPTg neurons were destroyed (range 50% to 93%). Of those rats 1 unilateral lesion was caused by 0.1M ibotenate (instead of the 0.09M ibotenate used for all other rats) but the second side of the same animal by 0.09M ibotenate. Despite this difference the rat was included into the behavioural analysis because histological analysis of the lesion size and ChAT+ count does not allow to differentiate this brain from the others (mean overall ChAT+ neuron loss = 70%; ChAT+ neuron loss on the side that received the differing toxin concentration = 78%). The percentage of surviving cholinergic PPTg neurons is shown in Figure 3.5.

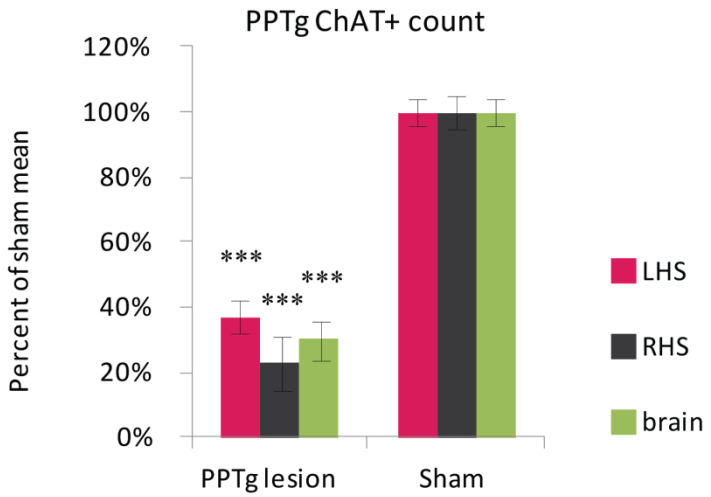

Figure 3.5. PPTg ChAT+ count as the percentage of the mean count in sham lesioned rats. *** indicates a significant difference between PPTg lesion group and shams with $p \leq 0.001$; LHS = left-hand side; RHS = right-hand side; brain = overall cell count left and right combined.

Univariate ANOVAs of the raw cell count of surviving ChAT+ neurons confirm that the lesion group had significantly fewer ChAT+ neurons than sham controls on the left-hand side ($F_{1,8} = 65.939$ $p < 0.001$; $\eta^2_p = 0.892$), right-hand side ($F_{1,8} = 42.393$ $p < 0.001$; $\eta^2_p = 0.841$) and accordingly when combining both sides to give an overall ChAT+ count ($F_{1,8} = 71.670$ $p < 0.001$; $\eta^2_p = 0.900$).

3.3. Behavioural results

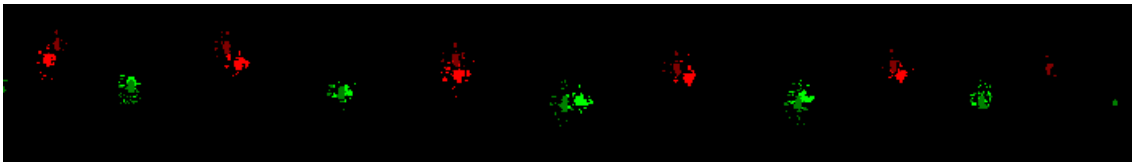

Figure 3.6. Illustration of a rat's footfall on the CW during pre-surgical baseline testing. The animal is crossing the walkway from the right to the left-hand side. Paws of the right-hand side are in green, paws of the left-hand side in red. Darker shades depict the hindpaws.

Based on the debate about whether or not PPTg lesions can cause motor problems as described in the introduction to this experiment, the focus here was on a possible deficit on individual gait parameters after PPTg lesions. In a long series of experiments no general locomotor deficits could be shown in rodents bearing PPTg lesions. However, effects such as hypo- and akinetic states and postural disturbances after PPTg lesions were described elsewhere. Consequently, the question here is whether deficits are detectable in PPTg lesioned rats on a more detailed level such as in individual gait parameters. In order to assess different aspects of gait – gait stability, speed, stride and coordination – the data were analysed individually and presented as such in the following. Normality (as examined with the Shapiro-Wilk test) was given on most of the test days. On rare occasions the data did not follow a normal distribution. Given this infrequence these seldom violations were tolerated in the analyses reported below. For all analysed parameters a pre-existing difference between groups could be ruled out (see Supplementary Table 3.1. for details). Repeated measures of the post-surgery data for the lesion group and the sham group showed in general no day effect. On those few occasions where there was a significant effect of day Bonferroni corrected planned pairwise comparisons did not reveal any significant differences between the individual testing

days. Because there was no clear effect of day, it was regarded as acceptable to pool data over days. (Data not shown)

3.3.1. Gait stability

BOS – The mean values of BOS (and other parameters) are presented in Figure 3.7. This is the only parameter where a mixed ANOVA showed a significant interaction of condition (pre vs post surgery) and group ($F_{1,8}$ = 15.262 p = 0.005; η^2_p = 0.656) and a significant effect of surgery ($F_{1,8}$ = 10.258 p = 0.013; η^2_p = 0.562) on BOS of the front limbs but no effect of group ($F_{1,8}$ = 0.299 p = 0.600). Exploring the simple main effect of group on the post-surgery data confirms the absence of a group difference ($F_{1,8}$ = 2.224 p = 0.174). The effect of surgery lies in the sham group. Their stance was significantly narrower after surgery ($F_{1,3}$ = 13.98 p = 0.033; η^2_p = 0.823), a training effect which the lesion group did not show ($F_{1,5}$ = 0.445 p = 0.534). A narrower stance, shorter duty cycles, increased swing speed and longer strides were observed in shams on several occasions during this project (and in the preceding Master's project (Gut, 2009)) after a prolonged period of time when the rats had a lot of exposure to the CW. These changes are opposite to deficits displayed by parkinsonian rats and are therefore considered *improvements*. Regarding the hind limbs both groups showed a significantly narrower BOS after surgery (lesion group: $F_{1,5}$ = 32.148 p = 0.002; η^2_p = 0.865; sham group $F_{1,3}$ = 364.079 p < 0.001; η^2_p = 0.992); the groups did not differ from each other ($F_{1,8}$ = 0.144 p = 0.714).

Duty Cycle – a repeated measures ANOVA of the duty cycle of the hind limbs in the lesion group revealed a significant effect, showing that the duration of contact with the walkway decreased after surgery, representing an adaptation to the task rather than an effect of the lesion ($F_{1,5}$ = 9.099 p = 0.030; η^2_p = 0.645). However, there were no differences between the groups regarding neither the duty cycle of the front limbs ($F_{1,8}$ = 4.782 p = 0.060) nor the hind

limbs ($F_{1,8}$ = 0.223 p = 0.650). The post-surgery data of the lesion group of the hind limb data was not normally distributed (p = 0.019).

Support – The majority of the rats' runs were supported by 2 legs at any given time. This percentage of diagonal support was decreased in the lesion group after surgery ($F_{1,5}$ = 15.231 p = 0.011; η^2_p = 0.753), but the two groups did not differ significantly from each other ($F_{1,8}$ = 0.003 p = 0.955). Similarly, the percentage of moments during a run when the rat was supported by 3 legs simultaneously was the same in both groups ($F_{1,8}$ = 1.870 p = 0.209) and in neither of the groups was this affected by surgery (lesion group: $F_{1,5}$ = 4.544 p = 0.086; sham group: $F_{1,3}$ = 2.704 p = 0.199). An additional analysis of the use of 4 paws for support shows that the change observed in the lesion group regarding the diagonal support did not mean a worsening of gait – that is an increased need of support using more than 2 legs. Here, likewise, the groups did not differ from each other ($F_{1,8}$ = 0.640 p = 0.447) nor were any of the groups affected by surgery (lesion group: $F_{1,5}$ = 1.960 p = 0.220).

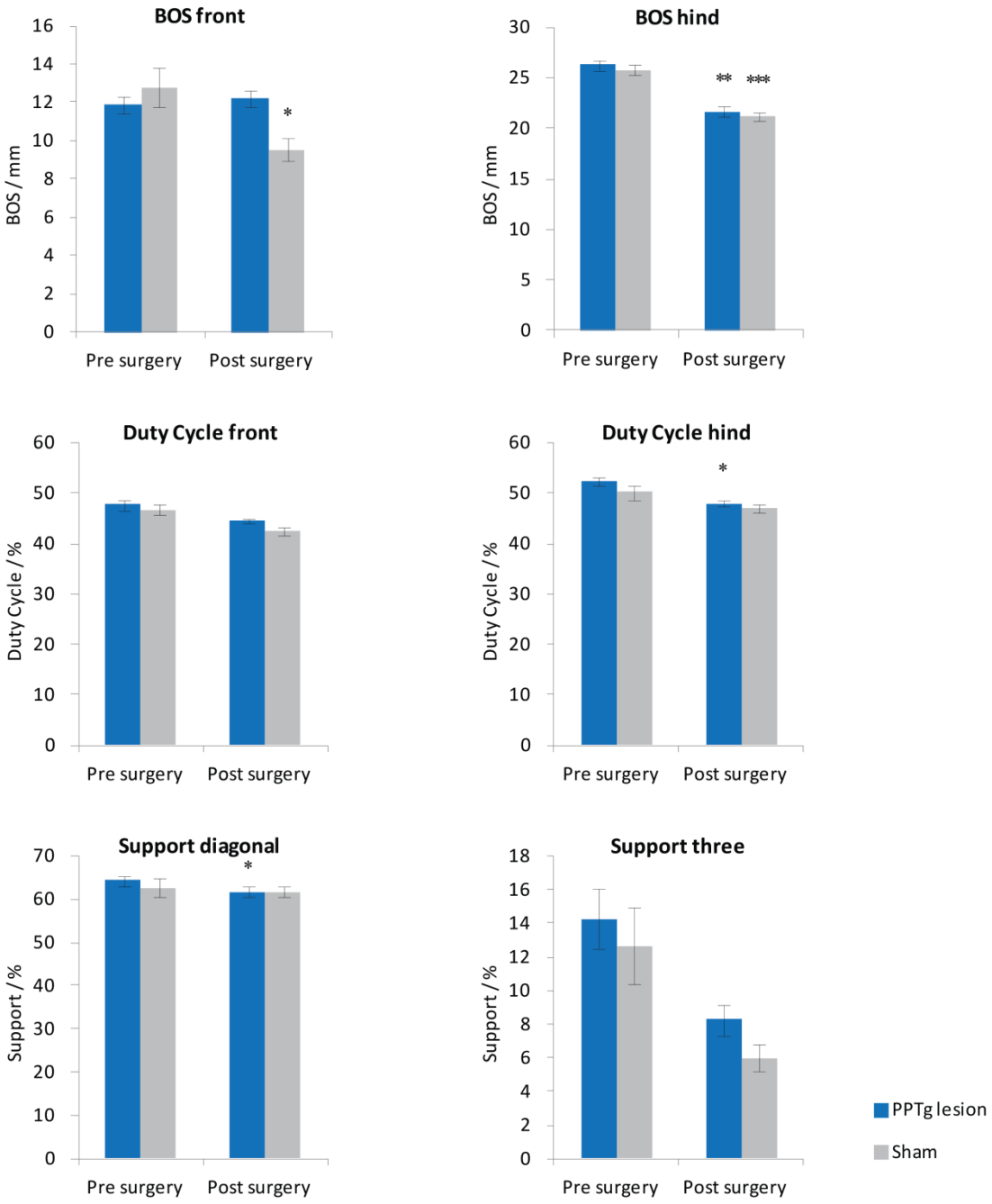

Figure 3.7. Performance on the CW – Parameters reflecting gait stability. Graphs show group means ± SEM of selected gait parameters; *** indicates a significant difference compared to pre surgery baseline with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$. The changes in gait in both, lesion and sham group, indicate an improvement rather than a worsening of gait as the changes were opposite to what is observed in parkinsonian rats. Please refer to the text for details.

3.3.2. Speed

Swing Speed – Repeated measures ANOVAs of the Swing Speed of front and hind limbs confirmed a slight reduction of the velocity of the legs during the swing phase in the lesion group (front: $F_{1,5}$ = 6.707 p = 0.049; η^2_p = 0.573; hind: $F_{1,5}$ = 6.660 p = 0.049; η^2_p = 0.571). However, this reduction was not enough to cause the groups to differ significantly from each other (front: $F_{1,8}$ = 2.938 p = 0.125; hind: $F_{1,8}$ = 2.745 p = 0.136).

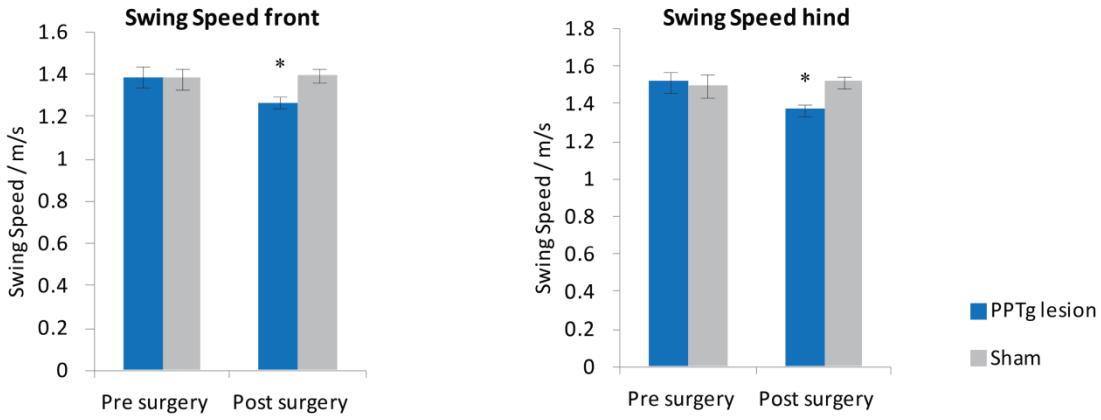

Figure 3.8. Performance on the CW – Swing Speed. Graphs show group means ± SEM of selected gait parameters; * indicates a significant difference compared to pre surgery baseline with p ≤ 0.05. Please refer to the text for details.

3.3.3. Stride

Stride Length – Both groups showed an inceased stride length after surgery, another training effect, which, however, was only significant in the front limb data of the lesion group ($F_{1,5}$ = 7.476 p = 0.041; η^2_p = 0.599). But most importantly lesion group and sham group did not significantly differ from each other (front: $F_{1,8}$ = 3.606 p = 0.094; hind: $F_{1,8}$ = 2.233 p = 0.173).

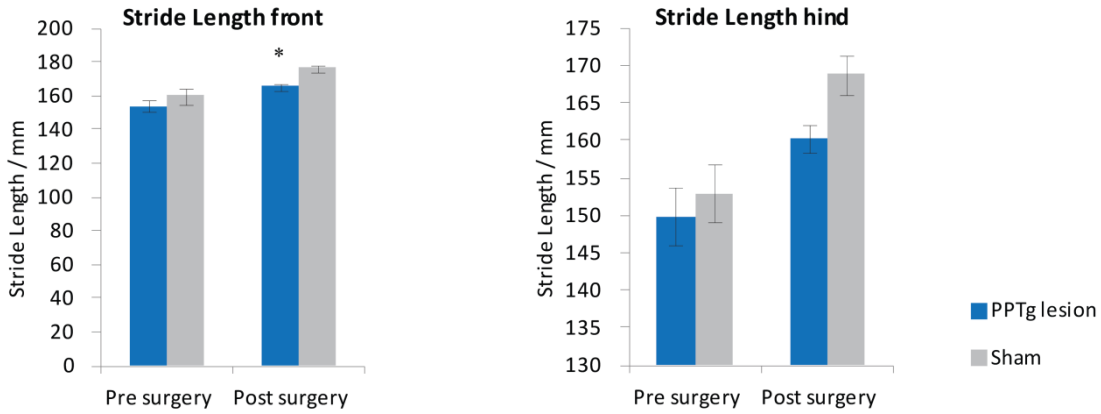

Figure 3.9. Performance on the CW – Stride Length. Graphs show group means ± SEM of selected gait parameters; * indicates a significant difference compared to pre surgery baseline with $p \leq 0.05$. Please refer to the text for details.

3.3.4. Coordination

Print position – The negative values of print position show that pre as well as post surgery, the rats placed their hind paws in front of the front paws during their runs. This distance between ipsilateral front and hind paws was further increased after surgery. Accordingly, repeated measures ANOVAs showed significant differences between pre and post surgery data of the left Print Position in the lesion and sham group (lesion group: $F_{1,5} = 11.701$ $p = 0.019$; $\eta^2_p = 0.701$; sham group: $F_{1,3} = 11.260$ $p = 0.044$; $\eta^2_p = 0.790$) and in the lesion group on the right hand side – the change in the sham group did not reach significance (lesion group: $F_{1,5} = 10.911$ $p = 0.021$; 0.686; sham group: $F_{1,3} = 5.687$ $p = 0.097$). Given the similar development of the Print Position in both groups post surgery the groups did not differ from each other on either side (left: $F_{1,8} = 4.619$ $p = 0.064$; right: $F_{1,8} = 0.226$ $p = 0.648$).

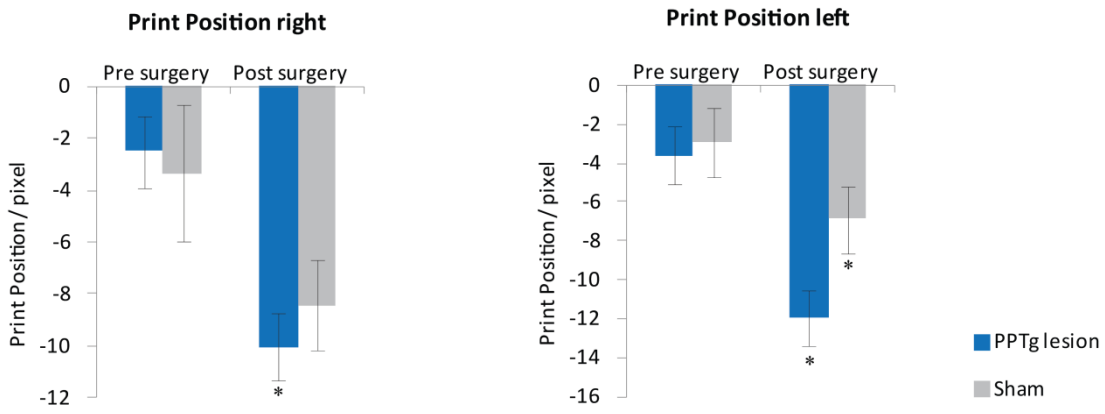

Figure 3.10. Performance on the CW – Parameter reflecting coordination. Graphs show group means ± SEM of selected gait parameters; * indicates a significant difference compared to pre surgery baseline with $p \leq 0.05$. Please refer to the text for details.

4. Discussion

In accordance with the experience of this and other labs the results show that complete and near-to-complete lesions of the PPTg do not cause gait deficits. So far it had been shown that similar lesions have no effect on more general motor behaviour as observed in the homecage, in locomotor test boxes or when performing tasks in the radial maze or operant boxes. Here it has been shown that this extends to individual gait parameters. It is important to make this addition given the association made by severals of gait deficits in PD and the PPTg.

The animals' gait did not differ in the individual parameters reflecting stability, speed, stride and coordination compared to sham lesioned animals. In some parameters changes could be observed compared to the pre surgery gait. These changes however do not reflect gait deficits as would be expected in parkinsonian animals or in fact PD patients (shortened stride, slower movement, increased stand) (Rodriguez-Oroz *et al.*, 2009). On the contrary, they show *improvement* in those parameters. This needs to be interpreted as a training effect that the animals show when becoming more familiar with the CW. The same effect had already been

observed previously in sham operated rats and even in parkinsonian rats when tested on the CW for more than 5 weeks (Gut, 2009). An extensive training before the first data collection before surgery and a re-training day after surgery were scheduled to counteract against this training effect, but it is clearly still apparent. Where this effect occurred it did so in both groups however, and on some occasions did not reach significance. However, on these occasions this was not indicative of group differences: in fact, the groups did not differ significantly from each other in any of the tested parameters.

In all animals the lesions were extensive. Additional damage was found anterior to the PPTg extending into SN and/or posterior to the last section of the PPTg, invading the area in and around the lateral and medial parabrachial nucleus. Nevertheless these extensive lesions did not have an effect on the quantitative metrics of gait – contrary to the view that the PPTg is a critical componant of locomotor circuitry and in contrast to the reported effect of extensive lesions of the PPTg and surrounding areas in monkeys.

In the General Introduction plausible explanations have been given to explain the notable differences between PPTg lesion studies in non-human primates and rodents in relation to motor behaviour. In a more recent paper the authors present data from PPTg lesioned macaques (without dopaminergic depletion) which displayed gait deficits in form of decreased step length and speed, postural changes, as well as rigidity in the extremities without affecting the global motor behaviour as observed in the home cage (Karachi *et al.*, 2010). The authors describe those PPTg lesions as cholinergic lesions because they used the selective urotensin II – conjugated diphtheria toxin (Clark *et al.*, 2007). However, the same concentration that has been used successfully in this lab to produce selective cholinergic lesions of the PPTg in rodents had no effect on gait in the macaque study. Only a concentration ten times stronger than that produced the reported effects. It is therefore very likely that the lesions made with this concentration were not selective but also destroyed other cell types in the PPTg and

surrounding area (as was observed in rodents with increasing concentrations of this toxin). Non-selective lesions would however be more comparable to the lesions produced in this rodent experiment. It is being argued that the organisation of the PPTg and its projections is different in rats compared to primates, one argument being the difference between the quadrupedal locomotion of rodents compared to the bipedal gait of monkeys (Alam *et al.*, 2011). Given the remarkable preservation of the PPTg through evolution with very similar structure and pattern of connections in all studied species from teleost fish to human [for reviews see (Martinez-Gonzalez *et al.*, 2011; Winn, 2008) this argument does not appear strong enough to explain such fundamental differences. However, despite a well preserved anatomical structure, interspecies differences have been pointed out with respect to the connectivity between the PPTg and different BG nuclei (Alam *et al.*, 2011). In this regard the rat PPTg receives mostly BG output from SN (Steininger *et al.*, 1992) whereas in monkeys the PPTg receives most afferents from the GPm (DeVito and Anderson, 1982). Entopeduncular efferents in the rat only target a smaller area of the PPTg, if at all (Steininger *et al.*, 1992). Afferent and efferent projections of SNr in return are more differentiated in the monkey than in the rat (Beckstead and Frankfurter, 1982). Nevertheless it remains unknown whether these differences can account for the apparently conflicting results found in lesion studies in rats and monkeys.

The PPTg clearly has motor properties but these are not separable from its sensory and attentional properties and its role in reinforcement processes, which the literature has shown (i) anatomically and (ii) functionally: (i) The functional heterogeneity is reflected in its anatomical/neurochemical, electrophysiological heterogeneity and its widespread connectivity with a great variety of structures [see General Introduction and (Alderson *et al.*, 2008; Ros *et al.*, 2010)]. Martinez-Gonzalez et al have shown that ascending and descending motor connections of the PPTg arise largely from separate, though intermingled, neurons of different

neurochemical compositions (Martinez-Gonzalez *et al.*, 2013). Given the different target sites of PPTg projections from individual cell groups within the PPTg and their possibility to contribute to the activation of different pathways, PPTg motor output has to be considered as highly differential making it inadvisable to speak about its gross effect in terms of activation or inhibition. This is in line with different degrees of change in the activity of the PPTg in PD models (Aravamuthan *et al.*, 2008; Breit *et al.*, 2001; Carlson *et al.*, 1999; Mitchell *et al.*, 1989; Orieux *et al.*, 2000). And this can potentially provide a further explanation why different lesion studies of the PPTg show different behavioural results. In none of the studies the lesion techniques cause a 100% depletion of all cell types of the PPTg, nor are the proportions of surviving neuronal types identical across those different experiments. In this experiment an average of 29.4% of cholinergic neurons remained intact. Even though the exact degree of depletion of the other cell types has not been established, the NeuN stain suggests a much higher degree of cell death compared to cholinergic neurons. In the study conducted by Karachi and colleagues they report a survival of cholinergic neurons of 61% (Karachi *et al.*, 2010). It is not implausible that the survival of different cell groups and types might contribute to the different results observed. In an earlier study by Dunbar and colleagues (Dunbar *et al.*, 1992) the use of two different excitotoxins, ibotenic acid and quinolinate, caused different lesions in respect to the cholinergic and non-cholinergic parts of the PPTg in rats, resulting in functional differences on behavioural level as well.

(ii) Functionally, and in further support of the findings of this experiment, electrophysiological data relate PPTg firing patterns to conditioned responses and reward related processes rather than to movement [cat: (Dormont *et al.*, 1998); primate: (Kobayashi and Okada, 2007; Okada *et al.*, 2009)], which fits very well with findings on the behavioural level showing the role of the PPTg in the process of learning [for example (Alderson *et al.*, 2002; Inglis, Allen*, et al.*, 1994; Inglis, Dunbar*, et al.*, 1994; Keating *et al.*, 2002; Keating and Winn, 2002)] specifically in

establishing of associations between actions and outcomes (Maclaren *et al.*, 2013; Thompson and Felsen, 2013; D. I. G. Wilson *et al.*, 2009). Kobayashi and colleagues have shown repeatedly in primates that patterns and rates of PPTg neuron firing are not just related to movements the macaques had to perform (visual saccades) in a reward task. Different populations of PPTg neurons responded to reward predicting stimuli and their delivery, mirroring further the expected reward value (Kobayashi and Okada, 2007; Okada *et al.*, 2009). Tonic increase and decrease during the task execution correlated with their response magnitude to larger or smaller reward cues (Okada and Kobayashi, 2013). Likewise when mice had to make a left or right-orientating movement following an odour cue in order to receive a reward, PPTg neuron activity was related to the direction selection and there were also overlapping populations firing in relation to movement direction and reward outcome (Thompson and Felsen, 2013). PPTg neurons have the ability to pass information onto systems more directly involved in the selection of actions by providing midbrain DA neurons with rapid information about incoming sensory stimuli. This happens fast enough [sensory response latencies between 4 – 80ms (Dormont *et al.*, 1998; Pan and Hyland, 2005)] to assume that it informs DA neurons about external sensory events [which have a response latency of 70-100 ms after stimulus presentation (Redgrave *et al.*, 2008)]. By influencing midbrain DA neurons and activity in corticostriatal pathways it assumes a role in the construction of goal-directed actions/movements. The BG have been proposed to be the seat of the ancient problem of selecting an action among behavioural alternatives (Redgrave *et al.*, 1999) and the PPTg needs to be understood as a part of this action selection process at a different, *lower*, level of the neuroaxis, interacting with the BG to make a decision on a specific movement. This highlights that the PPTg is not proposed to be non-involved in movement, but it stresses the nature of the involvement in movement – one that involves the process of selection. For general

locomotor behaviour, exploration, grooming, feeding, walking down the CW, the PPTg is not necessary. Chapter 5 discusses the differential role of anterior and posterior PPTg.

In conclusion, the data provide evidence that extensive PPTg lesions in rats do not cause gait deficits which agrees with the lack of locomotor deficits in earlier PPTg lesion studies but is difficult to reconcile with some lesion studies conducted in primates. When investigating the role of the PPTg in PD it needs to be understood within its strategic position and close relation with the BG, thalamus and cortex and equally with the rest of the brainstem and spinal cord. It needs to be taken into account that the functional relations between these structures change in the absence of normal DA availability in PD, that the firing activity of the PPTg itself changes as a consequence and that in addition the PPTg is also affected from degeneration of its neuronal population. For this reason the next step was to add partial PPTg lesions to the existing PD rodent model and investigate the effect of such lesions on gait.

Chapter 4

Development and examination of a refined model of Parkinson's disease

1. Introduction

1.1. A role of the PPTg in PD

Parkinson's disease has a broad spectrum of symptoms. Not all of these are motor symptoms and of those motor symptoms, not all respond well to DA replacement therapy. In more advanced stages, some PD patients show akinesia, gait deficits and postural impairments that are refractory to DA medication (Chastan *et al.*, 2009). Degeneration of PPTg neurons in PD – and in other pathological conditions that lead to parkinsonian-like symptoms; changes of PPTg functioning in PD models and PD patients; the anatomical position of PPTg and its involvement in BG functioning; as well as data from animal studies suggesting a role of PPTg in posture and gait (for more details please refer to the General Introduction) – all these facts strongly indicate an involvement of the PPTg in PD (Pahapill and Lozano, 2000).

1.2. Identification of the extent of PPTg degeneration

Loss of PPTg cholinergic neurons was shown many years ago (Hirsch *et al.*, 1987; Jellinger, 1988; Zweig *et al.*, 1989). Rinne and colleagues (Rinne *et al.*, 2008) specified in their study an average loss of cholinergic neurons similar to that reported by Zweig and colleagues (36%). Rinne and colleagues showed that in addition to cholinergic cell loss, non-cholinergic neuron number was also reduced by 23% and the overall reduction in the numbers of all neurons in the PPTg along the rostrocaudal axis was reported to be 27%. Cell degeneration and atrophy was found in surviving neurons, showing a 14% (all neurons) to 26% (cholinergic neurons only) decrease in cell size. In Parkinsonism the DA loss leads to an overactive inhibitory output from the BG output nuclei SNr and GPi onto the thalamus and PPTg. Increased metabolic activity in the PPTg has been interpreted as an indicator for this inhibitory input in primates and rats (Carlson *et al.*, 1999; Mitchell *et al.*, 1989). More apparently decreased cytochrome oxidase in MPTP treated monkeys (Gomez-Gallego *et al.*, 2007), decreased fluoro-2-DG in a rodent PET

study (Jang *et al.*, 2012) and decreased firing activity in parkinsonian rats (Florio *et al.*, 2007) indicate hypoactivity in the PPTg. On the other hand PPTg hyperactivity has been shown as well using extracellular recordings (Breit *et al.*, 2001; Breit *et al.*, 2005; Zhang *et al.*, 2008) and expression of cytochrome oxidase messenger RNA (Orieux *et al.*, 2000) in parkinsonian rats, a possible consequence of an overactive STN projecting onto the PPTg. Since different neuronal populations within the PPTg receive different afferents (Martinez-Gonzalez *et al.*, 2011) it is plausible to think that these populations can show different activities.

1.3. Need for a refinement of the existing model of PD

Lesion studies in rodents have consistently shown that the PPTg is not necessary for the generation of locomotion and the previous chapter has proven that this is also true for the generation of gait. Nevertheless, in particular the literature on PPTg-DBS in PD patients hypothesises that the functional disturbance and degeneration of the PPTg plays a role in the development of gait and postural dysfunctions in, especially those symptoms refractory to DA medication. While animal models bearing extensive PPTg lesions were described to show akinetic symptoms, Karachi and colleagues showed gait and postural deficits in macaques bearing DTx-UII-induced lesions in the PPTg. This group very recently described that additional PPTg lesions to MPTP-induced DA depletion caused an increase of gait deficits which did not respond to DA medication, in contrast to the hypokinetic symptoms that were observed before. This is the only study that investigates the effect of a combination of DA depletion and PPTg lesion on motor symptoms. In an earlier study DA depletion was induced by MPTP after lesioning of the PPTg, with the effect that hardly any parkinsonian symptoms developed (Matsumura and Kojima, 2001). Apart from this study, PPTg lesions were only added to nigral DA cell loss to study the electrophysiological effect on the BG circuit in rats (Breit *et al.*, 2006; Yan *et al.*, 2008). Given the clear indications for PPTg involvement in PD and especially in the search for a treatment option that puts the PPTg at the centre of attention, an animal model is

required that mimics closer the pathophysiological state found in PD patients by taking into account PPTg degeneration.

1.4. Decision on the model

An ideal model for PD needs to prove face validity – similarities in symptoms and underlying pathophysiology – and ideally a high degree of construct validity – similarities in the pathogenesis of the disease.

The most common animal models of PD are achieved by intracerebral injections of 6-OHDA, a catecholamine cell-specific neurotoxin, in rats and systemic administration of MPTP in primates and mice (Burns *et al.*, 1983; Sundstrom *et al.*, 1990; Ungerstedt, 1968). The 6-OHDA model shows considerable construct validity: while the exact mechanisms of DA neuron degeneration caused by this toxin are still being investigated it is known that 6-OHDA initiates degeneration through a combination of oxidative stress, mitochondrial respiratory dysfunction by acting on complex I and IV of the mitochondrial respiratory chain causing respiratory inhibition, and inflammation [for more details please refer to (Duty and Jenner, 2011)]. For this study face validity is of great importance. In rats, nigrostriatal lesions made by infusions of 6-OHDA, cause akinesia, rigidity and catalepsy, impairments in skilled paw use such as reaching and compensatory stepping (Jeyasingham *et al.*, 2001; Klein *et al.*, 2007; Lee *et al.*, 1996). Depending on the dose and injection site, moderate to severe degrees of DA neuron loss in the SN can be achieved, which remain higher to DA cell loss in the neighbouring VTA, with associated striatal DA depletion. Further similarities include changes in neuronal activities in the BG and related structures, such as an increased firing of the STN (Benazzouz *et al.*, 2002; Breit *et al.*, 2006) and overactive BG nuclei [GPi and SNr (Blandini *et al.*, 2000; Mitchell *et al.*, 1989)]. However, the use of 6-OHDA alone does not cause other pathological changes outside

the nigrostriatal system, which makes a refinement of the model necessary to include further pathological features, such as PPTg degeneration.

The choice of the optimal technique for the creation of the 6-OHDA model, which serves as a basis for the new model, is crucial. This model shows marked variability in the size of lesion and behavioural effects depending on the use of the toxin.

The literature shows that in order to achieve consistent and visible behavioural outcomes of 6-OHDA lesions it requires a substantial depletion of DA neurons. Partial lesions have proven to be less reliable. Thus, with lesions of below 80% destruction, animals did not show robust deficits in the cylinder-reaching test (Hefti *et al.*, 1980). It needs close to complete lesions for PD models to display deficits in adjusted stepping tests, forelimb placement tests (Kirik *et al.*, 1998; Olsson *et al.*, 1995). Paw preference in the cylinder-rearing test occurs in rats with about 90% lesions (Schallert *et al.*, 2000). This translate to PD gait deficits: in a previous experiment (Gut, 2009) it was shown that rats which received a high dose of 6-OHDA showed more robust and longer lasting deficits as measured on the CW than rats which received a lower dose of the same toxin (injected into the same striatal sites). Apart from the dose, Kirik and colleagues have shown that functional effects further depend on the site of infusion (Kirik *et al.*, 1998). Infusions can be made (a) into the SN directly, though this adds mechanical damage to the tissue caused by the infusion cannula and further increases the risk of a higher DA cell loss in the neighbouring VTA; or (b) into the MFB, which causes a rapid and complete lesion in the nigrostriatal pathway, but also the mesoaccumbens pathway; or (c) into the striatum, which causes a more progressive retrograde damage to the SN, the extent of which can be controlled by infusion technique and dose. While the dorsomedial parts of the striatum are reported to have more general effects on locomotion, the lateral parts have more pronounced effects on movement initiation, sensorimotor orientation, and skilled motor behaviour (Kirik *et al.*, 1998).

It is the lateral part of the caudate-putamen complex in rodents which corresponds to the human putamen, the striatal subregion with the most profound DA depletion in PD patients. Assessing the effect of 1, 2, 3 and 4 injection sites Kirik and colleagues found that rats that received 6-OHDA injections distributed over 4 sites of the lateral striatum along the rostro-caudal axis showed more pronounced deficits in forelimb stepping and skilled paw use than rats that received the same amount of toxin into fewer injection sites.

Many 6-OHDA-induced PD models are hemiparkinsonian, which allows for various tests of lateralised function including rotation in response to amphetamine or apomorphine (Cenci and Bjorklund, 1993; Metz *et al.*, 2005) or the cylinder paw preference test. Bilateral nigrostriatal lesions result in marked adipsia and aphagia, making increased care in the form of hand-feeding and monitoring water intake and body weight necessary (Sakai and Gash, 1994). In PD, degeneration of brain structures is not limited to one side, although the degeneration is not necessarily equally distributed (Brooks, 2010). For this reason, and especially when aiming to assess deficits that effect the whole body and not just one side, for this study it was necessary to use a bilateral model. Further, Roedter and colleagues have shown that the same amount of toxin injected bilaterally instead of unilaterally causes additional behavioural deficits in general locomotor activity and explorative behaviour, and also skilled forelimb use, indicating compensatory mechanisms of the unlesioned side, which is lost with bilateral lesions (Roedter *et al.*, 2001). A pilot study testing two unilaterally lesioned rats (using the same surgical techniques, toxin and dose as described below in the methods for the main experiment) on the CW confirmed the need for a bilateral model: the gait of those hemiparkinsonian rats did not differ from sham lesioned rats (see Table 4.1.).

Table 4.1. Summary of the effect of unilateral 6-OHDA lesions in comparison to sham lesion rats. Data show group means. Please note that the data derive from a pilot study using just two hemiparkinsonian rats. Hence, no SEM are presented. Data from sham lesion rats derive from the experiment described below.

	BOS		Duty Cycle		Support	
Post surgery	front	hind	front	hind	diagonal	three
Unilateral 6-OHDA lesion (N = 2)	10.95	26.33	46.41	52.66	58.45	15.03
Sham lesion (N = 8)	10.34	22.06	43.83	50.27	61.66	9.93

	Swing Speed		Stride Length		Print Position	
Post surgery	front	hind	front	hind	right	left
Unilateral 6-OHDA lesion (N = 2)	1.21	1.39	159.12	155.09	-2.98	-10.75
Sham lesion (N = 8)	1.26	1.42	160.82	154.74	-7.67	-8.07

1.5. Aim of this experiment

With the intention to subsequently test the effect of PPTg stimulation on gait parameters, this experiment aimed to refine the existing traditional 6-OHDA model and examine the differences in gait of parkinsonian rats with and without partial PPTg lesions. Based on the literature reviewed above it was decided to use a bilateral 6-OHDA model made by infusion of the toxin at four striatal sites in order to create a stable lesion of sufficient magnitude to cause a robust behavioural phenotype with consistent and long-lasting deficits. Partial PPTg lesions were planned to be made by microinfusions of ibotenic acid into 2 sites of the PPTg, as has been done routinely before (Bortolanza *et al.*, 2010; Walker and Winn, 2007; D. I. Wilson *et al.*, 2009). This, however, required a reduction of the toxin concentration, because the previous purpose of ibotenic acid infusion was to cause complete lesions of the PPTg. The concentration of the toxin was determined in pilot surgeries preceding the main experiment.

Furthermore, in accordance with the hypothesis that a lack or reduction of afferent stimulation of nigral DA neurons due to PPTg degeneration might contribute to DA degeneration, DA cell counts were examined in animals bearing partial PPTg lesions. Moreover, it is also hypothesised that a lack of functioning target neurons and altered afferent input from the BG might contribute to PPTg cell damage. Hence, cholinergic PPTg neuron counts in 6-OHDA treated rats were compared to sham operated animals.

2. Pilot 1: Determination of ibotenic acid concentration for partial lesions

2.1. Methods

Eight male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) with a mean pre-surgery weight of 346g (range 336g – 362g) underwent bilateral surgeries for partial PPTg lesions following the protocol described in the General Methods and below in the description of the main experiment. Four rats received 200 nl of ibotenic acid per infusion site (two per side) of a concentration of 0.04M and 4 the same volume of 0.06M. After the second surgery, the rats were perfused, coronal 30 µm sections were cut in a series of 1:4 of the PPTg area and stained for ChAT and NeuN (counterstained with cresyl violet) and assessed under a light microscope.

2.2. Characterisation of lesions

One rat died during recovery after the first ibotenate infusion. Hence, n = 3 rats constituted the final group lesioned with 0.06M ibotenic acid, n = 4 rats were lesioned with 0.04M ibotenic acid.

Figure 4.1. shows representative photomicrographs of lesions after 0.04M and 0.06M in anterior and posterior PPTg. Cell counts of ChAT+ neurons of the PPTg (of 6 brains; 1 brain of the 0.04M groups had to be excluded from cell counts because the sections were damaged) – averaged per section – are presented and compared in Figure 4.2. Univariate ANOVA of the

averaged cell count per section of surviving ChAT+ neurons revealed that the groups did not differ from each other ($F_{1,4}$ = 0.082 p = 0.789).

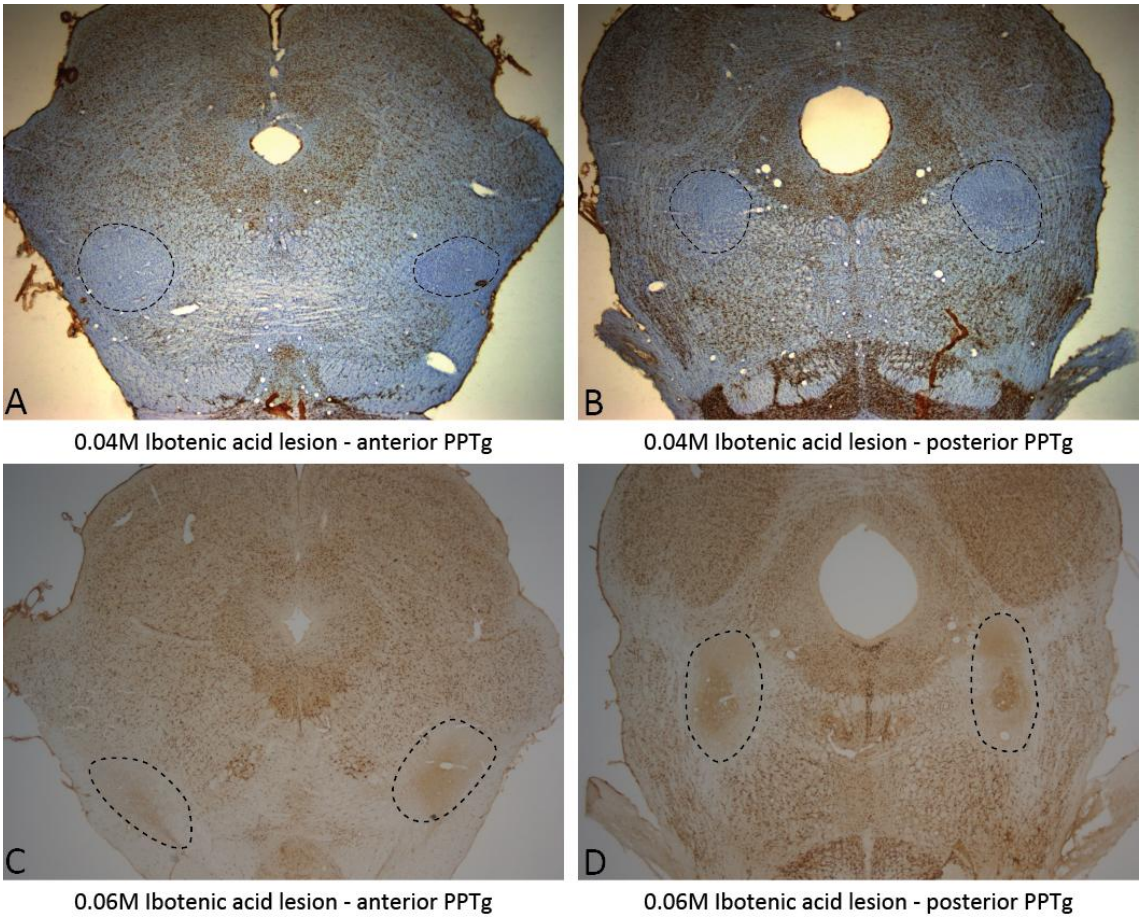

Figure 4.1. Example sections of a PPTg lesion caused by 0.04M ibotenic acid **(A and B)** and of a PPTg lesion cause by 0.06M ibotenic acid **(C and D)**. Photomicrographs show NeuN/Cresyl stained sections of anterior and posterior PPTg.

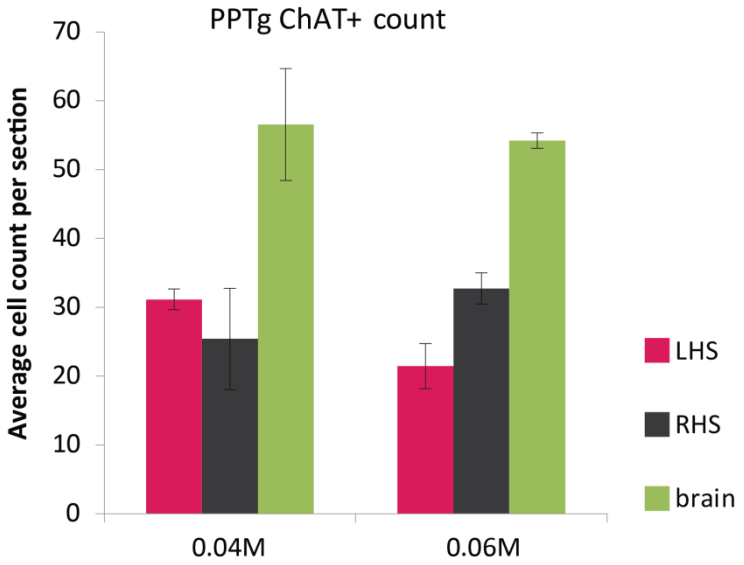

Figure 4.2. PPTg ChAT+ count averaged per section. Graph shows group means ± SEM. 0.04M and 0.06M group did not differ significantly from each other. LHS = left-hand side; RHS = right-hand side; brain = overall cell count left and right combined.

2.3. Conclusion

After visually inspecting the extent of lesions on the NeuN/Cresyl stain, and given the fact that the use of the slightly higher concentration of ibotenic acid did not cause a higher loss of cholinergic neurons, the decision was made to use a concentration of 0.06M of ibotenic acid in future experiments to create partial lesions that were to be added to DA depletion of SN. Lesions caused by 0.04M were smaller, to an extent that the risk was higher to miss the PPTg or not cause a big enough lesion that would have functional effect. In view of the complexity of the refined PD model it was intended to create – involving three surgeries per animal – and the risk that the toxin itself poses to the survival of the rat, it appeared advisable to minimise the risk of having to exclude rats from analysis due to unsatisfactory histological results.

3. Main experiment

3.1. Methods

3.1.1. Subjects

Forty-six adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used. Due to the time consuming care which those bilateral PD models require the experiment was split into 2 sets with 26 animals in the first set and 20 in the second. At the start of CW training the mean weight of the animals of the first set was 270g (range 247 – 289g) and of the second set 334g (range 310 – 363g). The animals were put on food control once they reached their target weight (described in the General Methods). All animals reached the target weight of 330g for the first surgery (1st set: mean weight 348g; range 335 – 359g; 2nd set: mean weight 335g; range 331 – 342g). The mean weights during pre-surgical baseline testing were 338g (range 280 – 352g) and 327g (range 319 – 336g) and a stable mean weight of 329g with a small range of 317 – 343g and 335g (range 329 – 343g) was maintained over the entire period of the experiment. For further details on housing please refer to the General Methods.

3.1.2. Experimental protocol

The experiment followed the timeline presented in Figure 4.3. In brief, the rats were trained on the CW for 10 days. All rats were proficient at that point to deliver acceptable runs on the CW. Over the following 4 days their pre-surgical performance was recorded. They then underwent 1, 2 or 3 consecutive surgeries, separated by a week, depending on the experimental group they belonged to. After a week of recovery the rats were then re-exposed to the CW and then tested for 7 days.

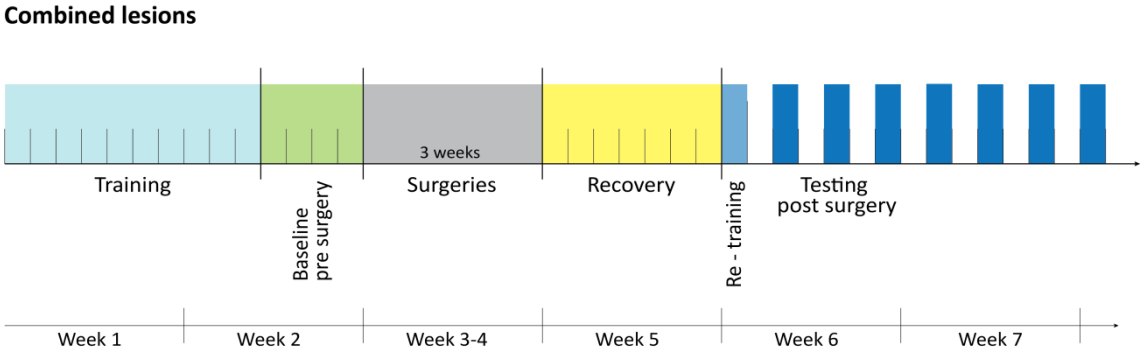

Figure 4.3. Experimental design. At the start of the experiment all animals were trained for 10 days on the Noldus CatWalk. They were then tested for 4 days to obtain a pre-surgical baseline. In order to comparing the traditional 6-OHDA model of Parkinson's disease to a refined model consisting of the same nigral DA depletion plus partial lesions of the PPTg, rats with partial PPTg lesions only and the corresponding sham controls, rats underwent 2 or 3 successive surgeries with 7 days in recovery in between. Seven days after the last surgery they were then re-trained. Post-surgical testing was performed every other day over 14 days.

3.1.3. Surgical procedures

Surgery followed the protocol as described in the General Methods. In brief:

Bilateral partial PPTg lesions

In two separate surgeries the PPTg was bilaterally lesioned by microinfusions of ibotenic acid into the anterior and posterior PPTg at the following coordinates (in mm): anterior PPTg: + 0.6 from IAL, ± 2.0 from midline and - 7.0 from dura; posterior PPTg: - 0.8 from IAL, ± 1.9 from midline and - 6.5 from dura. Infusions were always made anterior first but the order of infusions regarding the choice of side was counterbalanced. Rats were infused with 200 nl of 0.06M ibotenic acid per infusion site. Control rats received infusions of vehicle only (here: PB) into the same sites. At the end of surgery rats were treated with 1-1.5 ml of Hartmann's solution (i.p.) to account for the lack of fluid intake during the time of surgery. Furthermore,

they also received an i.p. injection of 0.2 ml Diazepam to reduce the risk of injury due to the immediate effect of the excitotoxin acting on the targeted brain stem sites.

Bilateral DA depletion of the SNc

Seven µg (free base weight) of 6-OHDA-hydrobromide dissolved in 2 µl of 0.1% L-ascorbic acid /0.9% saline were delivered at 4 sites into the striatum of both sides ot the brain at the following coordinates (in mm): (1) AP: + 1.3 (measured from Bregma), ML: ± 2.6 (measured from midline at the skull surface), DV: - 5.0 (measured from Dura); (2) AP: + 0.4, ML: ± 3.0 mm, DV: - 5.0; (3) AP: - 0.4, ML: ± 4.2, DV: - 5.0; (4) AP: - 1.3, ML: ± 4.5, DV: -5.0. Infusions were controlled by a Harvard microdrive pump. Control rats received infusions of vehicle only (here: 0.1% L-ascorbic acid /0.9% saline solution) into the same sites. Before being placed into a heated recovery cage rats were treated with 2-2.5 ml of Hartmann's solution (i.p.).

Combined lesions

Rats that received a combination of DA depletion and partial bilateral PPTg lesions underwent 3 separate surgeries. During the first 2 bilateral partial PPTg lesions were created as described above. Following a further 7 days of recovery the DA depleting procedure was performed.

3.1.4. Training and testing on the CW

Rats were trained for 10 days prior to baseline testing and surgery and tested as described in the General Methods. During the execution of the first set of the experiment inconsistencies of CW data during baseline testing required re-adjustments of software and hardware settings which delayed the data collection by a week. During this time the rats continued to be exposed daily to the CW. The order of rats trained and tested was randomised.

Gait parameters

The following gait parameters were examined: BOS, swing speed, stride length and duty cycle of both front and hind legs, print position of ipsilateral paws and the support formula of diagonal pairs of paws and three paws. For details on the gait parameters please see the General Methods.

3.1.5. Lesion assessment

The area of the PPTg and the SN were histologically assessed for overall neuronal damage and cholinergic cell loss and loss of dopaminergic neurons respectively following the methods as described in the General Methods. In brief, at the end of behavioural testing rats were transcardially perfused with a fixative, the brains removed and stored in sucrose solution until they sank. A series of 1:6 coronal 30 µm sections was cut on a freezing microtome.

PPTg

For PPTg assessment the corresponding sections were immunohistochemically stained for NeuN and ChAT, mounted on glass slides and examined under a light microscope. Overall neuronal damage was assessed visually on the NeuN/Cresyl stained sections and rated on a scale from 0 (no lesion) to 4 (extensive lesion). Lesions were accepted and rats included into the analysis when they received a rating of at least a 1 (small/partial lesion). Animals were excluded when both sides received a 4 (extensive lesion). Cholinergic cell survival was assessed by software assisted counts of ChAT+ neurons.

SNc

Sections corresponding to the SN were immunohistochemically double-stained for TH and Cb and similarly examined under a light microscope. All TH+ neurons within the dorsal and ventral tier of substantia nigra part compacta (SNc; SNcd and SNcv respectively), substantia nigra pars

medialis (SNm) and substantia nigra pars lateralis (SNl) were counted using a handheld cell counter.

3.1.6. CatWalk data analysis

In order to test the CW data for an effect of DA depletion on normal gait and to explore potential differences between the traditional and an refined model of PD mixed ANOVAs were performed across post-surgery testing days (within subject factor) and between groups (PPTg lesion group, combined lesion group, 6-OHDA group and sham controls, between subjects factor) and further examined for a simple main effect performing repeated measures ANOVAs for individual groups to compare the groups for an effect – or lack of it – of day on their performances. Preceding this, the data were assessed for pre-existing differences (mixed ANOVAs within pre surgical testing days and between groups). Normality of pre and post-surgery data was identified by the Shapiro-Wilk test and homogeneity of variance by Leven's test. The handling of violations of these assumptions is reported in the results section when they occurred. All tests were conducted using SPSS version 21 (IBM Corp., Armon, NY, USA) and results were considered statistically significant when $p \leq 0.05$.

3.2. Results

3.2.1. Histological analysis

The presented results were obtained from a total of 46 rats, of which 12 underwent surgery for bilateral partial PPTg lesions, 7 infusions of 6-OHDA, 17 underwent the 3 surgeries necessary for the combination of both lesion types and 8 received control infusions of vehicles in surgeries for either bilateral PPTg infusions (n = 3), infusions into the striatum (n = 2) and both (n = 3). Two animals were excluded before surgeries because they failed consistently to deliver acceptable runs on the CW by the time of baseline testing. One animal injured its paw

during the recovery stage after ibotenate infusion and had to be excluded as well. Due to a failed perfusion the resulting brain sections were too fragile to obtain a complete set of sections to analyse and one rat of the control group was not included in the histological analysis; however the available sections were inspected visually and no lesions were observable; therefore the behavioural data was considered for the other analyses

A total of 11 rats did not survive the recovery phase after infusion of ibotenate. After the loss of 4 rats in a row conducting the 2nd set of the experiment a new batch of toxin was used to exclude the possibility of problems with the toxin. Hence, n=7 rats constituted the final group of bilaterally PPTg lesioned rats, n = 7 rats were in 6-OHDA group (DA depleted rats), n = 7 rats formed the combined lesion group and n = 8 rats were in the control group. Figure 4.4. shows the lesions size ratings made on NeuN/Cresyl stained sections.

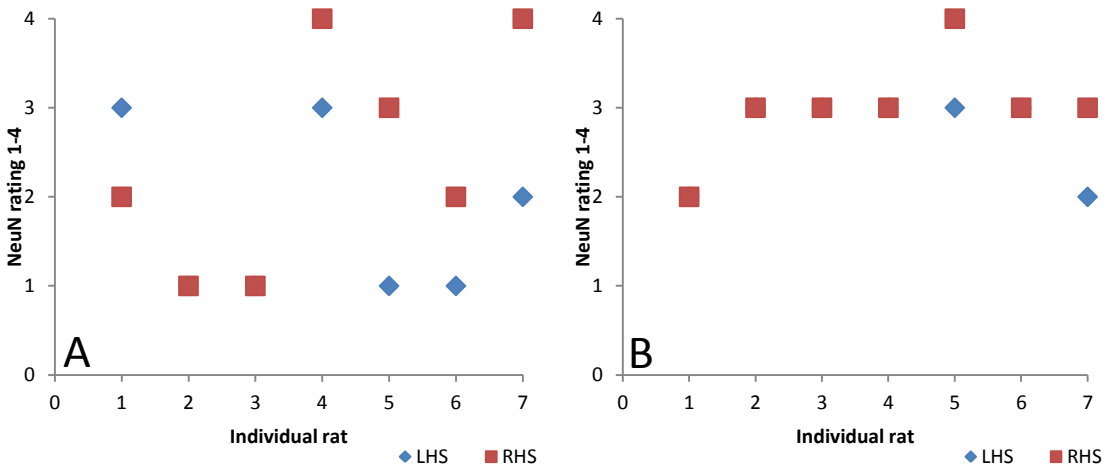

Figure 4.4. Lesion size for each rat of the PPTg lesion group (A) and combined lesion group (B) rated based on NeuN+ stained brain sections. Lesions were rated on a scale from 0 to 4 (0 = no lesion of the PPTg, 1 = small/ partial lesion of the PPTg, 2 = partial to full lesion of the PPTg, 3 = fully lesioned PPTg, 4 = extensive lesion covering the PPTg and surrounding tissue; square covering diamond).

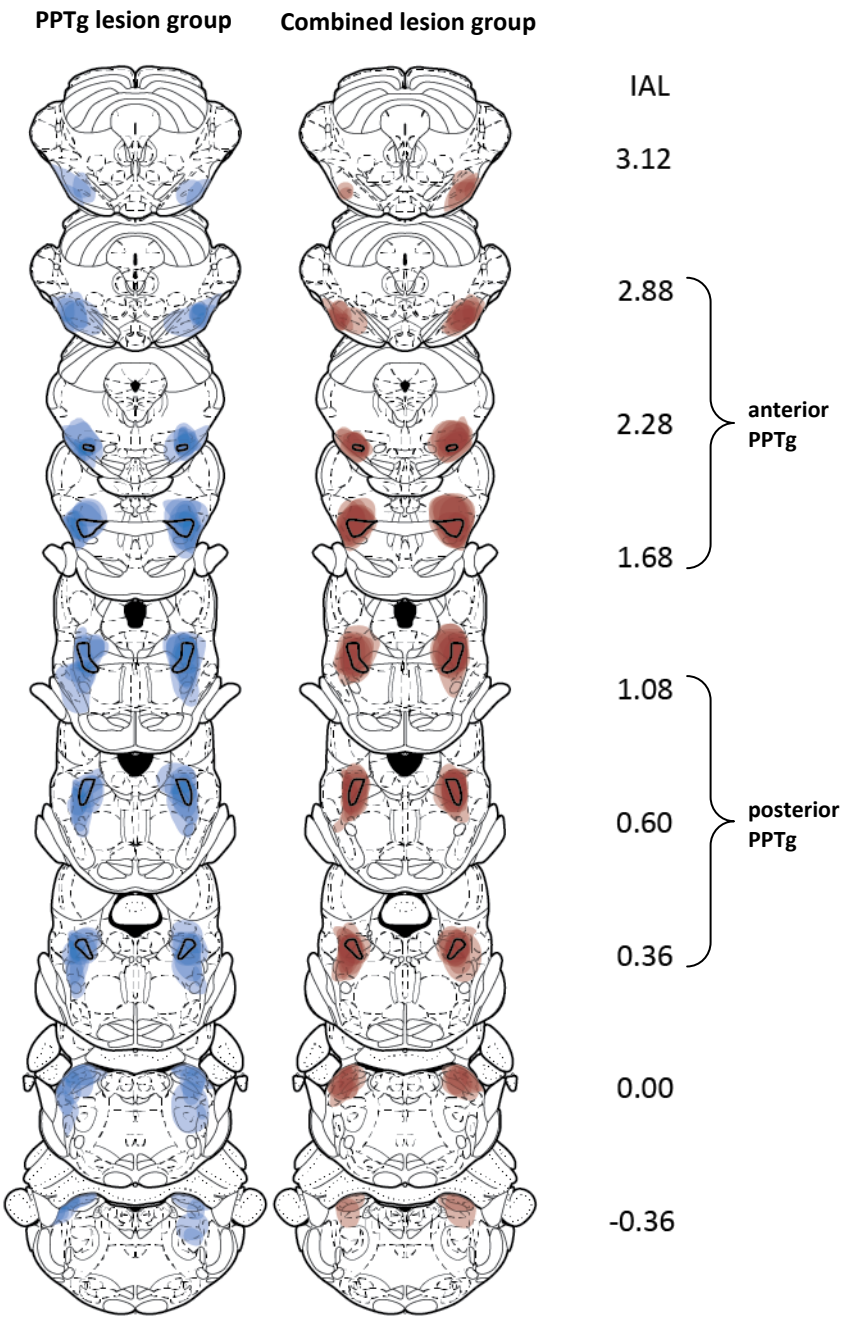

Figure 4.5. Representation of lesion extension along the rostro-caudal axis after ibotenic acid infusion on a selection of NeuN+ stained sections. The position of the schematics (adapted from Paxinos and Watson, 2005) on the rostro-caudal axis is represented as the distance from the interaural line (IAL; mm). Sections IAL + 2.88 mm through to IAL + 1.68 mm were identified as sections containing anterior PPTg neurons (identified by PPTg cholinergic neurons in shams and partially lesioned rats) and those from IAL + 1.08 mm to IAL + 0.36 mm represent sections containing posterior PPTg. Lesion damage is represented in coloured shading: blue for lesions in the PPTg lesion groups and red for lesions in the combined lesion groups.

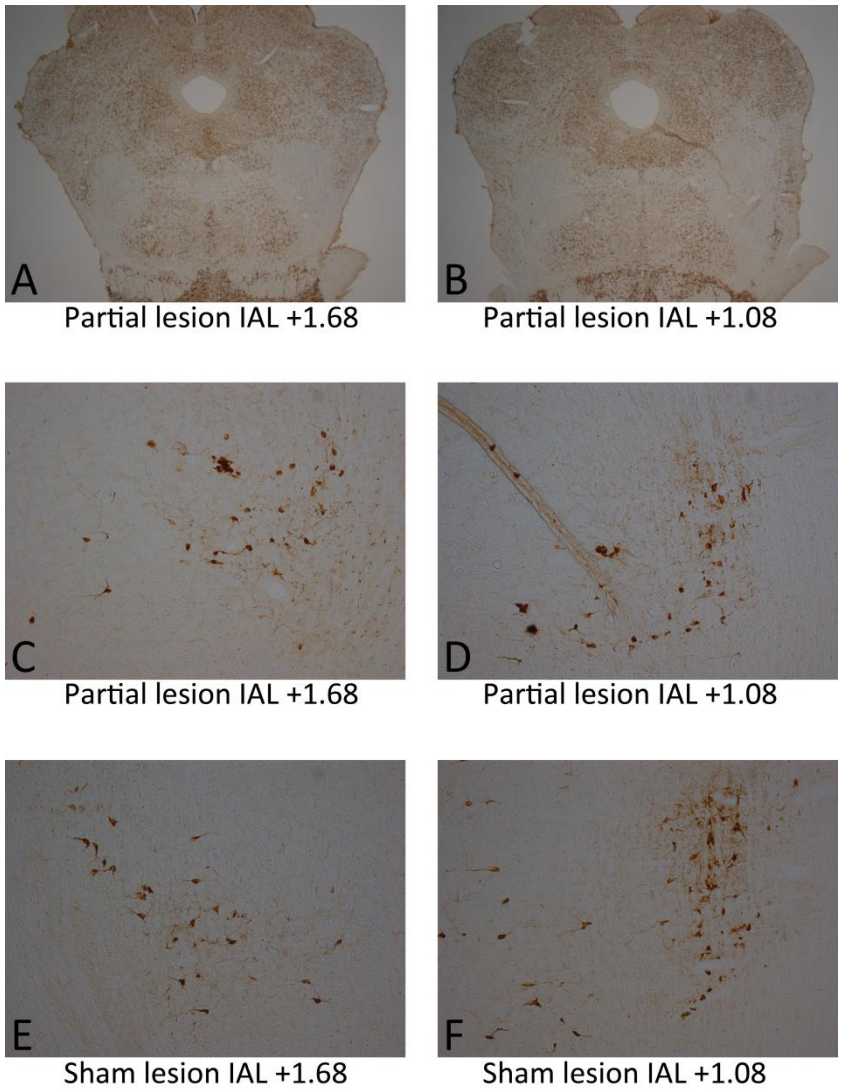

Figure 4.6. Representative histological photomicrographs of lesions in anterior and posterior PPTg of rats bearing partial lesions (**A and B**: NeuN stained; **C and D**: ChAT stained; **E and F**: ChAT stained sections of sham lesioned rat).

The PPTg lesion group showed a ChAT+ PPTg cell loss of 56% (range 33% to 73%) and in the combined lesion group 73% of cholinergic neurons were destroyed (range 56% to 96%). The 6-OHDA group, which did not receive any lesion inducing toxin infused into the PPTg showed a PPTg cholinergic cell survival of 97% (range 84% to 108%) when expressed as a percentage from the average sham cell count. The percentage of surviving cholinergic PPTg neurons is shown in Figure 4.7.

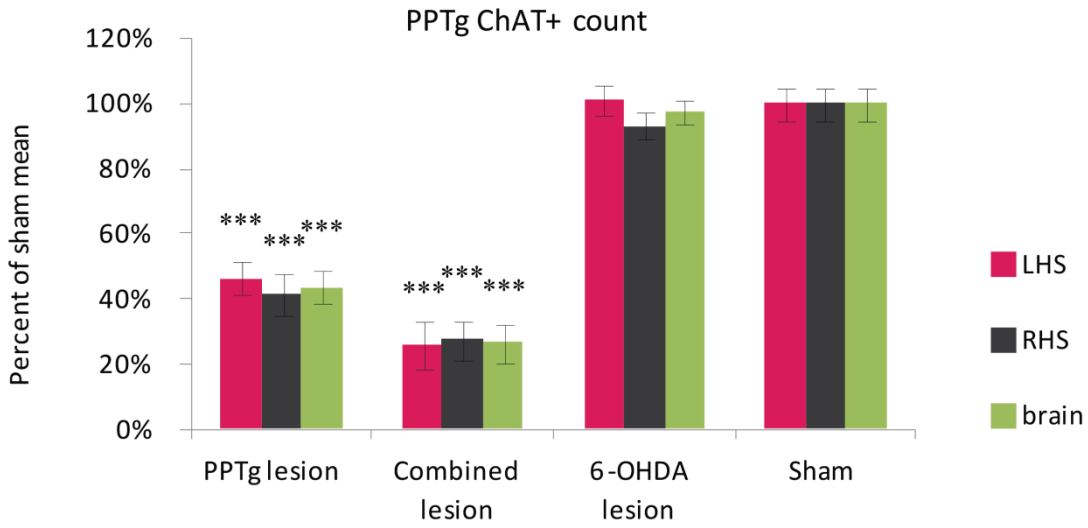

Figure 4.7. PPTg ChAT+ count as the percentage of the mean count in sham lesioned rats. Graph shows group means ± SEM. *** indicates a significant difference between PPTg lesion/ combined lesion group and shams with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$. PPTg and combined lesion groups did not differ from each other ($p = 0.095$) and neither did 6-OHDA and sham lesion groups ($p = 0.979$). LHS = left-hand side; RHS = right-hand side; brain = overall cell count left and right combined.

Univariate ANOVA of the raw cell count of surviving ChAT+ neurons confirm a significant difference between groups ($F_{3,24}$ = 56.235 $p < 0.001$; η^2_p = 0.875). Tukey corrected post hoc tests reveal that both groups, PPTg lesion group and combined lesion group had a significantly lower ChAT+ cell count in the PPTg compared to sham controls (PPTg lesion $p < 0.001$; combined lesion: $p < 0.001$). Furthermore, PPTg lesion group and combined lesion group did not differ from each other ($p = 0.095$), and neither did the 6-OHDA caused any observable destruction of cholinergic neurons in the PPTg: 6-OHDA group and sham controls did not differ from each other ($p = 0.979$).

TH+ cell count in SN

Both groups which received 6-OHDA infusions into the striatum showed a marked DA degeneration: TH+ count in the 6-OHDA group was reduced by 91% (range 88% to 96%; of average sham count) and in the combined lesion group by 93%. (range 92% to 95%). In the PPTg lesion group an average 90% of the TH+ neurons in SN were counted (range 69% to 112%). Statistically, as revealed by univariate ANOVA, the groups differed significantly from each other in their remaining nigral TH+ cell count. ($F_{3,24}$ = 238.817 $p < 0.001$; η^2_p = 0.968). Post hoc tests (Tukey corrected) showed that the differences between the sham controls and both 6-OHDA treated groups were significant (6-OHDA group: $p < 0.001$; combined group: $p < 0.001$). Both DA depleted groups did not differ from each other ($p = 1.000$). Likewise, there was no statistical difference with the PPTg lesion group and sham controls ($p = 0.230$) (see Figure 4.9).

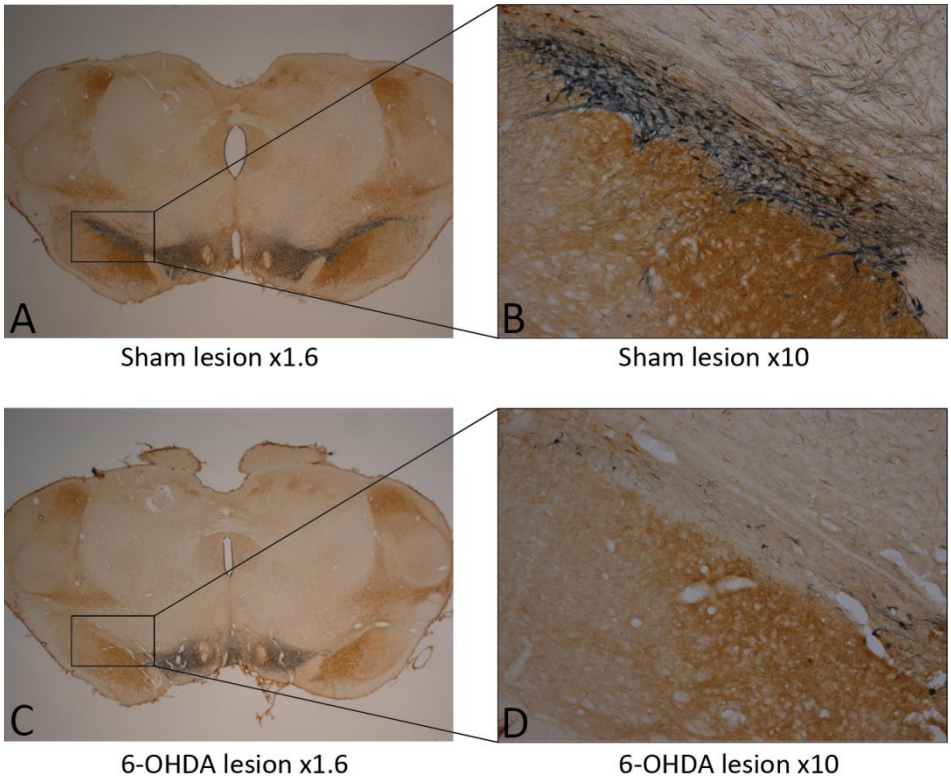

Figure 4.8. Representative histological photomicrographs of SN. **Panels A and B** show sections from a sham lesion rat; **C and D** show dopamine depletion after 6-OHDA infusions.

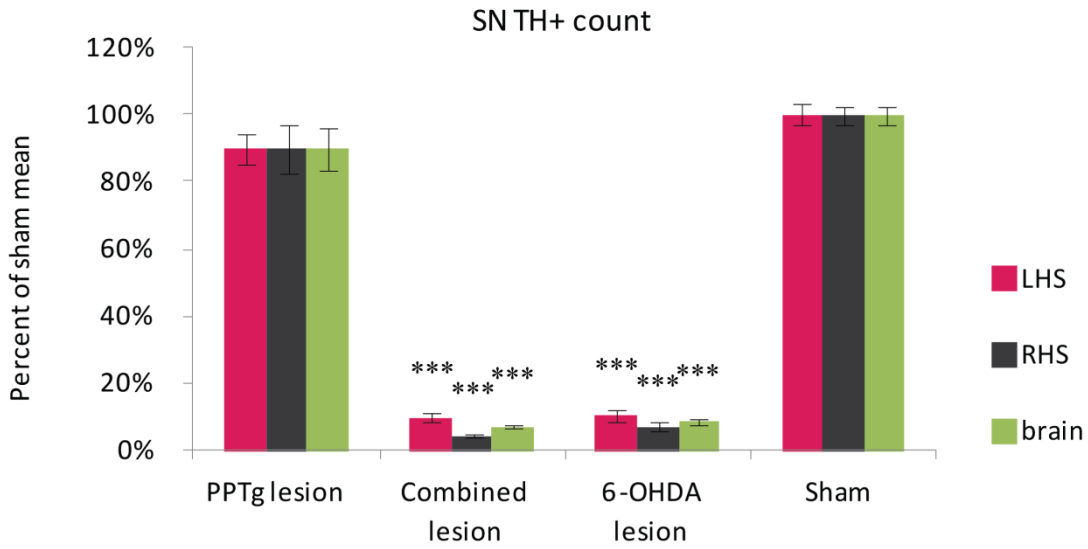

Figure 4.9. SN TH+ count as the percentage of the mean count in sham lesioned rats. Graph shows group means ± SEM. *** indicates a significant difference compared to shams with $p \leq 0.001$; LHS = left-hand side; RHS = right-hand side; brain = overall cell count left and right combined; 6-OHDA and combined lesion groups did not differ from each other ($p = 1.000$) and neither did PPTg and sham lesion group ($p = 0.230$).

3.2.2. Behavioural results

PPTg lesions on their own – at least in rodents – do not cause locomotor deficits or gait or postural deficits, as described in the previous chapter. Given its degeneration in PD and the fact that specific symptoms, such as gait and postural impairments, do not respond to DA medication this experiment aimed to assess whether additional partial PPTg lesions create a PD model whose deficits compared to sham operated rats are different to the traditional PD rodent model involving only DA depletion. This and the general feasibility of creating a refined model of PD by combining DA depletion and partial PPTg lesions were important questions to answer before pursuing to assess the effect of PPTg-DBS on gait and posture. The same aspects of gait – gait stability, speed, stride and coordination – were explored by analysing the data individually and presented as such in the following. Before any analysis, the data deriving from both individual sets of the experiment were inspected for differences. Great care had been taken to keep the experimental conditions as similar as possible so no differences due to confounding variables were expected. Given the small numbers in the groups, when taken separately for each set, the group means and ranges of each parameter were inspected for differences. On no occasion, neither in pre nor post-surgery performance, did the behavioural data allow identification of which set the data were derived from (Supplementary Table 4.1.). Normality as examined with the Shapiro-Wilk test was given on most of the test days. On rare occasions, the data did not follow a normal distribution. Given this infrequency these seldom violations were tolerated in the analysis, but reported below. Pre-surgery data was assessed for pre-existing differences, which could be ruled out for each analysed parameter (Supplementary Table 4.2.).

Traditional and refined model in comparison with sham controls

The main focus here was on the question of whether the PD model refined by additional PPTg lesions would be different than the traditional PD model when compared to sham controls.

Gait stability

BOS – The mean values of BOS (and other parameters) are presented in Figure 4.10. Mixed ANOVAs of front and hind limbs showed no interaction of post-surgery testing days and groups (front: $F_{18,150} = 1.394$ $p = 0.142$; hind: $F_{18,150} = 1.619$ $p = 0.062$) nor an effect of day in the front limbs, though this was present in the hindlimbs (front: $F_{6,150} = 1.776$ $p = 0.108$; hind: $F_{6,150} = 5.260$ $p < 0.001$; $\eta^2_p = 0.174$). Repeated ANOVAs for each group showed that this day effect was only in the PPTg lesion group and the sham controls (PPTg lesion group: $F_{6,36} = 5.652$ $p < 0.001$; $\eta^2_p = 0.485$; sham group: $F_{6,42} = 5.052$ $p = 0.010$ Greenhouse-Geisser corrected; $\eta^2_p = 0.419$) but not in the DA depleted models (combined group: $F_{6,36} = 0.603$ $p = 0.726$; 6-OHDA group: $F_{6,36} = 0.985$ $p = 0.449$). The PD models therefore failed to further decrease their BOS as both non-DA depleted groups did. There was a significant effect of group (front: $F_{3,25} = 27.154$ $p < 0.001$; $\eta^2_p = 0.765$; hind: $F_{3,25} = 20.219$ $p < 0.001$; $\eta^2_p = 0.708$). Post hoc tests specified that both, combined group and 6-OHDA group showed a significantly bigger BOS (front: combined group: $p < 0.001$; 6-OHDA group: $p < 0.001$; hind: combined group: $p < 0.001$; 6-OHDA group: $p < 0.001$). However, there was no significant difference between both PD models ($p = 0.683$).

Duty Cycle – A significant interaction of testing days and groups in the front limb data ($F_{18,150} = 2.334$ $p = 0.003$; $\eta^2_p = 0.219$) stems from day effects in PPTg lesion group ($F_{6,36} = 3.911$ $p = 0.004$; $\eta^2_p = 0.395$) and 6-OHDA group ($F_{6,36} = 3.172$ $p = 0.013$; $\eta^2_p = 0.346$). However, as post hoc tests reveal, this did not translate to significant differences between individual testing days in the 6-OHDA group and only the last of the testing days of the PPTg group reaching a significant decrease of stance duration, which can be attributed to a training effect as

explained before. More importantly there was no significant effect of group in this parameter (front: $F_{3,25} = 1.517$ $p = 0.235$; hind: $F_{3,25} = 2466$ $p = 0.086$). Post-surgery data of Duty Cycle hind were not normally distributed but this violation was tolerated.

Support – There was a group difference regarding the percentage of diagonal two-legged support during the animals' gait cycle ($F_{3,25} = 7.991$ $p = 0.001$; $\eta^2_p = 0.490$). Post hoc tests found that only the 6-OHDA group used significantly less diagonal support compared to sham controls ($p = 0.004$). Despite the observable decrease of diagonal support in the combined lesion group, 6-OHDA group and combined lesion group were not significantly different ($p = 0.097$); this change was not big enough to lead to a significant difference between the combined group and sham controls ($p = 0.583$). A decrease of two-legged support is usually compensated by an increase of three or more legged support. Indeed, the significant effect of group ($F_{3,25} = 5.536$ $p = 0.005$; $\eta^2_p = 0.399$) and further post hoc tests found that the three-legged support in the 6-OHDA group was significantly increased compared to sham controls ($p = 0.014$). The combined group showed the same trend as described for diagonal support: three-legged support seemed increased – and there was no significant difference between combined lesion group and 6-OHDA group ($p = 0.750$) – but not sufficiently to lead to a significant difference when compared to sham controls ($p = 0.132$). Post-surgery data of Support 3 were not normally distributed but this violation was not further accounted for in the analysis.

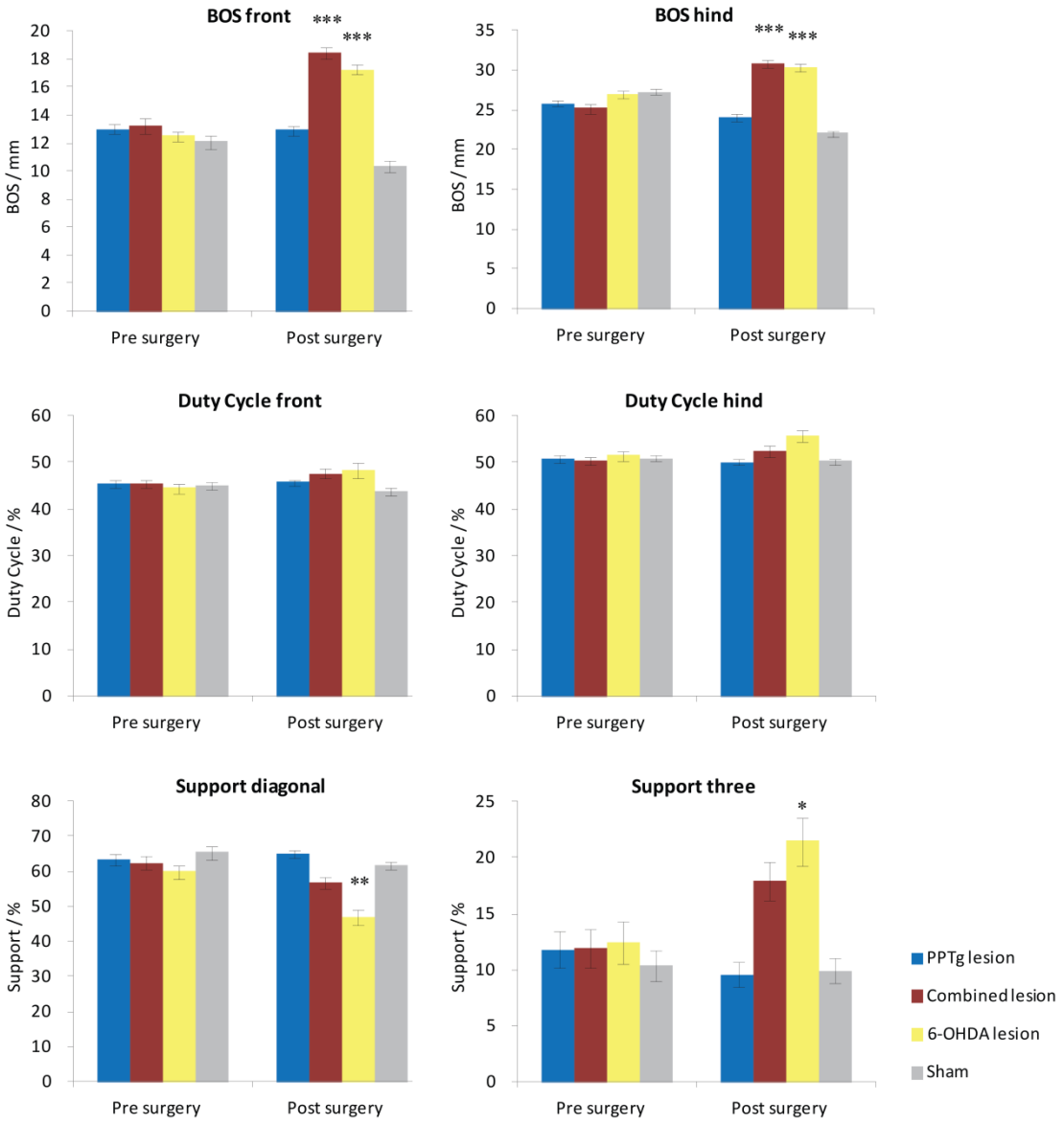

Figure 4.10. Performance on the CW – Parameters reflecting gait stability. Graphs show group means ± SEM of selected gait parameters; *** indicates a significant difference compared to Sham lesion group with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$. Despite the changes in the distribution of support in the combined lesion group failing to reach significance, in none of the selected parameters the combined and the 6-OHDA lesion groups differ from each other significantly. Neither did PPTg and sham lesion groups show significant differences. Please refer to the text for details.

Speed

Swing Speed – No day x group interaction nor any day effect was found by mixed ANOVAs of Swing Speed front and hind, but there were significant effects of group (front: $F_{3,25}$ = 26.540 $p < 0.001$; η^2_p = 0.761; hind: $F_{3,25}$ = 66.856 $p < 0.001$; η^2_p = 0.889). Both PD groups differed significantly from sham controls, showing a significantly reduced swing speed (both combined lesion group and 6-OHDA group compared to sham: $p < 0.001$ for front and hind limbs). Post-surgery data of the hind limbs was not normally distributed; this was tolerated in the analysis.

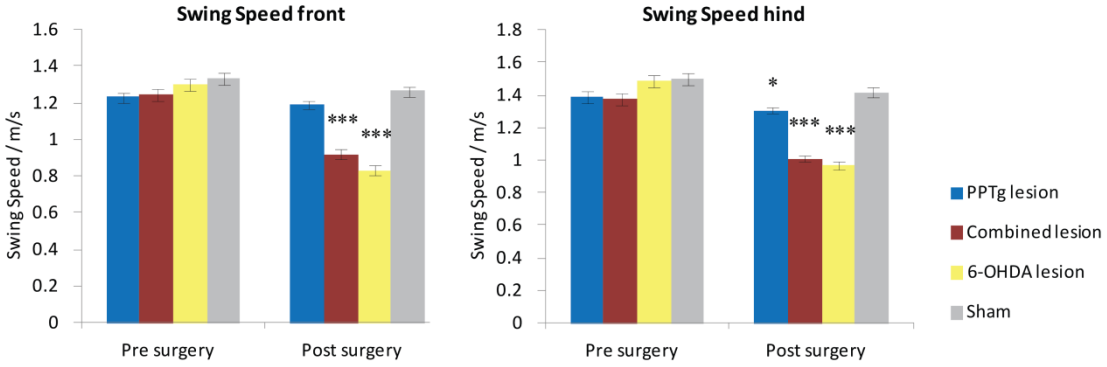

Figure 4.11. Performance on the CW – Swing Speed. Graphs show group means ± SEM of selected gait parameters; *** indicates a significant difference compared to Sham lesion group with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$. The combined and the 6-OHDA lesion groups did not differ from each other significantly. Neither did PPTg and sham lesion groups show significant differences. Please refer to the text for details.

Stride

Stride Length – Mixed ANOVAs on Stride Length of front and hind limbs found significant day x group interactions (front: $F_{18,150} = 2.038$ $p = 0.026$, Greenhouse-Geisser corrected; $\eta^2_p = 0.196$; hind: $F_{18,150} = 1.873$ $p = 0.046$, Greenhouse-Geisser corrected; $\eta^2_p = 0.184$) which, followed up by repeated measures ANOVAs for each group, meant that sham controls and PPTg lesioned rats changed across the testing days (front: PPTg lesion group: $F_{6,36} = 4.975$ $p = 0.001$; $\eta^2_p = 0.453$; sham control group: $F_{6,42} = 2.470$ $p = 0.039$; $\eta^2_p = 0.261$; hind: PPTg lesion group: $F_{6,36} = 6.170$ $p < 0.001$; $\eta^2_p = 0.507$; sham control group not reaching significance: $F_{6,42} = 2.147$ $p = 0.068$) but not the DA depleted animals of both PD groups (front: combined lesion group: $F_{6,36} = 0.420$ $p = 0.861$; 6-OHDA group: $F_{3,36} = 1.358$ $p = 0.258$; hind: combined lesion group: $F_{6,36} = 0.400$ $p = 0.874$; 6-OHDA group: $F_{6,36} = 1.068$ $p = 0.400$). Most importantly there were significant effects of group (front: $F_{3,25} = 13.795$ $p < 0.001$; $\eta^2_p = 0.623$; hind: $F_{3,25} = 11.156$ $p < 0.001$; $\eta^2_p = 0.572$). The front limbs of rats from the combined lesion and the 6-OHDA group made significantly shorter steps (combined lesion group: $p = 0.021$; 6-OHDA group: $p < 0.001$). The shortened steps of hind paws in the combined lesion group did not reach significance ($p = 0.068$), the steps of 6-OHDA rats were significantly shorter than sham controls ($p < 0.001$); however there was no significant difference between 6-OHDA group and combined lesion group in the hind limbs data either ($p = 0.134$).

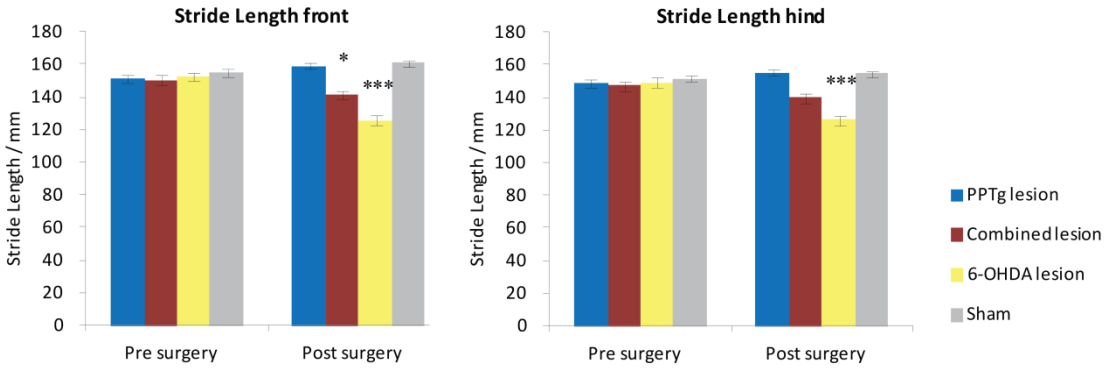

Figure 4.12. Performance on the CW – Stride Length. Graphs show group means ± SEM of selected gait parameters; *** indicates a significant difference compared to Sham lesion group with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$. Despite the decrease in stride length of the hind limbs in the combined lesion group failing to reach significance, the combined and the 6-OHDA lesion groups did not differ from each other significantly. Neither did PPTg and sham lesion groups show significant differences. Please refer to the text for details.

Coordination

Print position – Mixed ANOVAs found no day x group interactions (RHS: $F_{18,150} = 1.588$ $p = 0.129$, Greenhouse-Geisser corrected; LHS: $F_{18,150} = 1.189$ $p = 0.277$) or effects of day (RHS: $F_{6,150} = 0.605$ $p = 0.623$, Greenhouse-Geisser corrected; LHS: $F_{6,150} = 1.316$ $p = 0.253$) or group (RHS: $F_{3,25} = 0.873$ $p = 0.468$; LHS: $F_{3,25} = 1.057$ $p = 0.385$) in the Print Position data. This parameter was unaffected by DA manipulation.

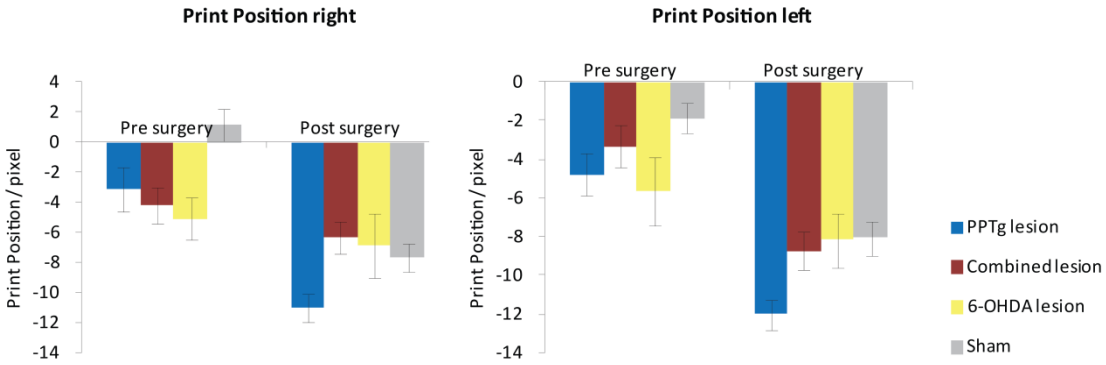

Figure 4.13. Performance on the CW – Parameter reflecting coordination. Graphs show group means ± SEM of selected gait parameters. Please refer to the text for details.

The effect of partial PPTg lesions

In none of the analysed parameters, bar Swing Speed of the hind limbs, did the PPTg lesion group bearing bilateral partial PPTg lesions differ significantly from the sham controls (please refer to Table 4.2. for details).

Table 4.2. Summary of the effect of partial PPTg lesions, 6-OHDA lesions and combined lesions on CW gait parameters. The data show group means ± SEM. There was no significant difference between combined lesion group and 6-OHDA lesion group and no significant difference between PPTg lesion group and sham lesion group. * $p < 0.001$ versus sham lesion group. † $p < 0.005$; ‡ $p < 0.05$.

	BOS		Duty Cycle		Support		Swing Speed		Stride Length		Print Position	
	front	hind	front	hind	diagonal	three	front	hind	front	hind	right	left
Partial PPTg lesion (N = 7)	12.91 ± 0.31	24.04 ± 0.51	45.70 ± 0.58	50.14 ± 0.77	65.27 ± 1.08	9.56 ± 1.11	1.19 ± 0.02	1.30‡ ± 0.02	159.07 ± 1.88	155.36 ± 1.88	-11.06 ± 0.95	-12.03 ± 0.81
Combined lesion (N = 7)	18.43* ± 0.38	30.80* ± 0.43	47.70 ± 0.85	52.45 ± 1.10	56.73 ± 1.60	17.90 ± 1.73	0.92* ± 0.03	1.01* ± 0.02	141.4‡ ± 2.87	139.57 ± 2.90	-6.33 ± 1.04	-8.72 ± 0.96
6-OHDA lesion (N = 7)	17.25* ± 0.38	30.35* ± 0.53	48.39 ± 1.59	55.69 ± 1.29	47.08† ± 2.25	21.44‡ ± 2.21	0.83* ± 0.03	0.97* ± 0.03	125.8* ± 3.10	126* ± 2.57	-6.90 ± 2.15	-8.19 ± 1.39
Sham lesion (N = 8)	10.34 ± 0.36	22.06 ± 0.39	43.83 ± 0.72	50.27 ± 0.66	61.66 ± 1.17	9.93 ± 1.10	1.26 ± 0.03	1.42 ± 0.03	160.82 ± 2.06	154.74 ± 1.94	-7.67 ± 0.95	-8.07 ± 0.89

3.3. Discussion

		Different to sham?			Different to pre-surgery?
		PPTg	combined	6-OHDA	sham
BOS	front	No	bigger	bigger	no
	hind	No	bigger	bigger	no
Swing Speed	front	No	slower	slower	no
	hind	slower (but significantly faster than other groups)	slower	slower	no
Stride Length	front	No	shorter	shorter	no
	hind	No	No	shorter	no
Duty Cycle	front	No	No	No	no
	hind	No	No	No	no
Print Position	right	No	No	No	further
	left	No	No	No	no
Support	diagonal	No	No	less	no
	three	No	No	more	no

Table 4.3. Summary of analysed parameters. Post-surgery data of rats bearing partial PPTg lesions, combined lesion group and 6-OHDA lesion group compared to sham lesion group. Further the table shows the results of pre- to post-surgery data comparison of the sham lesion group. Cells shaded in red show differences compared to the sham lesion group that reflect a deficit of the performance. Green shading reflects improvements. Blue frames reflect the absence of significant differences.

The experiment presented in the previous chapter showed that the PPTg itself is not a crucial structure for the generation of normal gait. Rats bearing complete and near-to-complete lesions of the PPTg did not present differences in the analysed gait parameters reflecting stability, speed, stride and coordination compared to sham lesion rats. This is also true for partial PPTg lesions. Only the swing speed of the hind legs was slower than in the control group, but significantly faster than both parkinsonian models of this experiment. Furthermore, the addition of a partial PPTg lesion did not cause any of the analysed gait parameters to differ significantly from the traditional Parkinson's model – but in comparison with the sham lesion control group the deficits in stride length of the hind limbs and in the distribution of support of the combined lesion group were not significantly different. Potentially this could mean that the additional PPTg lesion might have attenuated the effect of the DA depletion on specific gait parameters. Two explanations could be plausible: a) In PD (models) the STN is hyperactive (Bergman *et al.*, 1994; Breit *et al.*, 2007; Hassani *et al.*, 1996; Remple *et al.*, 2011): a partial PPTg lesion might cause a decrease of excitatory input to the STN. Or b) caused by the loss of a part of its neuronal population compensatory mechanisms within the PPTg could have led to an compensatory increase of activity in those remaining PPTg neurons which are under over-inhibitory control from the BG. Whatever mechanism might be involved, it could have partially re-established a balance within the BG circuit.

Parkinson's patients who suffer more frequently from falls than others ("fallers") were reported to show a higher cell loss in the PPTg (Karachi *et al.*, 2010) and a decreased thalamic innervation of cholinergic afferents (Bohnen *et al.*, 2009) than non-fallers. Falls can be caused by postural deficits and impaired balance, as well as being the consequence of progressively decreased step length and occurrences of FOG (Schaafsma *et al.*, 2003). In this context, it seems surprising that the additional PPTg lesions in this study did not lead to further impairments in comparison to the traditional 6-OHDA model. One reason might be that the

induced destruction of a part of the PPTg spared enough neurons to maintain the same level of impairment as caused by DA depletion alone. This is however unlikely, comparing the average cell loss of 73% of cholinergic neurons (range 56% to 96%) in this experiment to the degree of PPTg degeneration in PD patients described in the literature. Another reason might be the nature of deficits that might be expected with additional PPTg lesions: if the main effect is to be expected in balance rather than gait *per se*, the CatWalk might not be the most direct means to measure it. Balance deficits and consequently falls can be the result of impaired sensorimotor integration – including deficits in proprioceptive processing, inability to switch between sensory modalities, impaired interlimb coordination and asymmetries (Boonstra *et al.*, 2008; Lewis and Byblow, 2002; Vaugoyeau *et al.*, 2011). To test if postural adjustments can be processed adequately or quickly enough in the response to changed postural demands, a more adequate test could be the rotarod test. The beam walking test might be a good alternative for assessing motor coordination deficits (Stanley *et al.*, 2005).

The results presented here are discordant with the only other study that examines the behavioural effect of a combination of PPTg lesions and DA degeneration in the SN. The Paris group describe a MPTP-model which, as they describe, showed worsening of DOPA-unresponsive gait and balance disorders after the addition of bilateral PPTg lesions. Bilateral lesions were made either by the excitotoxin ibotenic acid or Dtx-UII, which, injected into the upper brainstem, is selective for cholinergic neurons if given in the right concentration (Clark *et al.*, 2007). The authors reported a slight increase in balance deficits (an increase from 0 to 1 on a semi-quantitative scale from 0 to3) and adverse postural changes such as an increased knee angle and elevated pelvis, which however were marginal and not significant. In fact, examining the data as presented in the paper (Table 1; Grabli 2013), the only significant effect of the additional PPTg lesion is an improvement of global activity, step speed and rigidity compared to the MPTP-induced state pre-PPTg lesion, which does not reach baseline

performance. Unfortunately, the authors do not offer data for the ibotenic acid and DTx-UII groups individually, but offer graphs that show a more severe effect after ibotenic acid. The histological data do not offer either a differentiation of the effect of both toxins: the cholinergic cell loss is only given averaged for both groups. Surprisingly, the loss of non-cholinergic neurons in both groups did not differ significantly from each other: the DTx-UII group showed a loss of 6% and the ibotenic acid group 10%. One last difficulty regarding the interpretation of this study lies in the fact that no timescale of the experimental design is offered. It is unclear when and how quickly after surgeries the animals were tested. It is surprising that the monkey that showed slight balance deficits after PPTg lesion surgery showed improved balance scores after a subsequent further dose of MPTP. It seems that the actual results – reflecting no further deficits after additional PPTg lesions – do not differ much from the results presented in this chapter. However, given the issues described above this interpretation has to remain cautious.

As described in the General Introduction there is no agreement if there is a relationship between DA loss in SN and PPTg degeneration. According to Braak the degeneration in both structures occurs around the same stage of the disease (Braak *et al.*, 2003); the literature offers support for a destructive influence of a defective PPTg onto SN neurons, others have shown PPTg degeneration after MPTP-induced DA cell death in aged monkeys (Karachi *et al.*, 2010), and there is evidence for no influence at all (Heise *et al.*, 2005). Very recently, Pienaar and colleagues (Pienaar and van de Berg, 2013) have shown that the destruction of SN DA neurons in the unilateral 6-ODHA model caused no decrease in the total number of ChAT+ neurons in the PPTg, confirming the results of Heise and colleagues. This goes in line with the findings here. However, by means of further stereological analysis of NeuN+ neurons of that unilateral PD model the authors could reveal a significant loss of non-cholinergic neurons. Since quantitative histological analysis in this study was restricted to the cell count of ChAT+

neurons in PPTg it cannot be excluded that other techniques might lead to similar findings. In a similar way to this 'dying-back'-theory (Pienaar and van de Berg, 2013) of PPTg neurons, others argue that nigral DA neurons might degenerate due to altered input from the PPTg, given that DA neurons have been shown to need to be stimulated in order to survive (Michel *et al.*, 2013). Here, however, partial PPTg lesions similar to the degree of degeneration in PD patients did not cause an decrease of TH+ neurons in SN compared to sham lesioned rats.

In pilot surgeries preceding the experiments lesions caused by 0.04M ibotenic acid produced small lesions. The risk of not causing enough damage was considered high with this concentration and the decision was made to use a concentration of 0.06M. Examining the extent of lesions in NeuN/Cresyl stained brain sections in this experiment showed that it might be advisable to reduce not the concentration, but the volume of the toxin to decrease spread of toxin in the tissue and constrain the affected area more.

Apart from the effect of additional PPTg lesions on gait parameters in the rodent PD model and the effect of partial PPTg lesions on nigral DA neurons and *vice versa*, this experiment aimed to assess the feasibility of a combined lesion model in view of subsequent experiments that were aiming to assess the effect of PPTg-DBS in a rodent model that mimics the physiological state of PD closer than the traditional model. It was important to ascertain that rats bearing combined lesions could survive three surgeries and would be able to complete the behavioural tests. Further, it was important to assess the degree of care – especially feeding – those rats would require, in order to plan the next experiments. Given that they tolerated the surgeries well (once recovered from the ibotenic acid infusions) and required the same amount of care as the traditional bilateral 6-OHDA model, this refined model could be taken forward to the stimulation studies.

Chapter 5

The effect of anterior and posterior PPTg-DBS on gait and L-DOPA induced dyskinesias

1. Chapter Introduction

Since 2005 DBS of the PPTg has constituted an experimental treatment option in attempts to address axial symptoms of Parkinsonism. The hope was to find an alternative to the established target sites for DBS, which fail (or lose efficacy with disease progression) to address symptoms refractory to DA medication – gait and postural disturbances, FOG – and to offer an option to patients not considered good candidates for STN-DBS. Despite initial reports of beneficial effects of PPTg-DBS (Mazzone *et al.*, 2009; Mazzone *et al.*, 2005; Moro *et al.*, 2010; Plaha and Gill, 2005; Stefani *et al.*, 2007) the results of subsequent studies have been at best only modest (Ferraye *et al.*, 2010; Snijders *et al.*, 2008). In addition, the focus has moved from axial symptoms to other domains: cognition (Alessandro *et al.*, 2010; Costa *et al.*, 2010; Stefani *et al.*, 2010; Zanini *et al.*, 2009), sleep (Lim *et al.*, 2009; Romigi *et al.*, 2008; Thevathasan *et al.*, 2010) and physiological functions (Aviles-Olmos *et al.*, 2011; Xiang *et al.*, 2011). To date, the PPTg is not a safe or standard option in clinical settings (Potter-Nerger and Volkmann, 2013).

1.1. Hypothetical basis for the consideration of the PPTg as a target for DBS

The hypothesis that successful correction of the axial symptoms of PD could be achieved with PPTg-DBS stems from two assumptions: (i) dysfunction in the output nuclei of the BG has a pathological impact on the PPTg that might be corrected by local low frequency stimulation (LFS); and (ii) the PPTg is a locomotor control structure, a main component of the MLR (Munro-Davies *et al.*, 1999; Nandi *et al.*, 2002; Pahapill and Lozano, 2000). This second assumption has been discussed in Chapter 3 and it has been shown that in accordance with evidence from the literature, the PPTg is not a motor controlling structure: full – or partial – lesions of the PPTg did not lead to changes in rats' gait.

There is clear evidence for changed neuronal activity of the PPTg in PD models and it is plausible that altered input from the BG output nuclei plays an important role. However, limiting the BG-PPTg relationship in PD to an over-inhibitory input resulting in reduced glutamatergic output to the spinal cord would present an over-simplified understanding of the role of the PPTg in PD and further the role of the PPTg as a target of DBS.

1.2. Precise target site within a heterogeneous structure

The PPTg contains a diverse population of cholinergic, GABAergic and glutamatergic neurons, each distributed differently (Martinez-Gonzalez *et al.*, 2011; Ros *et al.*, 2010). The pattern of afferent and efferent connections differs: BG output is directed predominantly to anterior parts (the pars dissipatus) and sensory input to posterior parts (pars compactus) (Winn, 2008). Consequently, there are clear functional differences between anterior and posterior PPTg (Alderson *et al.*, 2008). This raises questions whether the consequences of PPTg-DBS are dependent on location within the PPTg, as well about the nature of effects that might be expected. Clarity about this is an essential precursor to decisions about which patients might be considered best candidate for PPTg-DBS and to which PPTg sites DBS should be targeted.

1.3. L-DOPA induced dyskinesias

After chronic treatment with L-DOPA, motor complications develop which can interfere with normal behaviour and can be more debilitating than the PD symptoms themselves. Dyskinesias include different types of movement disorders such as chorea-ballism, dystonia, myoclonus, tics and tremor (Guridi *et al.*, 2012). L-DOPA induced dyskinesias (LID) are defined as involuntary, purposeless, irregular but potentially repetitive movements, which are mainly choreic in nature (Voon *et al.*, 2009). They generally coincide with peak L-DOPA plasma concentrations [‘on’ period dyskinesias (Guridi *et al.*, 2012)] but also appear as diphasic dyskinesias occurring both before the onset of L-DOPA benefit and as the effect is wearing off

(Obeso *et al.*, 2000). The pathogenetic mechanisms are not completely agreed on (Winkler *et al.*, 2002) but the degree of nigrostriatal damage and the use of L-DOPA – chronic, pulsatile and overall duration – are known to be determining factors. This non-physiological, intermittent stimulation of denervated DA receptors is thought to cause processes of striatal plasticity that change striatal synaptic functions and hence the excitability of the striatum, introducing changes in striatopallidal circuits such as a reduction in main firing frequencies in GPi and STN (causing an increase in thalamocortical drive leading to dyskinesias) (Guridi *et al.*, 2012; Thanvi and Lo, 2004). Given the complexity and interconnectivity of the BG with other structures it is likely that not only changes in striatal response to L-DOPA are involved in LIDs. A recent study showed that outside the BG there was a response to LIDs, measured by immediate-early gene expression, suggesting a possible involvement of structures such as the motor cortex, the dorsal hippocampus, the cuneiform nucleus and the PPTg (Bastide *et al.*, 2014). FosB expression was also noted in pedunculopontine and zona incerta of dyskinetic PD mice models (Ding *et al.*, 2007). What is the nature of the effect of degeneration of those structures on LIDs? There is a vast literature employing rodent models of LIDs [for a review see (Duty and Jenner, 2011; Iderberg *et al.*, 2012)] eliciting abnormal involuntary movements (AIMs) as a rodent manifestation of dyskinesias and examining the mechanisms responsible for treatment related dyskinesias in PD. However, there is no assessment of LIDs in PD models that include extranigral degeneration. There is evidence of what might be considered as dyskinesias in rats with PPTg damage. For example, PPTg lesioned rats showed enhanced stereotypic orofacial movements in response to either systemic or intra-ventrolateral striatal injections of d-amphetamine compared to sham operated animals (Inglis, Allen*, et al.*, 1994) (Allen and Winn, 1995). Further, if the degeneration of the PPTg influences the motor performance of PD patients it is likely that it also has an effect on motor symptoms induced by L-DOPA. Could stimulation of the PPTg correct LIDs? Chronic DBS of the EPN and Pf improved

dyskinesia scores in hemiparkinsonian rats that showed LIDs after chronic administration of L-DOPA (Alam *et al.*, 2014), while STN stimulation has been shown to be pro-dyskinetic (Jouve *et al.*, 2010).

1.4. Deep brain stimulation in clinical practice

DBS stimulation in patients is – apart from the risk that the surgery involves – much more complex than treating the symptoms with medication. The most important factor for a beneficial outcome is patient selection. The decision for implantation of DBS electrodes has to be made carefully and to withstand a critical cost-benefit analysis. This evaluation in regards to a treatment of the cardinal features of PD, essential tremor and dystonia by targeting STN, thalamus and the GPi can nowadays be based on many years (and patients) of experience. For STN stimulation a patient has the best chance of a positive effect if the diagnosis is idiopathic PD with symptoms of bradykinesia, rigidity and/or tremor and if these symptoms are L-DOPA responsive. In this case, debilitating dyskinesias and severe motor fluctuations can be the reason for consideration of DBS and can be improved. Contraindications are a high age (>70), cognitive deficits (because these might be indicative of an atypical PD syndrome), axial L-DOPA-resistant symptoms such as speech difficulties, FOG, psychiatric symptoms and general surgical contraindications like cardiovascular or respiratory problems. The main objective should be to achieve an increase of quality of life and therefore the real life situation of the patient and the expectations should be guidance in the decision. Consequently, extensive behavioural assessment of the symptoms, the psychiatric state, the response and possible dependence of DA medication (Ardouin *et al.*, 2009; Thobois *et al.*, 2010), cognitive assessment, often L-DOPA challenges and brain scans all have to precede the decision for the DBS surgery. This requires an extensive interprofessional team of neurologists, neurosurgeons, neuropsychologists and internists (Bronstein *et al.*, 2011). Post-operative management can be complex and time consuming as well. It often takes several months until the optimal settings

are found and they require close monitoring to be able to respond to changes due to disease progression (Castrioto *et al.*, 2013).

Several companies offer stimulation systems which are becoming increasingly sophisticated. A major improvement was the development of devices carrying rechargeable batteries. New advances will include closed-loop modulation systems, which allow adjustments of stimulation parameters based on a feedback system – these have so far been used in DBS for epilepsy (Smith *et al.*, 2010). Implantation techniques depend on training and experience of the surgeon and the capacities of the hospital. Different brain navigation systems exists (whether frame-based or frameless) and pre-operative assessment for the determination of target coordinates and calculation of the trajectory are based on different techniques including MRI and CT scanning, ventriculography, and intraoperative neurophysiological monitoring. Surgeons also differ in the use of general or just local anaesthesia. Confirmatory MRI scans after implantation in the operating theatre can detect problems of misplaced electrodes or haemorrhages immediately which ideally can be addressed during the same procedure (though not all centres can offer this). Bilateral electrodes are implanted during one or two procedures and the extension cables and pulse generators can be implanted the same day or any time after this (Benabid *et al.*, 2009).

Programming is intricate because the available range of frequency, pulse width and amplitude allows a large number of possible combinations. In addition, the electrodes are often quadrupolar, offering a variety of contact configurations as well. The main aim should be to achieve an optimal clinical benefit, avoiding adverse side effects. Since the use of re-chargeable devices, current consumption is not a very important factor to consider anymore.

1.5. DBS in behaving rodent models

The rat brain is of course considerably smaller than the human brain and in a laboratory setting the surgical equipment generally does not include possibilities of *in vivo* scanning and intraoperative confirmation of electrode placement. Neither can stimulation settings be adjusted until an optimal benefit is achieved; on the contrary, an experiment requires strategic and consistent decisions on independent variables and maximal control of possible confounding variables. Therefore, many technical considerations have to be taken into account when deciding on materials and techniques. In the literature, most DBS studies in behaving rats are acute (Benazzouz *et al.*, 2000; Gubellini *et al.*, 2009; Schulte *et al.*, 2006; Spieles-Engemann *et al.*, 2010). For stimulation, the rats are mostly implanted with electrodes which are then connected to a pulse generator. An interposed swivel prevents twisting of the connecting cables and allows relatively free movement of the rat. Using this technique, rats in an STN-DBS study were tested for PPI of startle (Lindemann *et al.*, 2012) or during PPTg-DBS, rats were tested in different tasks including the cylinder test, adhesive removal test and open field exploration (Rauch *et al.*, 2010). Other solutions are implantable devices (Paulat *et al.*, 2011), encapsulated in silicon rubber and sterilized before implantation. However, this technique poses a greater risk for infections and in case of device failure there is no direct access to it. Others have developed devices that can be carried by the rat in a backpack (Nowak *et al.*, 2011). This, however, limits the rat's mobility and can increase stress and anxiety. Forni and colleagues developed a microstimulator that can be attached and detached to a support base on the skull, which is connected to the implanted electrodes (Forni *et al.*, 2012). This solution appears to be the most elegant, because it gives the rat completely free movement as long as the device is well balanced and not too heavy. It is constantly accessible for control and maintenance, the battery can be changed (which allows chronic stimulation over a long period of time), it decreases the risk of inflammation and mechanical irritation of

subcutaneous tissue and, in the case of device failure, it can be repaired or exchanged. For these reasons, the decision in the present study was to create a headstage for the stimulation device. The microstimulator was designed by a colleague of the Strathclyde Institute of Pharmacy and Biomedical Sciences Richard Pinnell. (For more details please see below.)

For this study, first a series of methodological aims needed to be achieved before the experimental questions could be addressed:

Methodological aims:

(i) In order to accommodate a headstage on the skull of the rats that would be able to hold the DBS device securely in place for chronic stimulation, the size of the rats needed to be increased. Hence, the well-established PPTg coordinates for smaller rats required verification and, if necessary, adjustments. (ii) Small electrodes and a headstage offering secure fixture of the device but also quick release of it had to be developed and surgical techniques established. (iii) The stimulation system – electrodes, headstage, device, battery – had to be tested for its effect on normal behaviour of the rats and its overall durability in a long experimental protocol.

Experimental Aims:

The experimental aim of this study was to define more accurately what the potential for PPTg-DBS might be in order to make predictions about what effects might be expected from chronic stimulation of particular parts of the PPTg. Taking forward the refined PD model described in the previous chapter two experiments are presented here that describe the effects of DBS of different sites within the PPTg – namely the anterior and the posterior part of the structure - on gait and L-DOPA induced side effects. Before, methodological considerations and the respective pilot studies are summarised.

2. Pilot 2: Coordinates pilot

2.1. Introduction

The coordinates for lesions of anterior and posterior PPTg as used in the previous experiments have been employed in this lab for many years and the rate of successful toxin infusions into the correct site was high. However, in order to increase the stability of the headstage in this experiment the decision was made to use bigger animals than before (minimum of approximately 380g instead of 330g) to have a bigger surface to work on and a thicker skull to hold the weight of the stimulation device. Changing the animals' weight required verifying the accuracy of the coordinates.

2.2. Methods and procedure

A total of eight rats at a mean weight of 385g (range 379g – 393g) were used to test and correct the coordinates for ibotenic acid infusions into anterior and posterior PPTg in two sets of two rats and one set of four.

1) n = 2 rats received unilateral lesions using 200 nl of 0.06M ibotenic acid into the previously used target sites with the following coordinates (in mm): anterior PPTg: + 0.6 from IAL, ± 2.0 from midline and - 7.0 from dura; posterior PPTg: - 0.8 from IAL, ± 1.9 from midline and - 6.5 from dura.

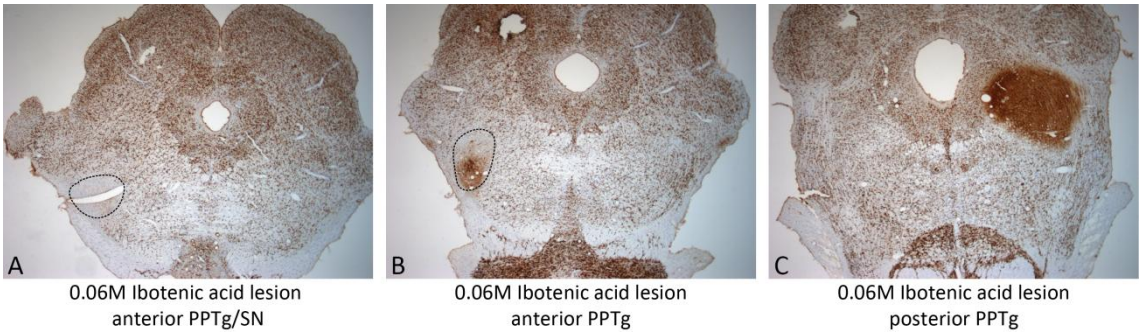

Figure 5.1. Representative histological photomicrographs of lesions in anterior and posterior PPTg after infusion of 200nl of 0.06M ibotenic acid per site at previously used coordinates (see text for details). Sections are all NeuN/Cresyl stained. **(A)** The lesion covers the very rostral part of the PPTg and reaches probably into SN; **(B)** The medial-lateral and dorsal-ventral position in anterior PPTg looks good. **(C)** The posterior lesion sits too high. The brown patch is surprising but appears repeatedly in these pilots, however have hardly ever been seen – or only in very extensive, full lesions – in previous (and later) experiments. The reason can be that these rats were perfused only 2 days after the surgery, whereas usually there is a longer period of time between lesion and perfusion. The dark stain is likely the product of an immediate but transient inflammatory response to the injection.

After perfusion, 2 days after surgery, the brains were sectioned and stained for NeuN/Cresyl to determine the lesion placement (see Figure 5.1.). Based on the findings, the decision was made to move the anterior coordinate backwards by 1 mm and the posterior electrode 2 mm more ventrally. Given that the surgery is performed at an angle with the nose of the rat elevated, going “deeper” also means going further caudally.

2) n = 2 rats therefore received unilateral lesions using 200 nl of 0.06M ibotenic acid using the following coordinates (in mm): anterior PPTg: + 0.5 from IAL, ± 2.0 from midline and - 7.0 from dura; posterior PPTg: - 0.8 from IAL, ± 1.9 from midline and - 6.7 from dura.

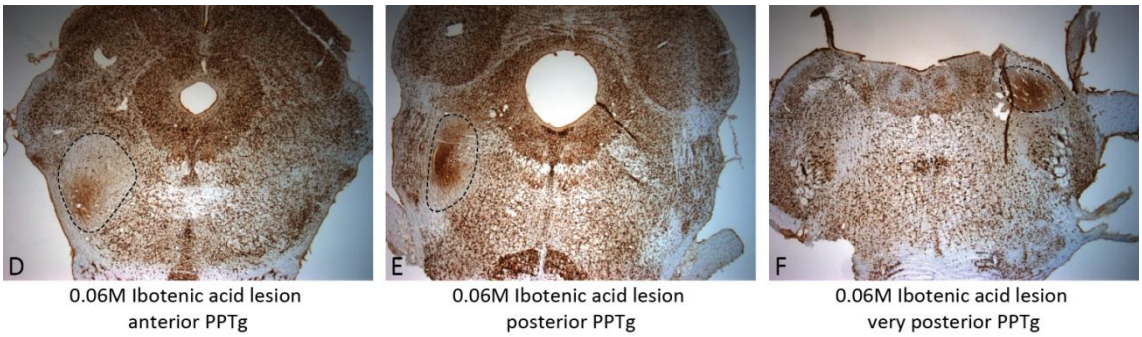

Figure 5.2. Representative histological photomicrographs of lesions in anterior and posterior PPTg after infusion of 200nl of 0.06M ibotenic acid per site at adjusted coordinates which brought the anterior infusion further caudal and the posterior lesion more ventral/caudal (see text for details). Sections are all NeuN/Cresyl stained. **(D)** Lesion placement is good, the lesion size however is on the large side. **(E)** The dorsal-ventral position is much better. **(F)** The posterior lesion reaches into several sections caudal of the PPTg).

The placement of the lesions of the posterior infusion appeared to be reaching too far caudally of the end of the PPTg. In addition, especially one lesion was more extensive than expected and in comparison to others. This obviously could have been due to an inexact volume of the toxin and therefore creating a bigger lesion. Nevertheless, two more changes were piloted based on these observations:

3) n = 2 rats received infusions of 200 nl ibotenic acid into the same (changed) coordinates used in 2) however at a lower concentration of 0.04M. In n = 2 rats 200 nl of 0.06M ibotenic acid were infused, moving the posterior coordinate 1 mm further rostrally, keeping the previously changed deeper dorsal-ventral measurement.

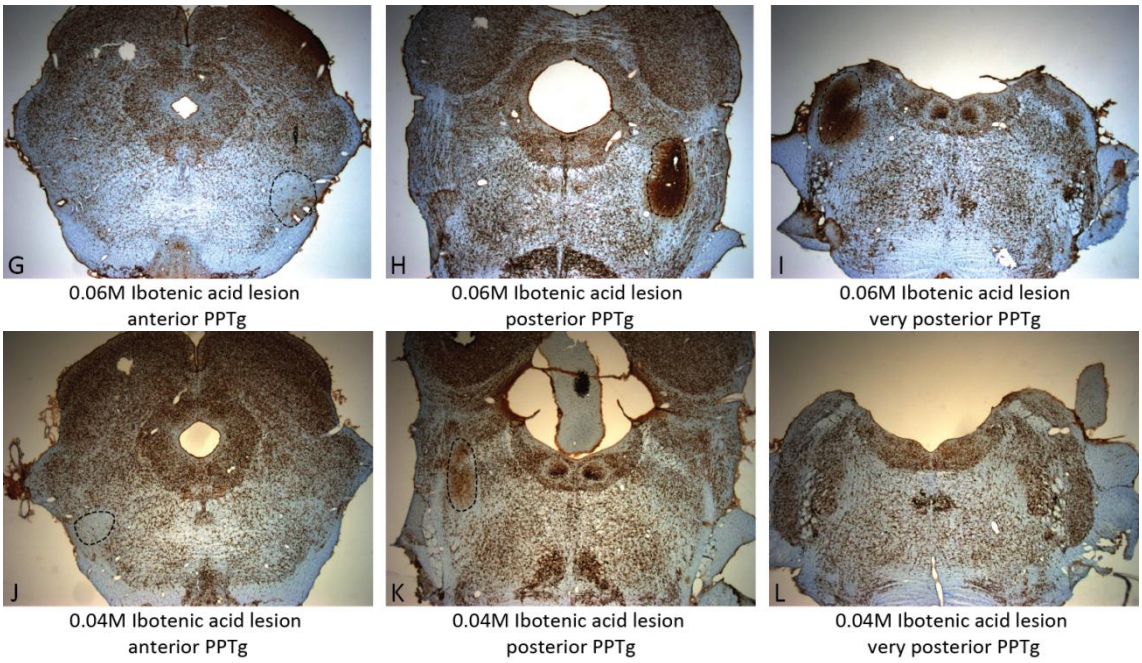

Figure 5.3. Representative histological photomicrographs of lesions in anterior and posterior PPTg after infusion of 200nl of 0.06M ibotenic acid per site at further adjusted coordinates which brought the posterior infusion further rostral (**G – I**; see text for details);and after infusion of 200nl of 0.04M ibotenic acid at coordinates used in (ii). Sections are all NeuN/Cresyl stained. **(G)** Lesion placement is good. **(H)** Lesion reaches a bit too far ventral. **(I)** The posterior lesion still reaches into several sections caudal of the PPTg. **(J)** Position is good, lesion size is small. **(K)** Good placement, too small. **(L)** No further lesion behind the caudal end of the PPTg.

The purpose of reducing the toxin concentration was to avoid creating a large lesion that went much further caudal than the PPTg. This was successfully done with 0.04M of ibotenic acid, but the overall lesion size was considered too small. The coordinates as adjusted and used with 0.06M ibotenic acid produced satisfactory lesion placements, though still slightly caudal.

Nevertheless, the decision was made to use these coordinates, because moving the posterior infusion further rostral would have carried the risk of overlapping lesions of anterior and posterior infusions.

The final coordinates therefore were (in mm): anterior PPTg: + 0.5 from IAL, ± 2.0 from midline and - 7.0 from dura; posterior PPTg: - 0.7 from IAL, ± 1.9 from midline and - 6.7 from dura.

3. Pilots 3 – 5: Development and pilots of the DBS system (pulse generating device, headstage and electrodes)

3.1. Introduction

3.1.1. Device

As described above critical features in the pulse generating device were the possibility to easily detach the device for maintenance and battery exchange, a wireless operation of the device, and a size and weight which allowed to place the device onto the rats' head rather than implanting it subcutaneously. Richard Pinnell, a PhD student under the supervision of Professor Judith Pratt, was developing a wireless DBS device in the course of his studies. Together we developed a head mountable fixture for the device, which was small enough to fit on the rats' skull offering enough stability to hold the device.

Further, specific decisions had to be made regarding the configurations and technical features of the device. (i) Constant-voltage or constant-current: Constant-voltage (or voltage control) stimulators maintain a constant voltage emission, and if the stimulus wave form is a rectangular voltage pulse, the actual current pulse does not have a rectangular shape and will vary. The fluctuations in resistance due to different and changing compositions of brain tissue can have an important effect on the actual current. Constant-voltage devices consume less battery power. Constant-current (or current control) stimulators: the amplitude of the current pulse is independent of the electrode-tissue impedance, because the voltage curve will be

constantly adjusted to create a consistent current flow. (ii) Mono-phasic vs biphasic: Mono-phasic pulses result in a charge build-up at the electrode/tissue interface, which causes damage to the tissue. An alternative are biphasic pulses with a second pulse going in the other direction (anodic pulse) of the same size to achieve a charge balance at the electrode poles (Butson and McIntyre, 2007; Hanson *et al.*, 2008). In the clinical setting, the stimulators deliver pulses followed by a repolarising pulse of smaller amplitude. (iii) Channels: the original device was designed to record from one channel (corresponding to two electrodes) and stimulate through a second one. By eliminating the option for electrophysiological recording space was freed on the central processing unit for further control over stimulation settings (pulse shape, frequency, pulse width, intensity) without having to increase the size of the device. Furthermore, it offered a second channel for possible simultaneous stimulation of two brain structures; an option also discussed in the literature (Ferraye *et al.*, 2011; Stefani *et al.*, 2007). (iv) Ranges: the device needed to offer programmability of stimulation parameters covering at least 20 – 130 Hz for the stimulation frequency, 60 – 500 µs for the pulse width and 30 (or as low as possible) – 400 µA stimulation amplitude. (v) Safety: the original device very cleverly offered the possibility to change stimulation parameter wirelessly, without having to detach the device from the animal's head. However, for safety reasons (for complete control over a continuous and stable stimulation without changes to the settings over the entire time of chronic stimulation) the default code of the device's CPU needed to be easily programmable to ensure that no change of stimulation settings would occur in the case of a device failure and consequential reset. Further, the device required a switch, which would offer a quick interruption of the stimulation activity.

3.1.2. Electrodes

The choice of the electrode design needed to reflect the need for a small electrode that causes the least amount of mechanical damage as possible. Furthermore it had to deliver current to the tissue precisely and the stimulation-induced damage – inflammation, glial scar formation – kept to a minimum. Electrochemical reactions at the interface of electrode and tissue are unavoidable (Gimsa *et al.*, 2005). The risk of damaging tissue depends on the electrode properties, stimulation waveform, charge per phase, charge density, and total stimulation time (Harnack *et al.*, 2004). The right choice of material can significantly reduce the amount of damage. Stainless steel has the advantage of being strong but unfortunately causes significant damage to neuronal tissue within a wide region around the tip of the electrode after high frequency stimulation (HFS) (Harnack *et al.*, 2004). In fact, stainless steel showed the lowest resistance to corrosion (for direct current) in comparison with platinum, platinum/iridium and tungsten (Stevenson *et al.*, 2010) and damaged volume induced by Pt/Ir electrodes was significantly lower in comparison with stainless-steel electrodes (Gimsa *et al.*, 2005; Harnack *et al.*, 2004). Platinum/Iridium alloys in a proportion of 90:10 is a commonly used material with a higher biocompatibility. It also evokes tissue responses, though these were described as being less than with other materials such as stainless steel, titanium, tungsten (Geddes and Roeder, 2003).

No electrodes are available which represent a comparable downscaled version of multi-polar electrodes used in human DBS. One option is to use a monopolar electrode, which consists of an electrode with one pole (usually the cathode) at the target site in the brain and an extracranial anode. This type of electrode might influence a larger area than a bipolar electrode. Bipolar electrodes have an increased target specificity compared to the spherical field created with a unipolar electrode. A bipolar electrode can either consist of two separate

unipolar electrodes with a specific pre-defined distance between which the current travels. A further option is a concentric electrode, which is made of a cannula through which a wire is threaded. The tip of the cannula and the tip of the wire make the two poles. The limitation of the parallel wires is the difficulty in maintaining consistently the same distance between the two poles. Concentric electrodes have the lowest stimulus artefact and their biggest advantage is that they do not have any directionality – orientation of the electrode while implanting it is of no issue.

Impedance depends on the electrode material, the nature of the tissue, the surface area and the stimulation parameter. It decreases with increasing frequency and current density. The impedance of the electrode-tissue interface determines the actual charge delivered to the tissue, the volume of neural activation (Butson and McIntyre, 2005), the distribution of current density on the electrode and therefore consequence like neural excitation, tissue damage and electrode corrosion (Wei and Grill, 2009). *In vivo* impedance quantification of electrode-electrolyte interface was not possible in this study. However *in vitro* identification of impedance in aCSF was performed to obtain a point of reference for the comparison with other electrodes and to test consistency of these homemade electrodes.

3.2. Establishing materials and methods

3.2.1. Device

The microstimulator consisted of two printed circuit boards (PCBs) containing the transceiver module, voltage amplifier, constant-current generator and microcontroller. The microcontroller could be programmed for the required frequency (range 0.1 Hz – 5 KHz) and pulse width (range 10 µs – 500 ms). A variable resistor offered the possibility of manual adjustment of the devices intensity output individually for each channel (range 30 µA – 1.5 mA). It was designed to deliver constant current square waves: with either anodal, cathodal, or

biphasic pulses. For system configuration (parameter setting) the programme software IAR Embedded Workbench IDE was used by connecting the device to the computer via a debug-interface module and a custom-built reprogrammer circuit (built by Richard Pinnell). For quick interruption of the stimulation circuit a slide-switch was interposed. The final dimensions of the device were 1.5 cm x 3 cm x 0.5 cm at a weight of 5g.

3.2.2. Batteries

The use of button cell lithium batteries allowed keeping the weight and the size of the pulse generator system small, however at the cost of having to replace the batteries regularly for chronic stimulation. The choice fell on CR2032 (see Figure 5.4.) because of their flat shape that would only add a few millimetres (about 5 mm) to the device's dimensions. In a series of test running the device at different stimulation parameters using this battery I could confirm that when running the device at 25 Hz, 160 µs at a biphasic mode with the stimulation intensity set relatively high [about 500 µA following the results of (Rauch *et al.*, 2010)] the battery would last for a guaranteed 76h before the device's performance became unstable. At a higher frequency of 130 Hz and a pulse width of 400 µs this threshold dropped to 60h. Using two channels of the device (which would correspond to four electrodes) a guaranteed stable performance lay between 24 and 48h. On the basis of these tests the decision was made to change the batteries every other day to avoid irregularities of the chronic stimulation. The battery added about 3.5g to the weight of the stimulation device.

3.2.3. Headstage

Chronic stimulation was possible with a connector stage mountable to the skull, which connects to the implanted electrodes. The home cages were modified to prevent snagging. In several pilot surgeries (Pilot 3) the headstage was finalised and adjusted in a further pilot during which the wearability was tested over a prolonged period of time.

Figure 5.4. (Pilot 3) Photographs illustrating the development of the headstage design. **(A)** The first design was small. However, it did not offer enough support for the device. Especially after decreasing the friction between headstage and device: initially the device was sitting too tightly on the pins, which made it difficult and stressful for the rat to attach and remove the device without risking to the adhesion of the headstage to the skull. **(B)** Also, because of this, there was a gap between the device and the base of the headstage. **(C)** shows the device and the first prototype of the headstage. **(D)** The second version featured a case for the device to sit in and a screw, which allowed securing the device to the headstage **(E)**. The final version of the headstage **(F)** had an additional second screw on the opposite side (not visible here). **(F)** also shows the final device and the battery.

The DBS device had to be well tolerated by the rat in order to assure its well-being; further gait parameters should not be affected by simply wearing it (in OFF mode) to not confound the results. For this reason in Pilot 4 three rats received implants of electrodes into the PPTg and headstages as presented in Figure 5.4.E. The first of these animals was kept for three weeks and monitored if the headstage had an effect on the rat's wellbeing and if it adapted well to manipulations of the headstage when attaching and detaching the device. The rat did not receive any stimulation. The headstage and device were tolerated well and did not modify the spontaneous activities of the animal such as feeding, sleeping and moving and neither did the attached device. Figure 5.5. shows how well the rat adapted to extra height on its head when sleeping.

Figure 5.5. (Pilot 4).The rats adapted well to the device in their basic activities.

The other two rats also had implanted electrodes connected to headstages. After a week of recovery, the rats were trained on the CW and then tested, first without wearing the device for two days and then with the device attached (no stimulation) for another two days. The testing protocol followed the CW testing protocols described in the General Methods and the other experimental chapters. Figure 5.6. shows that wearing the device itself – without stimulation –

caused no difference in the rats' gait measured on the CW. Univariate ANOVAs confirmed the absence of any significant difference between the rats carrying the device and not (BOS front: $F_{1,6}$ = 0.847 p = 0.393; BOS hind: $F_{1,6}$ = 0.173 p = 0.692; Stride Length front: $F_{1,6}$ = 0.081 p = 0.786; Stride Length hind: $F_{1,6}$ = 0.002 p = 0.963; Swing Speed front: $F_{1,6}$ = 0.249 p = 0.635; Swing Speed hind: $F_{1,6}$ = 3.437 p = 0.113; Regularity Index: $F_{1,6}$ = 1.000 p = 0.356). Pilot 3 also served to refine the surgical techniques (elevated nose for electrode implant to avoid damage to the sinus situated right above the PPTg) and determination of the required length of the electrode and of the connecting wires. Further, craniotomies were performed above the striatum as required for striatal infusion of 6-OHDA to test the skull stability. These extra holes in the skull did not seem to have an effect on the stability of the headstage.

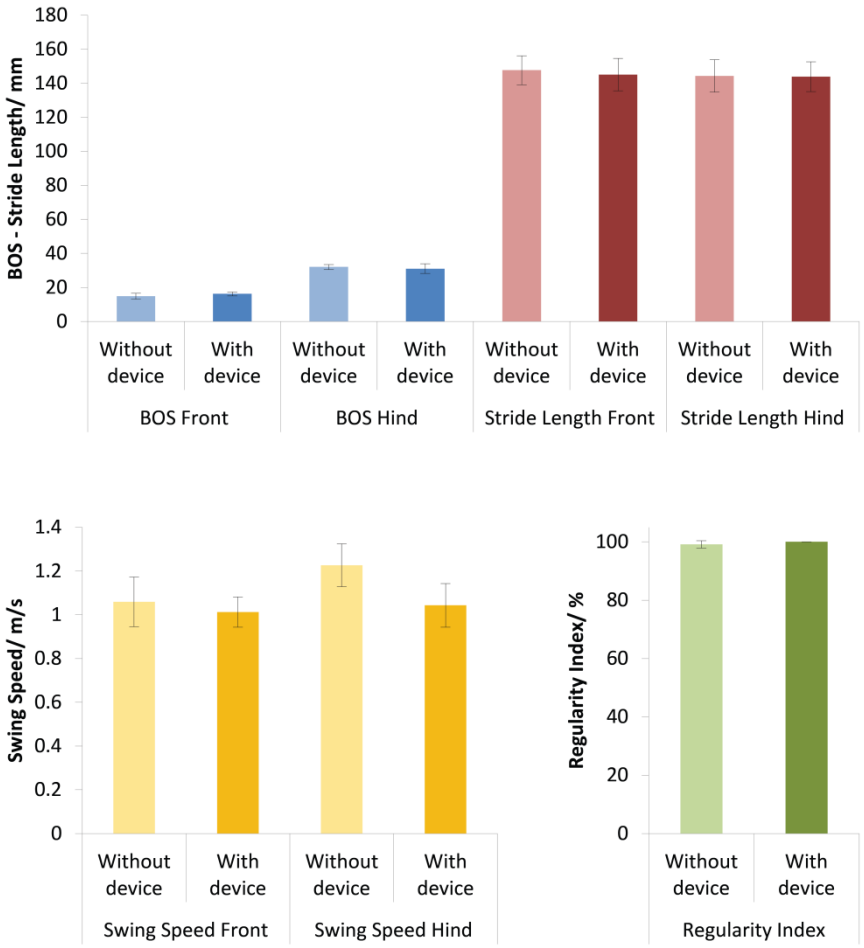

Figure 5.6. (Pilot 4). Selected gait parameters of rats without and with the device attached to the headstage. Graphs show group means ± SEM.

3.2.1. Electrodes

The electrodes were made in the lab using Teflon coated platinum/iridium (90:10) wire (uncoated diameter 50 µm, coated diameter 114 µm; Science Products GmbH, Hofheim, Germany) threaded through a 30 ga stainless steel cannula (Plastics One, Roanoke, VA, USA) protruding 500 µm. The cannula was insulated with polyolefin 2:1 heat shrink. Both the cannula and the wire were bared of the insulation material at the extremity leaving a bared tip of 250 µm. The exposed surface area of each pole was therefore approximately 0.041 mm^2 for the exposed tip of the inner wire and 0.236 mm^2 for the exposed end of the cannula. This electrode has a total diameter of 300 µm (cannula) + 50 µm (insulation). Short pieces of wire connect both, the cannula and the wire, to connector pins, which were to be connected to the headstage during implantation surgery. Before use the electrodes were tested fully immersed in 0.9% saline solution to check for breakages of the insulation. Before deciding on this very thin Pt/Ir wire, which allowed the use of a 30 ga cannula, it was tested, whether it could withstand the resistance of brain tissue without bending, by pushing it into clay at the above described parameters (500 µm protruding from a 30 ga cannula).

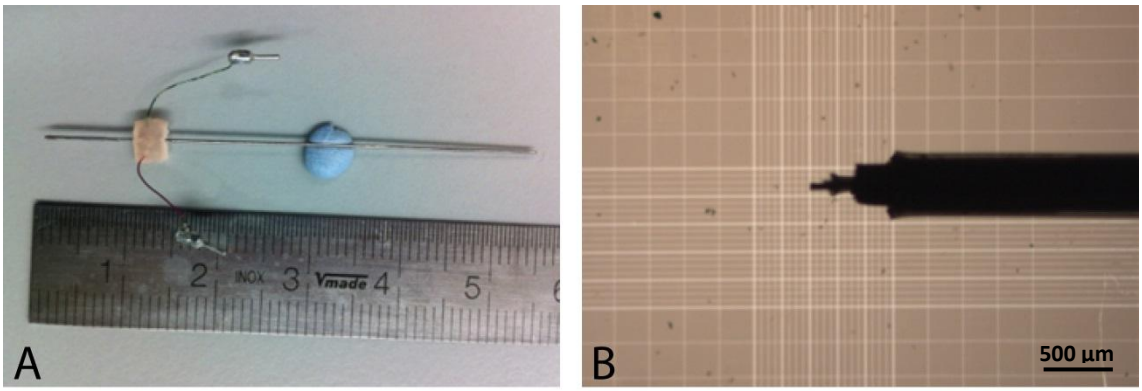

Figure 5.7. Photograph and photomicrograph of concentric electrodes. **(A)** The electrode is attached to a guide cannula by means of dental cement. The green and pink wires connect the cannula and Pt/Ir wire respectively to the headstage.

To determine the impedance of the electrodes a sample of 6 electrodes were randomly selected and measured performing *in vitro* stimulation with the electrode tip immersed in aCSF (148mM NaCl, 2.7mM KCl, 1.2mM $CaCl_2$, 0.85mM $MgCl_2.6H_2O$) at room temperature using a Solartron 1260 impedance analyser (Solartron Analytical, Farnborough, UK). The electrodes were stimulated in constant current mode at stimulation parameters used in the experiment at (a) 25 Hz, 160 µs and 50 µA and (b) 25 Hz, 160 µs and 100 µA. The electrode impedance for 50µA was 22.3 ± 1.6 kΩ and for 100 µA the impedance was 21.2 ± 1.4 kΩ [calculated from the measured reactance and resistance using the equation |Z|= sqrt((Z'^2)+(Z''^2)), with Z' = resistance and Z'' = reactance]. This impedance is much lower than the impedance tested in vitro by Forni and colleagues (2012) who used parallel, bipolar, Pt/Ir electrodes. However, the impedance evaluated in vivo - 16.5 ± 1.3 kΩ - was very similar to the impedance measured here. Here, aCSF was used for *in vitro* measurements, whereas Forni and colleagues used a 0.9% saline solution. The aCSF solution might have a better conductance than the saline solution. More importantly, the measurements of the impedance showed, by the small SEM, the consistency of the construction of these homemade electrodes.

A final set of four pilot surgeries (Pilot 5) were conducted to assess the effect of stimulation and the feasibility of chronic stimulation. Two rats were implanted with bilateral electrodes (here still all stainless steel concentric electrodes) into anterior PPTg using coordinates established in Pilot 2. After a minimum of 7 days of recovery, the stimulation-induced side effects for each animal were determined at a stimulation of 25 Hz (frequency) and 160 µs (pulse width) and the intensity adjusted accordingly. Both rats were stimulated at a final amplitude of 96 µA. One of the animals was stimulated over 12 days before it lost its device. In the meantime, it had lost the battery once as well. The other rat lost the device after 5 days; further, the switch had broken off. This pilot showed that the general behaviour, once a stimulation intensity was found that did not cause any side effects, was not affected by the

stimulation and the rats were able to feed, drink and groom themselves. The spontaneous activity in the homecage did not seem to be altered. Further, it showed the exposure of the device to possible damage in the homecage. For this reason the decision was made to wrap the device into parafilm (Bemis Company, Inc., Neenah, USA) after setting of parameters was completed and the battery attached. This protected the device from water and dust and held the battery in place.

Figure 5.8. (Pilot 5). Final design of headstage and stimulation device. The device is wrapped in parafilm for protection. The red LEDs indicate ongoing stimulation of bilateral electrodes.

The final two rats underwent further pilot surgeries to finalise the electrode design (length of the electrode and use of Pt/Ir). Both started chronic stimulation 9 days after surgery at 25 Hz, and 160 µs. Unfortunately, one rat lost its headstage after 27 days. The other rat was perfused after 43 days. During this time both rats were successfully stimulated for 10 days without causing changes in the rat's general behaviour and wellbeing. During the rest of the time they were kept with the device attached to the headstage to assess long-term headstage stability.

3.2.2. Stimulation parameters

Stimulation parameters for the experiments described in this chapter were:

(i) Pulse shape: Biphasic square constant current waves to avoid charge build-up at the electrode/tissue interface as described above.

(ii) Frequency: 25 Hz. Based on the literature more beneficial results were achieved with LFS rather than HFS in the PPTg (Jenkinson *et al.*, 2004; Mazzone *et al.*, 2005).

(iii) Pulse width: 160 µs and 500 µs. A crucial question regarding the mechanisms behind DBS is the question whether soma or axons are mainly affected by the stimulation. The truth is that different factors determine current spread and therefore the effect of DBS: the characteristics of the stimulated tissue play a crucial role in current spread: axon diameter, whether axons are myelinated, axon orientation with respect to the electrode, and what is closest, soma or axon. Chronaxie describes a tissue-excitability parameter and is the determining factor for the choice of pulse duration. It is the minimum time required to stimulate an excitable tissue at double the strength of the rheobase (minimal current amplitude that is required for depolarisation threshold of the cell membrane). Pulse width and amplitude are inversely exponential: With higher amplitude the pulse width can be shorter. In addition, the type of neural element influence the chronaxie and it was suggested that longer pulse widths influence the cell soma, whereas shorter pulse widths mainly affect axons (Kern and Kumar, 2007). For this reason a shorter pulse width of 160 µs (with change of direction after 80 µs) was chosen as described by Rauch and colleagues (Rauch *et al.*, 2010), and a longer pulse width of 500 µs.

(iv) Intensity: Individual differences and slight deviations during implantation surgery can affect the effect of the stimulation. In PD patients, all parameters are individually set and modified until the best clinical benefit is achieved. Here, frequency and pulse width were

maintained constant, but the stimulation intensity was set individually according to the stimulation induced side effects observable in the rats. (Please refer to the General Methods for details.)

4. Main experiments: Anterior and posterior PPTg-DBS

4.1. Methods

4.1.1. Subjects

Forty-two adult male Lister-Hooded rats (Harlan Olac Ltd, Bicester, UK) were used in two experiments. The first stimulation experiment targeted the posterior PPTg (pPPTg-DBS); the second experiment targeted the anterior PPTg (aPPTg-DBS). At the start of baseline testing the mean weight of the animals of the pPPTg-DBS experiment was 385g (range 378 – 390g) and 380g (range 363 – 390g) of the aPPTg-DBS experiment. The animals were put on food control once they had reached their target weight of 380g so all animals maintained their weight for the first surgery (pPPTg-DBS: mean weight = 384g; range 380 – 388g; aPPTg-DBS: mean weight = 381g; range 367 – 387g). A stable mean weight of 380g with a range of 371 – 389g and 379g (range 366 – 396g) was maintained over the entire period of the experiment. For further details on housing, please refer to the General Methods.

4.1.2. Experimental protocol

In both experiments parkinsonian models (traditional and refined) and sham controls with implanted DBS electrodes into anterior or posterior PPTg underwent a series of different testing conditions after surgery (represented schematically in Figure 5.9.). After 11 days of CW training a post-surgical baseline was taken (3 days), then their response to levodopa was observed on 2 consecutive days. After a washout period of 2 days rats were re-tested on the CW before DBS was switched on. Stimulation was chronic and lasted 6 days: rats were tested

on day 4, 5 and 6 during that period. After a day of rest from stimulation, DBS was switched on again, maintaining the same frequency but changing the pulse width. Testing followed the same pattern as before. After testing on day 6 of stimulation, the DBS was left on. The following 2 days the rats were tested for their reaction to the combination of L-DOPA and DBS. DBS was then switched off and the rats given a day of rest before being re-tested for the last time.

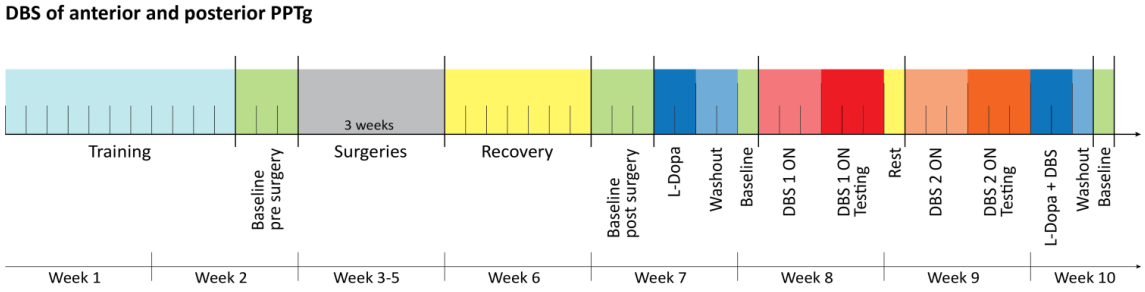

Figure 5.9. Experimental design. At the start of the experiment all animals were trained for 11 days on the Noldus CatWalk. They were then tested for 3 days to obtain a pre-surgical baseline. Rats then underwent 1 or 3 successive surgeries for 6-OHDA infusion, PPTg lesion surgeries and DBS electrodes implantation with 7 days in recovery in between procedures. 7 days after the last surgery they were then re-tested on the CW under post-surgical baseline condition, after L-DOPA administration, under two different chronic DBS-ON conditions and DBS-ON + L-DOPA condition. For more details please refer to the text.

4.1.3. Surgical procedures

Surgery followed the protocol as described in the General Methods. In brief:

Bilateral DA depletion of the SNc

7 µg (free base weight) of 6-OHDA-hydrobromide dissolved in 2 µl of 0.1% L-ascorbic acid /0.9% saline were delivered to 4 sites in the striatum of both sides at the following coordinates (in mm): (1) AP: + 1.3 (measured from Bregma), ML: ± 2.6 (measured from midline at the skull

surface), DV: - 5.0 (measured from Dura); (2) AP: + 0.4, ML: ± 3.0, DV: - 5.0; (3) AP: - 0.4, ML: ± 4.2, DV: - 5.0; (4) AP: - 1.3, ML: ± 4.5, DV: - 5.0. Infusions were controlled by a Harvard microdrive pump. Control rats received infusions of vehicle only (here: 0.1% L-ascorbic acid /0.9% saline solution) into the same sites. After surgery all rats were placed into a heated recovery cage and given 2-2.5 ml of Hartmann's solution (i.p.).

Combined lesions

Rats that received a combination of DA depletion and partial bilateral PPTg lesions underwent 3 separate surgeries. In 2 surgeries separated by a week the PPTg was bilaterally lesioned by microinfusions of ibotenic acid into the anterior or posterior PPTg at the following coordinates (in mm): anterior PPTg: + 0.5 from IAL, ± 2.0 from midline and - 7.0 from dura; posterior PPTg: - 0.7 from IAL, ± 1.9 from midline and - 6.7 from dura. Infusions were always made anterior first but the order of infusions regarding the choice of side was counterbalanced. Rats were infused with 200 nl of 0.06M ibotenic acid per infusion site. Control rats received infusions of vehicle only (here: PB) into the same sites. At the end of surgery rats were treated with 1-1.5 ml of Hartmann's solution (i.p.) to account for the lack of fluid intake during the time of surgery. Furthermore, they also received an i.p. injection of 0.2ml Diazepam to reduce the risk of injury due to the immediate effect of the excitotoxin. Following a further 7 days of recovery the DA depleting procedure was performed as described above.

Electrode implantation

Both PD models received implantations of concentric bipolar electrodes into either the anterior or posterior PPTg. These procedures were conducted during the same surgery as the infusion of 6-OHDA for DA depletion. After the last infusion of 6-OHDA into the striatum the skull was cleaned and dried and bone wax (Henry Schein Animal Health, Dumfries, UK) was used to close the burr holes above the striatum. Unless burr holes had already been made in

previous procedures they were now drilled above the anterior or posterior PPTg on both sides using the same coordinates as stated for the PPTg lesion procedure. Five to 6 stainless steel round-head machine screws with sharply cut threads (0-80 x 1/8 and 0-80 x 3/32); Plastics One, Roanoke, VA, USA) were screwed into the skull. The first electrode (for details about the electrode design and material please see Section 3.2.1. of this chapter) was then carefully lowered into place (anterior or posterior PPTg) and fixed onto the skull and neighbouring screw using a methyl methacrylate based dental acrylic (Simplex Rapid, Kemdent Works, Wiltshire, UK). The electrode was detached from the guiding cannula and the procedure repeated for the implantation of the second electrode. Anode and cathode of the electrodes were then carefully connected to the headstage (see details about the headstage in Section 3.2.3 of this chapter) and the headstage was then fixed with more dental cement to the skull. Rats were given an i.p. injection of 2.5 ml Hartmann's solution (Baxter Healthcare Ltd, Norfolk, UK) at the end of the procedure.

4.1.4. Training and testing on the CatWalk

Rats were trained for 11 days prior to baseline testing and surgery and tested as described in the General Methods. Red LEDs on the device corresponding to the connected electrodes indicated functioning of the device. This blinking red light often caused the recording of CW-runs to start before the rat had entered the start box of the glass walkway. On those occasions the clips were manually cut to match with the actual start of the run of the rat.

4.1.5. Gait parameters

The following gait parameters were examined: BOS, swing speed, stride length and duty cycle of both front and hind legs, print position of ipsilateral paws and the support formula of diagonal pairs of paws and three paws. For details on the gait parameters please see the General Methods.

4.1.6. Stimulation protocol

Details on intensity settings, battery changes and regular checks can be found in the General Methods. In brief; DBS devices were programmed and tested on an oscilloscope. The stimulation intensity was then manually set to the lowest point possible (~30 µA). Then, the threshold for stimulation-induced behavioural side-effects was determined for each rat individually in a step-wise procedure. Typical side effects were slowing or an arrest of exploratory behaviour up to "freezing" and "staring", erected whiskers and/or ears. Once one or several of these side effects were detected the intensity was reduced by 20% of this side effect inducing intensity and re-tested. Once satisfactory stimulation intensity was determined the device was securely fixed onto the headstage and switched on. The rat was then returned to its homecage. Final average stimulation intensities were in the pPPTg-DBS experiment: DBS1: 107 µA (range 72 µA – 136 µA); DBS2: 105 µA (range 72 µA – 152 µA; for both settings the intensity was lower for 6-OHDA group than for the other groups); in the aPPTg-DBS experiment: DBS1: 78 µA (range 56 µA – 120 µA); DBS2: 61 µA (range 40 µA – 72 µA). The devices were checked at least twice a day by inspecting if the red LEDs were indicating ongoing stimulation. Every other day the battery of the device was changed.

4.1.7. L-DOPA injections and L-DOPA induced abnormal involuntary movements

For assessment of L-DOPA induced AIMs in both PD models rats received injections of L-DOPA (12 mg/kg/ml L-3,4 – Dihydroxyphenylalanine methylester [free base weight] plus 15 mg/kg/ml Benserazide hydrochloride [free base weight] in sterile 0.9% saline i.p.). Immediately after the injection they were returned to their homecage but kept under close observation (with the top of the cage removed) to classify and quantify L-DOPA induced AIMs. Ratings of behaviour were made using a modified version of the Creese-Iversen scale (Creese and Iversen, 1973) (for more details please refer to the General Methods).

4.1.8. Histological assessment

The area of the PPTg and the SN were histologically assessed for overall neuronal damage, cholinergic cell loss and loss of dopaminergic neurons respectively, and placement of DBS electrodes (following the protocols described in the General Methods). In brief, at the end of behavioural testing rats were transcardially perfused with a fixative, the brains removed and stored in sucrose solution until they sank. A series of 1:6 coronal 30 µm sections was cut on a freezing microtome.

PPTg lesions

For PPTg assessment the corresponding sections were immunohistochemically stained for NeuN and ChAT, mounted on glass slides and examined under a light microscope. Overall neuronal damage was assessed visually on the NeuN/Cresyl stained sections and rated on a scale from 0 (no lesion) to 4 (extensive lesion). Lesions were accepted and rats included into the analysis when they received a rating of at least a 1 (small/partial lesion). Animals were excluded when both sides received a 4 (extensive lesion). Cholinergic cell survival was assessed by software assisted counts of ChAT+ neurons. The quantification of ChAT+ neurons of the part of the PPTg in which the electrodes were implanted was not possible. Therefore only ChAT+ cell loss of the non-implanted portion of the PPTg was analysed.

SNc lesions

Sections corresponding to the SN were immunohistochemically double-stained for TH and Cb and similarly examined under a light microscope. All TH+ neurons within the dorsal and ventral tier of substantia nigra part compacta (SNc; SNcd and SNcv respectively), substantia nigra pars medialis (SNm) and substantia nigra pars lateralis (SNl) were counted using a handheld cell counter.

Electrode placement

Electrode tip location in the DBS experiments was determined by examining electrode track evidence on ChAT+ stained and counterchecked on NeuN/Cresyl stained sections where there were not enough ChAT+ neurons visible to make a judgement.

4.1.9. Data analysis

FOG

The occurrence of FOG was analysed by means of the Chi-square test of independence to test whether one of the groups that suffered from FOG was more likely to do so than the other.

CW data

Preceding any analysis, the data were assessed for pre-existing differences (univariate ANOVA between groups) and the effect of the different testing conditions on sham controls was assessed (repeated measures ANOVA within conditions). The effect of DA depletion and DA depletion plus additional PPTg lesions, as well as the effect of DBS was explored by mixed ANOVAs across condition (pre surgery, post-surgery, DBS1 and DBS2, within subject factor) and between groups (combined lesion group, 6-OHDA group and sham controls, between subjects factor) and further examined for simple main effects performing univariate ANOVAs between groups and repeated measures ANOVAs in the case of significant group x condition interactions. Normality of the data was identified by the Shapiro-Wilk test and homogeneity of variance by the Leven's test. The handling of violations of these assumptions is reported in the results section when they occurred. All tests were conducted using SPSS version 21 (IBM Corp., Armon, NY, USA) and results were considered statistically significant when $p \leq 0.05$.

L-DOPA induced AIMs

Mixed ANOVAs with group as between subject factor and severity scores 1 – 6 as within factor were conducted to assess the data for interactions between severity state of dyskinesia and

groups. Simple main effects were further tested in the case of a significant interaction. In order to directly compare the effect of anterior and posterior PPTg-DBS mixed ANOVAs with group as between subject factor and ON vs OFF condition as within subject factor were conducted on relevant dyskinesia scores.

4.2. Results

4.2.1. Histological analysis

Electrode placements

Despite presenting and discussing the results of the 2 experiments together in one chapter they will be analysed and illustrated separately in the following. A total of 42 rats were used, 21 in the anterior PPTg-DBS experiment and 21 in the posterior PPTg-DBS experiment. In both experiments 6 rats each underwent surgeries for the traditional PD model, 9 each underwent the 3 surgeries necessary for the refined model (combination of DA depletion and PPTg lesion) and 6 each received control infusions of vehicles – 3 striatal infusions and 3 infusions into striatum and PPTg.

The following flow chart summarises the numbers and reasons for exclusion of subjects (Figure 5.10.)

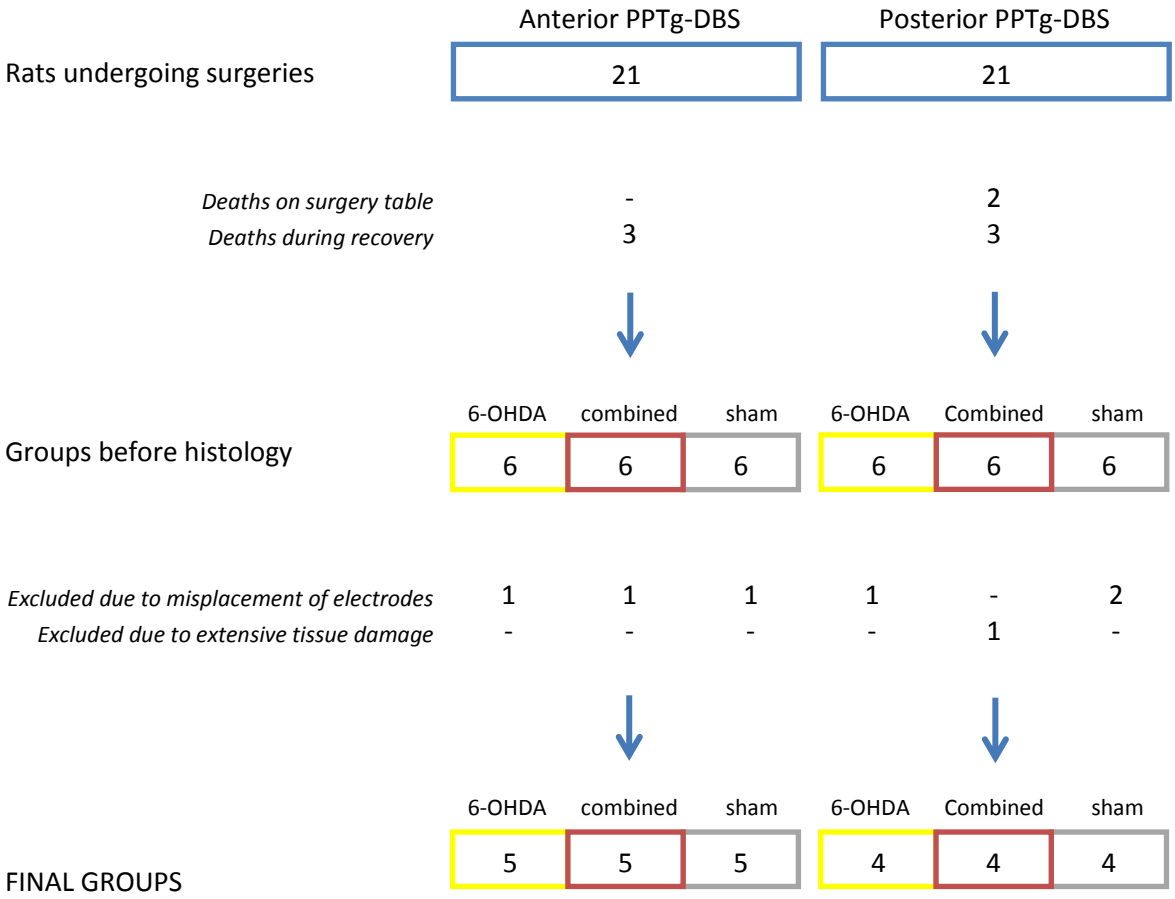

Figure 5.10. Flow chart of excluded subjects.

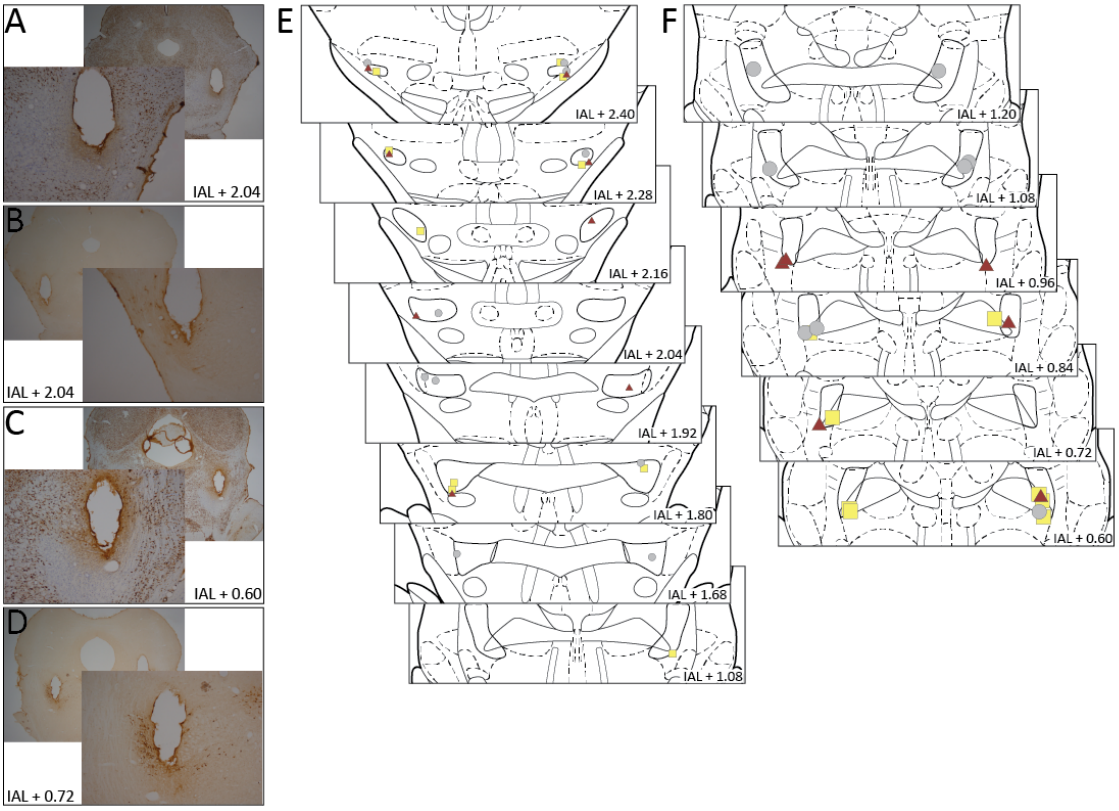

Figure 5.11. Electrode placements. Representative photomicrographs of electrode tracks in anterior **(A and B)** and posterior **(C and D)** PPTg. NeuN stained sections **(A and C)** are taken from combined lesion group and show cell loss of the PPTg area. ChAT stained sections **(B and D)** are from 6-OHDA lesion group showing cholinergic PPTg neurons. Schematic drawings (adapted from Paxinos and Watson, 2005) representing implantation sites of electrode tips in Experiment Three **(E)** and Experiment **(F)**. Note that one rat of the combined lesion group of Experiment Four was not included into the DBS testing, and is therefore not included in these schematics. Red triangles represent sites of electrode tips in the combined lesion groups, yellow squares of sites in the 6-OHDA lesion groups and grey circles of sites in the sham lesion groups.

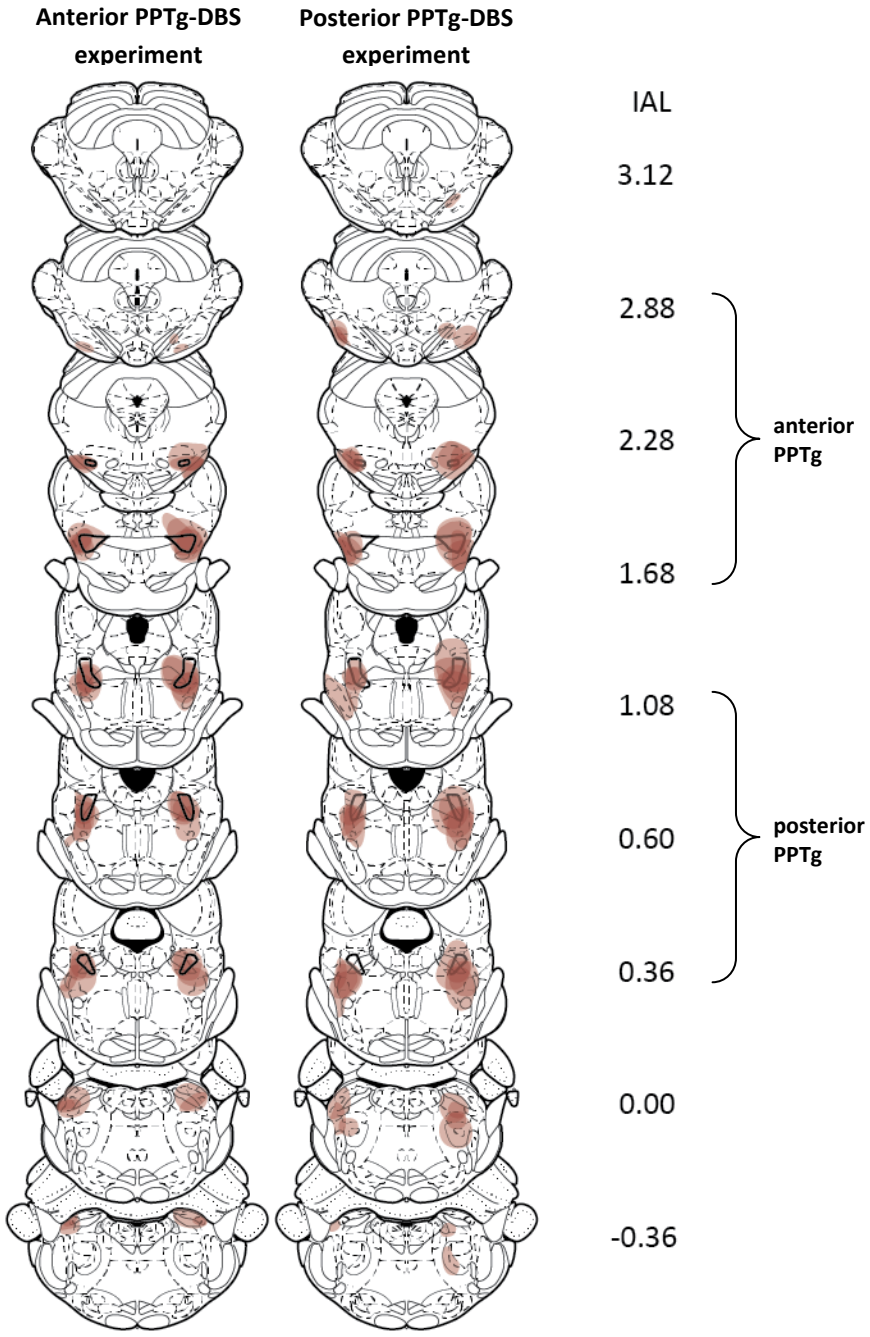

Figure 5.12. Representation of lesion extension along the rostro-caudal axis after ibotenic acid infusion on a selection of NeuN+ stained sections. The position of the schematics (adapted from Paxinos and Watson, 2005) on the rostro-caudal axis is represented as the distance from the interaural line (IAL; mm). Sections IAL + 2.88 mm through to IAL + 1.68 mm were identified as sections containing anterior PPTg neurons (identified by PPTg cholinergic neurons in shams and partially lesioned rats) and those from IAL + 1.08 mm to IAL + 0.36 mm represent sections containing posterior PPTg. The column on the left hand side represents lesions from the aPPTg-DBS experiments and the one on the right hand side shows lesion sizes of the pPPTg-DBS experiment. Lesion damage is represented in red shading.

PPTg lesions and ChAT+ cell count

Anterior PPTg-DBS experiment

All of the remaining animals bearing PPTg lesions of the combined lesion group had a NeuN/Cresyl lesion rating of at least 1 and no higher than one 3 (see Figure 5.13).

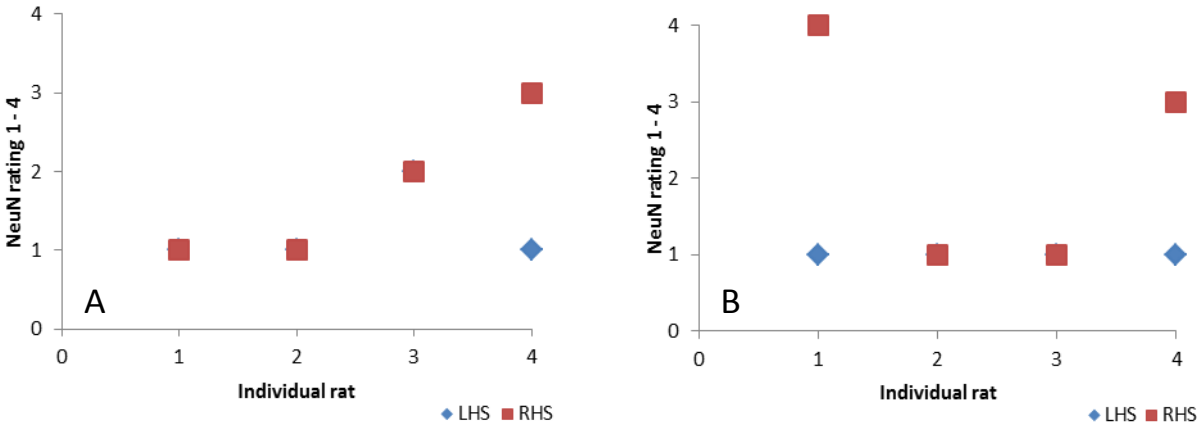

Figure 5.13. Lesion size for each rat of the combined lesion group of the aPPTg-DBS experiment **(A)** and pPPTg-DBS experiment **(B)** rated based on NeuN+ stained brain sections. Lesions were rated on a scale from 0 to 4 (0 = no lesion of the PPTg, 1 = small/ partial lesion of the PPTg, 2 = partial to full lesion of the PPTg, 3 = fully lesioned PPTg, 4 = extensive lesion covering the PPTg and surrounding tissue). Where only one symbol can be seen RHS and LHS received the same lesion size rating.

Because of the implanted electrode in anterior PPTg only ChAT+ cell loss of posterior PPTg was analysed and is presented here. The loss in the combined lesion group was 56% (range 37% to 80%). Cholinergic cell survival in posterior PPTg of the 6-OHDA group was 93% (range 78% to 106%). The percentage of surviving cholinergic PPTg neurons in its posterior part is shown in Figure 5.13.

Univariate ANOVA of the raw cell count of surviving ChAT+ neurons confirms a significant difference between groups ($F_{2,11}$ = 18.862 p < 0.001; η^2_p = 0.774). Tukey corrected post hoc tests reveal that the combined lesion group had a significantly lower ChAT+ cell count in the posterior PPTg compared to sham controls (p < 0.001). Furthermore, the 6-OHDA infusion did not cause any loss of cholinergic neurons in the PPTg: 6-OHDA group and sham controls did not differ from each other (p = 0.758).

Posterior PPTg-DBS experiment

Four rats formed the combined lesion group and had a NeuN/Cresyl lesion rating of at least 1 and no more than one side was scoring a 4 (see Figure 13 above).

Of rats with the electrodes implanted in posterior PPTg only the cell count of anterior PPTg was analysed and compared between groups. The average cell loss was 51% (range 35% to 67%). In the 6-OHDA group the cholinergic cell survival in anterior PPTg was 100% (range 74% to 122%) expressed as a percentage from the mean cell survival of sham controls (see Figure 14 below).

Univariate ANOVA of the raw cell count of surviving ChAT+ neurons found a significant difference between groups ($F_{2,9}$ = 8.210 p = 0.009; η^2_p = 0.646). Tukey corrected post hoc tests showed that the infusion of ibotenate caused a significant loss of ChAT+ cells in anterior PPTg of the combined lesion group (compared to sham controls p = 0.016). The cell count in the 6-OHDA group did not differ from sham controls (p = 1.000).

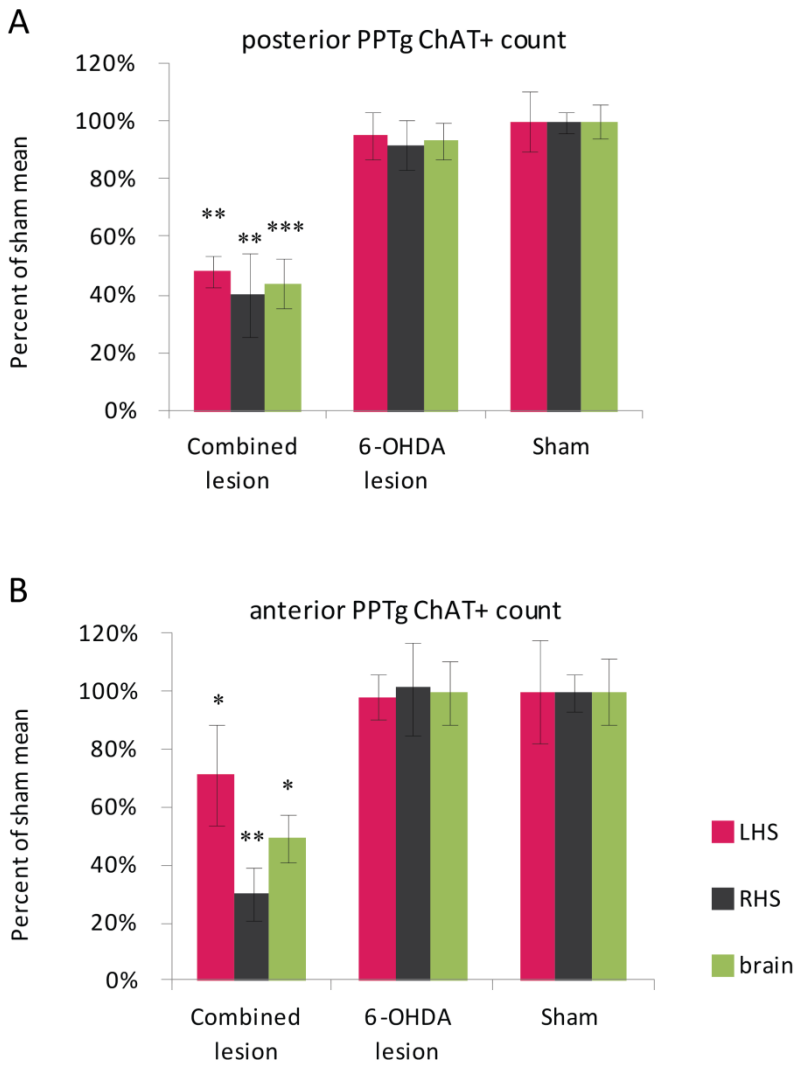

Figure 5.14. PPTg ChAT+ count as the percentage of the mean count in sham lesioned rats. **(A)** shows cell count of the posterior PPTg (aPPTg-DBS experiment) and **(B)** shows cell count of the anterior PPTg (pPPTg-DBS experiment). Graphs show group means ± SEM. *** indicates a significant difference between combined lesion group and shams with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$. 6-OHDA and sham lesion groups did not differ from each other (aPPTg-DBS: $p = 0.758$ and pPPTg-DBS: $p = 1.000$). LHS = left-hand side; RHS = right-hand side; brain = overall cell count left and right combined.

TH+ cell count in SN

Anterior PPTg-DBS experiment

6-OHDA infusions into the striatum caused an extensive DA depletion in SN: TH+ count in the 6-OHDA group was reduced by 94% (range 92% to 96%; of average sham count) and in the combined lesion group by 97% (range 95% to 99%; of average sham count). A univariate ANOVA found significant differences between groups ($F_{2,11}$ = 2211.938 $p < 0.001$; η^2_p = 0.998). The differences between the sham controls and both 6-OHDA treated groups were significant (6-OHDA group: $p < 0.001$; combined group: $p < 0.001$). Both DA depleted groups did not differ from each other ($p = 0.248$).

Posterior PPTg-DBS experiment

The DA depletion in the posterior PPTg-DBS experiment was similarly extensive in both groups which received 6-OHDA infusions into the striatum: TH+ count in the 6-OHDA group was reduced by 93% (range 92% to 95%; of average sham count) and in the combined lesion group by 94% (range 89% to 96%; of average sham count). The groups differed significantly from each other in their remaining nigral TH+ cell count ($F_{2,9}$ = 311.796 $p < 0.001$; η^2_p = 0.986). Post hoc tests (Tukey corrected) showed that the differences between the sham controls and both, the 6-OHDA group ($p < 0.001$) and the combined lesion group ($p < 0.001$) were significant. Both DA depleted groups did not differ from each other ($p = 0.971$).

Figure 5.15. SN TH+ count as the percentage of the mean count in sham lesioned rats. **(A)** shows cell count the aPPTg-DBS experiment and **(B)** from the pPPTg-DBS experiment; Graphs show group means ± SEM. *** indicates a significant difference compared to shams with $p \leq 0.001$; LHS = left-hand side; RHS = right-hand side; brain = overall cell count left and right combined; 6-OHDA and combined lesion groups did not differ from each other (aPPTg-DBS: $p = 0.248$; pPPTg-DBS: $p = 0.971$).

4.3. Behavioural results

4.3.1. Behavioural results - FOG

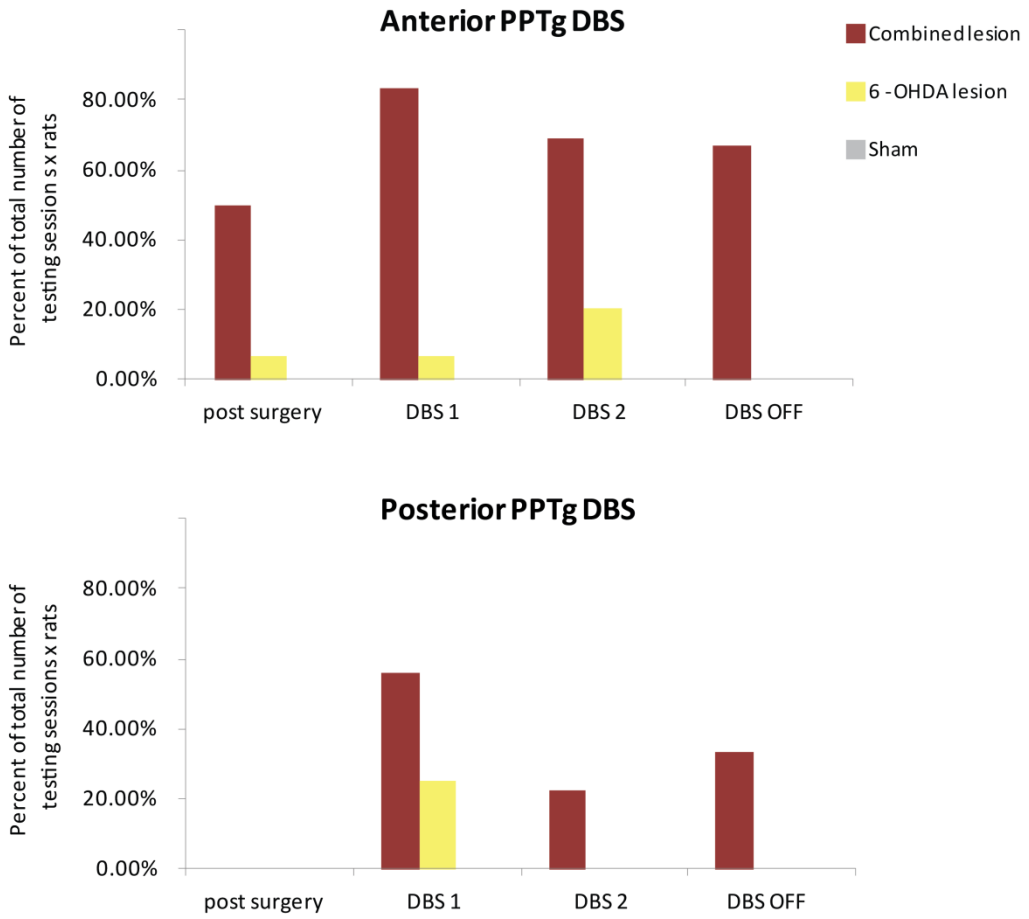

Figure 5.16. Frequency count of FOG episodes that resulted in < 3 runs per session or considerable difficulties in CW testing expressed as a percentage of the total number of testing occasions (number of testing sessions per condition x number of rats).

Testing sessions during which it was notably difficult for the rats to complete acceptable runs (for the criteria of acceptable runs, please refer to the General Methods) or where rats did not complete all 3 required runs due to severe starting hesitation – FOG – were noted and are reported as a percentage of all available testing occasion of each condition respectively (post-

surgery baseline, DBS1, DBS2, DBS off, Figure 5.16.). Chi-square tests of independence were performed to examine the relationship between the occurrence of freezing and experimental group. In the anterior PPTg-DBS experiment in each testing condition there was a statistically significant association between these variables (post-surgery baseline: $\chi^2(2) = 13.680$ $p = 0.001$; $V = 0.571$; DBS1: $\chi^2(2) = 27.756$ $p < 0.001$; $V = 0.823$; DBS2: $\chi^2(2) = 24.333$ $p < 0.001$; $V = 0.617$; DBS OFF: $\chi^2(2) = 7.200$ $p = 0.027$; $V = 0.775$). Rats of the combined lesion group were more likely to show freezing in all 4 conditions. In the posterior PPTg-DBS experiment no FOG occurred with solely the electrode in place but with the DBS OFF. A statistically significant association between group and the occurrence of FOG was only present in the DBS1 condition ($\chi^2(2) = 8.649$ $p = 0.013$; $V = 0.013$); the combined lesion group was more likely to freeze than the 6-OHDA group. FOG occurred also in the DBS2 and DBS OFF condition in the combined group, but not 6-OHDA group; however these frequencies were not high enough to reach statistical significance (DBS2: $\chi^2(2) = 5.677$ $p = 0.059$; DBS OFF: $\chi^2(2) = 2.933$ $p = 0.231$)

4.3.2. Behavioural results – Gait

Because the unexpected occurrence of FOG made it difficult in specific testing conditions to record sufficient acceptable runs (as defined in the General Methods) a total of 2 instead of 3 runs was accepted to calculate the mean of the behavioural data for the individual testing session if the animal was not capable to perform 3 runs within a reasonable time.

Pre surgery comparison

Univariate ANOVAs confirmed that there were no pre-surgery differences in any of the observed parameters (please refer to the Supplementary Table 5.1.). Violation of the assumption of normality in the sham lesion group of *BOS* front data and in the combined group of *BOS* hind data were tolerated.

Due to the FOG that occurred in the combined lesion group after surgery in the DBS OFF condition the group size was reduced to n = 3 at the time of post-surgery baseline testing.

Confirming previous results: Is the 6-OHDA group different to sham controls?

Anterior PPTg-DBS

Gait parameters in the 6-OHDA group which reflecting gait stability – *BOS, Duty Cycle* and *Support* – show differences in comparison with sham controls. The *BOS* was increased after surgery, the stance duration longer and less support was distributed over two diagonal paws and instead the time of three paws contacting the ground simultaneously was increased. Only the *Duty Cycle* of the hind paws showed the same trend but did not reach significance (BOS front: $F_{2,10} = 19.094$ $p < 0.001$; $\eta^2_p = 0.792$; BOS hind: $F_{2,10} = 10.982$ $p = 0.003$; $\eta^2_p = 0.687$; Tukey corrected post hoc tests front: $p = 0.001$; hind: $p = 0.012$; Duty Cycle front: $F_{2,10} = 10.718$ $p = 0.003$; $\eta^2_p = 0.682$; Tukey corrected post hoc tests front: $p = 0.004$; Support diagonal: $F_{2,10} = 9.456$ $p = 0.005$; $\eta^2_p = 0.654$; Support three: $F_{2,10} = 7.131$ $p = 0.012$; $\eta^2_p = 0.588$; Tukey corrected post hoc tests diagonal: $p = 0.006$; three: $p = 0.012$).

The *Swing Speed* of the hind limbs was slower in the 6-OHDA (Swing Speed hind: $F_{2,10} = 14.251$ $p = 0.001$; $\eta^2_p = 0.740$; Tukey corrected post hoc tests hind: $p = 0.002$). The decrease of speed of the front paws (compared to pre-surgical baseline: $F_{3,12} = 20.498$ $p < 0.001$; $\eta^2_p = 0.837$, Tukey corrected post hoc test: $p = 0.014$) did not reach significance in comparison with sham controls, probably due to the reduction of *Swing Speed* in shams. The *Stride Length* in the 6-OHDA group was significantly shorter than the *Stride Length* of sham controls (Stride Length front: $F_{2,10} = 8.183$ $p = 0.008$; $\eta^2_p = 0.621$; hind: $F_{2,10} = 5.948$ $p = 0.020$; $\eta^2_p = 0.543$; Tukey corrected post hoc tests front: $p = 0.007$; hind: $p = 0.021$). There was no difference between shams and the 6-OHDA regarding the *Print Position* ($F_{2,10} = 0.211$ $p = 0.814$).

Posterior PPTg-DBS

The effect of 6-OHDA lesions on gait parameters in the posterior PPTg-DBS experiment was by and large similar. *BOS* of the front limbs, *Duty Cycle* of the hind limbs, *Support*, *Swing Speed* and *Stride Length* showed the same parkinsonian characteristics (BOS front: $F_{2,9}$ = 24.697 p < 0.001; η^2_p = 0.846; Tukey corrected post hoc tests front: p = 0.002; Duty Cycle hind: $F_{2,9}$ = 8.282 p = 0.009; η^2_p = 0.648; Tukey corrected post hoc tests hind: p = 0.012; Support diagonal: $F_{2,9}$ = 5.356 p = 0.29; η^2_p = 0.543; Tukey corrected post hoc tests diagonal: p = 0.025; Support three: $F_{2,9}$ = 7.413 p = 0.013; η^2_p = 0.622; Tukey corrected post hoc tests three: p = 0.010; Swing Speed front: $F_{2,9}$ = 8.852 p =0.007; η^2_p = 0.663; hind: $F_{2,9}$ = 17.080 p = 0.001; η^2_p = 0.791; Tukey corrected post hoc tests front: p = 0.006; hind: p = 0.001; Stride front: $F_{2,9}$ = 15.700 p = 0.001; η^2_p = 0.777; hind: $F_{2,9}$ = 14.932 p = 0.001; η^2_p = 0.768; Tukey corrected post hoc tests front and hind: p = 0.001). During post-surgical testing the sham controls showed an increase in distance between front and hind paws on both sides (with the hind paw placed in front of the front paw) which can be interpreted as an increase in interpaw coordination. The 6-OHDA only showed attenuated changes of this distance which resulted in a significant difference on the righ-hand side compared to sham controls (Print Position right: $F_{2,9}$ = 7.856 p = 0.011; η^2_p = 0.636; Tukey corrected post hoc test p = 0.009).

Confirming previous results: Is there a difference between the traditional PD model and the refined model (Does the additional PPTg lesion make a difference)?

Anterior PPTg-DBS

None of the gait parameters measured in this experiment were affected by the addition of partial PPTg lesions; there were no differences between the 6-OHDA group and the combined lesion group (BOS front: p = 0.528; hind: p = 0.500; Support diagonal: p = 0.974; three: p = 0.827; Swing Speed hind p = 0.952; Stride Length front: p = 0.626; hind: p = 0.920).

Posterior PPTg-DBS

Most of the gait parameters here were not changed by additional PPTg lesions either (BOS front: p = 0.216; Support diagonal: p = 0.029; three: p = 0.163; Swing Speed front: p = 0.177; hind: 0.285; Print Position right: p = 0.082). Interestingly, in this experiment the group with the additional PPTg lesion showed improvement in a few gait parameters. The stance duration of the hind limbs became shorter and the *Stride Length* longer, reaching sham control levels (Duty Cycle hind: p = 0.024; Stride Length front: p = 0.038; hind: p = 0.05).

Does the DBS ameliorate the lesion deficits in the combined lesion group and 6-OHDA group? Do the stimulation parameters of DBS1 and DBS2 affect the gait differently?

Anterior PPTg-DBS

Conducting repeated measures ANOVAs for both, the 6-OHDA group and the combined lesion group, followed up by post hoc tests if appropriate, the data were tested for an effect of DBS compared to the post-surgery deficit. As presented above, the combined lesion group suffered from severe FOG already in the post-surgery testing condition which increased further during the DBS ON conditions. For this reason the group size dropped down to n = 2 and in addition, of the 3 testing sessions conducted per DBS condition, several did not lead to usable data, reducing the information even in these n = 2 considerably. Therefore the effect of DBS on animals of the combined lesion group who did not freeze or froze but still managed to deliver acceptable runs cannot be confidently assessed. However, conducting repeated measures ANOVAs on the collected data from the combined lesion group showed that there was no difference between conditions in any parameter – the stimulation of anterior PPTg had no effect on gait in the combined lesion group (*BOS*: front: $F_{3,3} = 3.711$ p = 0.155 hind: $F_{3,3} = 3.337$ p = 0.174; *Duty Cycle*: front:$F_{3,3} = 0.184$ p = 0.901; hind: $F_{3,3} = 2.166$ p = 0.271; *Support*: diagonal: $F_{3,3}$ 0.498 p = 0.709; three: $F_{3,3} = 0.294$ p = 0.829; *Swing Speed*: front: $F_{3,3} = 0.682$ p =

0.682; hind: $F_{3,3}$ = 1.183 p = 0.447; *Stride Length*: front: $F_{3,3}$ = 0.377 p = 0.778; hind: $F_{3,3}$ = 1.174 p = 0.449; *Print Position*: RHS: $F_{3,3}$ = 6.667 p = 0.077; LHS: $F_{3,3}$ = 1.376 p = 0.400).

In the 6-OHDA group anterior PPTg stimulation had a detrimental effect on a number of gait parameters, in many cases significant effects were only achieved with the settings of DBS2; other parameters were not affected in any way. The *BOS* of the front limbs further increased compared to the post-surgical baseline deficit with DBS1 and DBS2.

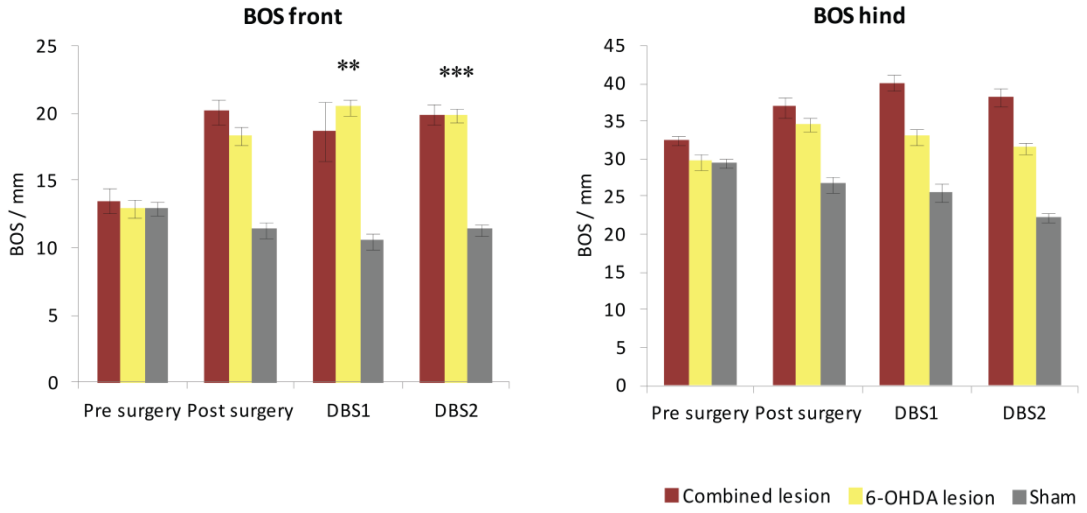

Figure 5.17. Anterior PPTg-DBS experiment: BOS. Comparison between combined lesion group, 6-OHDA group and sham control group of pre and post surgery performance, and performance in DBS ON conditions DBS1 and DBS1 on the CW. Displayed are group means ± SEM. For statistical significances please refer to the text. *** indicates a significant difference compared to post surgery data with p ≤ 0.001; ** indicates p ≤ 0.005 and * indicates p ≤ 0.05.

The stance duration of the front limbs increased as well; the hind limbs showed the same trend but the effect was not significant. The same was observed regarding the *Support* parameter: The *Support* over diagonal paws decreased and over three paws increased significantly with DBS2 while this trend could be observed applying stimulation with DBS1 settings.

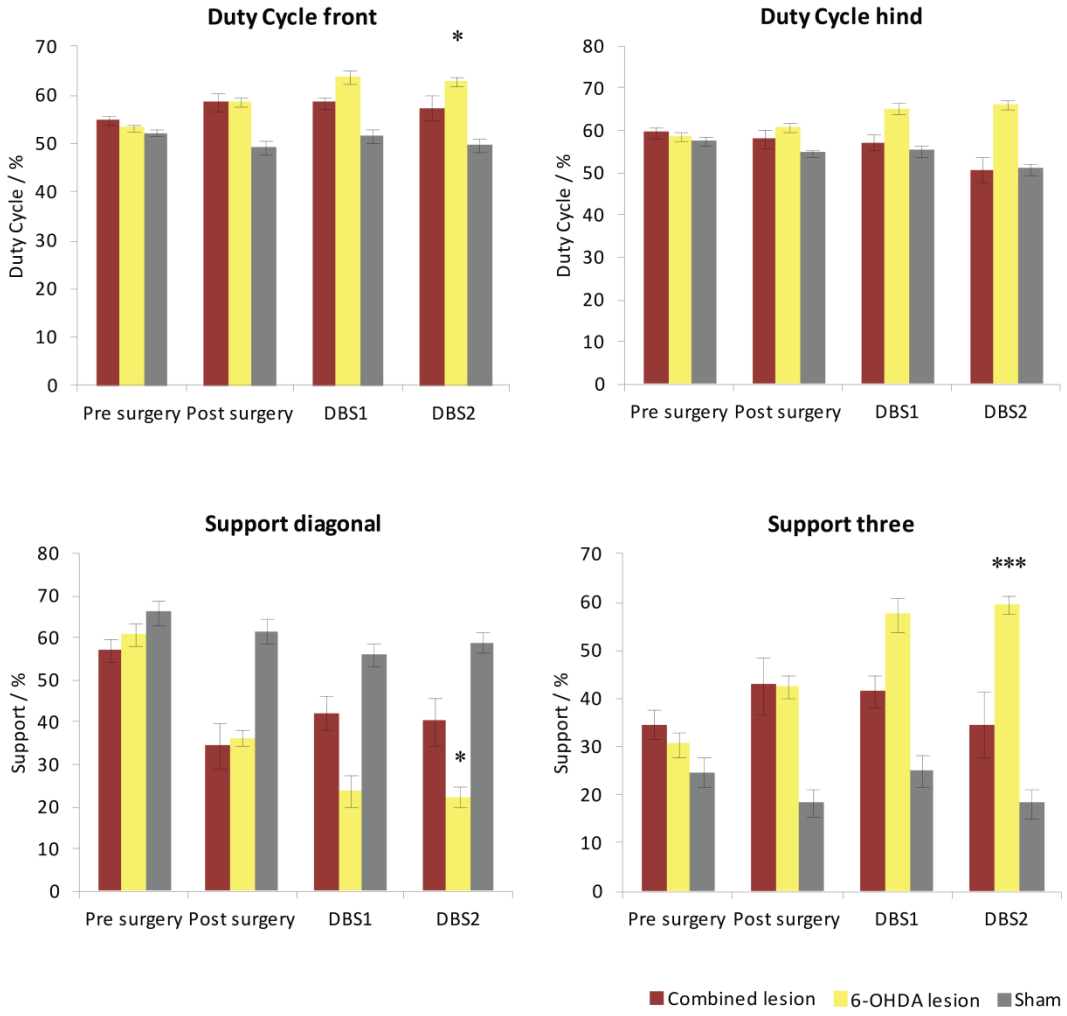

Figure 5.18. Anterior PPTg-DBS experiment: Duty Cycle and Support. Comparison between combined lesion group, 6-OHDA group and sham control group of pre and post surgery performance, and performance in DBS ON conditions DBS1 and DBS1 on the CW. Displayed are group means ± SEM. For statistical significances please refer to the text. *** indicates a significant difference compared to post surgery data with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$.

The *Stride Length* of the hind limbs was significantly shortened with DBS2 stimulation. DBS2 but not DBS1 stimulation slowed the *Swing Speed* and the shortened the *Stride Length* of the front limbs observably, without however reaching significance.

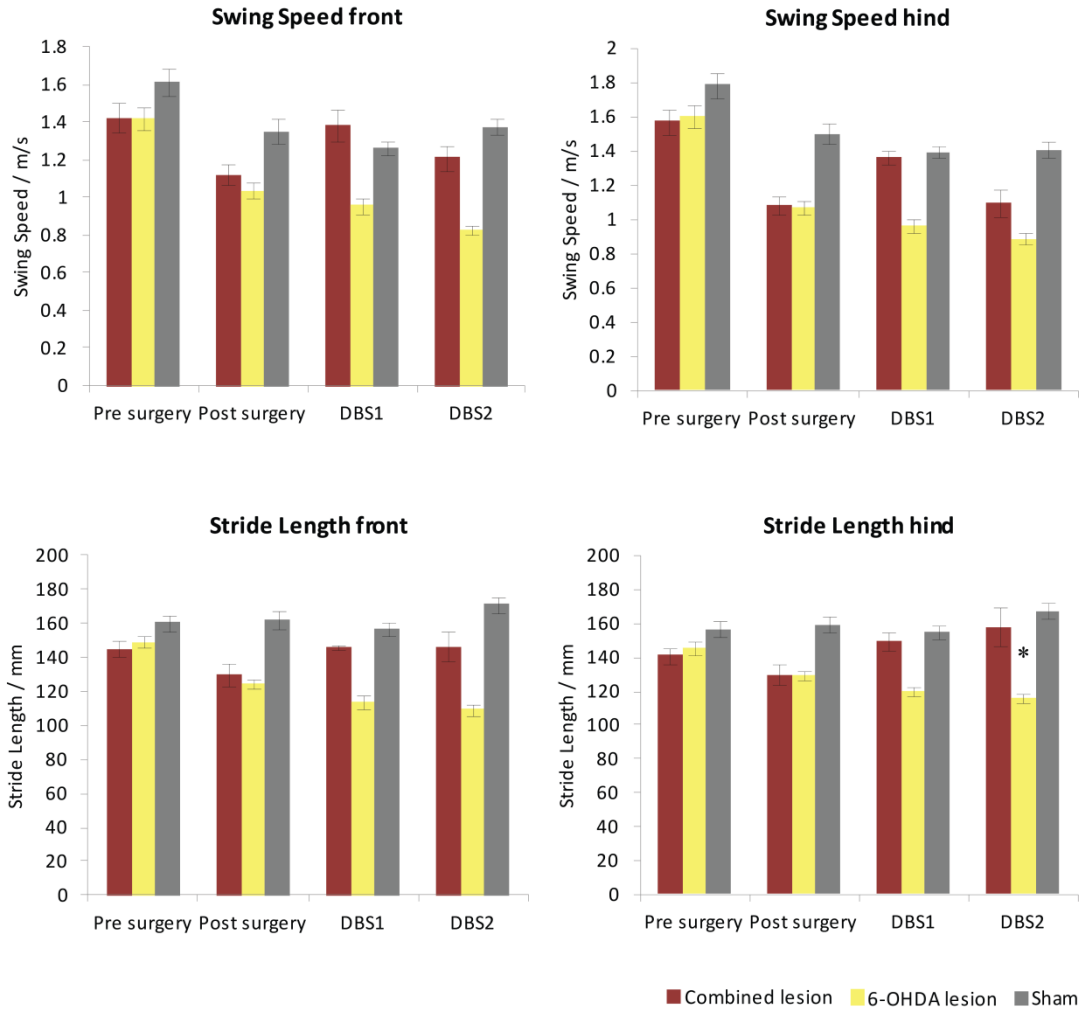

Figure 5.19. Anterior PPTg-DBS experiment: Swing Speed and Stride Length. Comparison between combined lesion group, 6-OHDA group and sham control group of pre and post surgery performance, and performance in DBS ON conditions DBS1 and DBS1 on the CW. Displayed are group means ± SEM. For statistical significances please refer to the text. *** indicates a significant difference compared to post surgery data with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$.

The stimulation of anterior PPTg did not change the Print Position significantly.

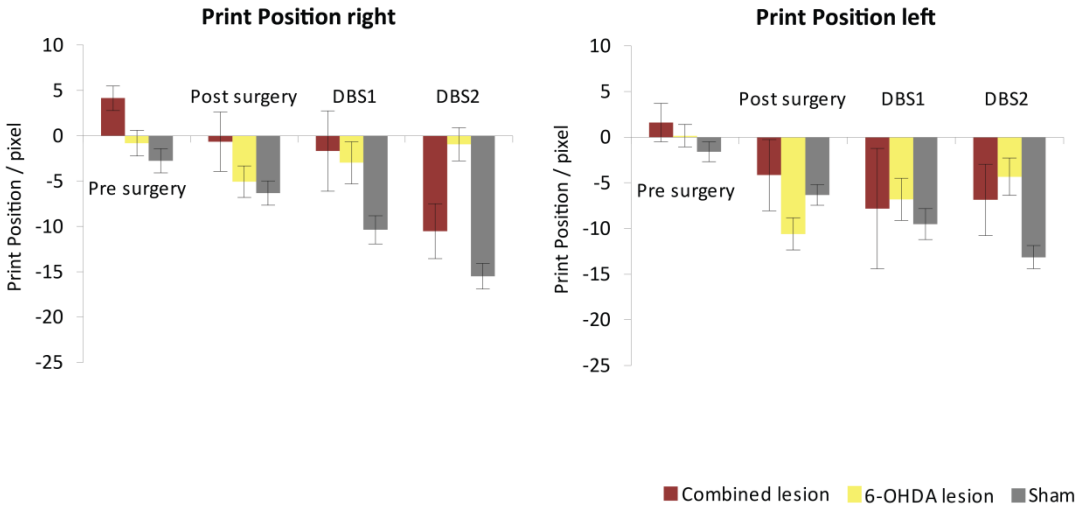

Figure 5.20. Anterior PPTg-DBS experiment: Print Position. Comparison between combined lesion group, 6-OHDA group and sham control group of pre and post surgery performance, and performance in DBS ON conditions DBS1 and DBS1 on the CW. Displayed are group means ± SEM. For statistical significances please refer to the text. *** indicates a significant difference compared to post surgery data with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$.

(BOS front: $F_{3,12} = 148.367$ $p < 0.001$; $\eta^2_p = 0.974$; DBS1: $p = 0.004$; DBS2: $p = 0.046$; Duty Cycle front: $F_{3,12} = 14.322$ $p < 0.001$; $\eta^2_p = 0.782$; hind: $F_{3,12} = 6.931$ $p = 0.018$ Greenhouse-Geisser corrected; $\eta^2_p = 0.634$; front: DBS2: $p = 0.010$; Support diagonal: $F_{3,12} = 18.557$ $p < 0.001$; $\eta^2_p = 0.823$; three: $F_{3,12} = 20.694$ $p < 0.001$; $\eta^2_p = 0.838$; diagonal: DBS2: $p = 0.039$; three: DBS2: $p = 0.001$; Stride Length hind: $F_{3,12} = 8.039$ $p = 0.003$; $\eta^2_p = 0.668$; hind: DBS2: $p = 0.040$).

Posterior PPTg-DBS

FOG in the posterior PPTg-DBS experiment was less severe and did not lead to a severe loss of data regarding the animals' gait as in the anterior PPTg-DBS experiment. In the combined lesion group the group size was reduced to n = 3 due to the loss of one animal before the start of DBS ON. The freezing that occurred allowed in all bar one rat of one testing day the collection of sufficient data.

Neither stimulation with a short (DBS1) nor with a long pulse width (DBS2) had an effect on any of the measured gait parameters in the combined lesion group. The 6-OHDA group, however, could benefit from stimulation of posterior PPTg. In most of the cases stimulation with DBS1 showed improvements which did not constitute significant changes in comparison with the post-surgical baseline deficit (the effect did not withstand post hoc corrections). Stimulation of the same gait parameters during the DBS2 condition then had a significant beneficial effect: The stance duration of the hind paws decreased, the *Support* with three paws decreased and the two-legged diagonal *Support* increased (almost reaching significance), and the *Stride Length* became longer.

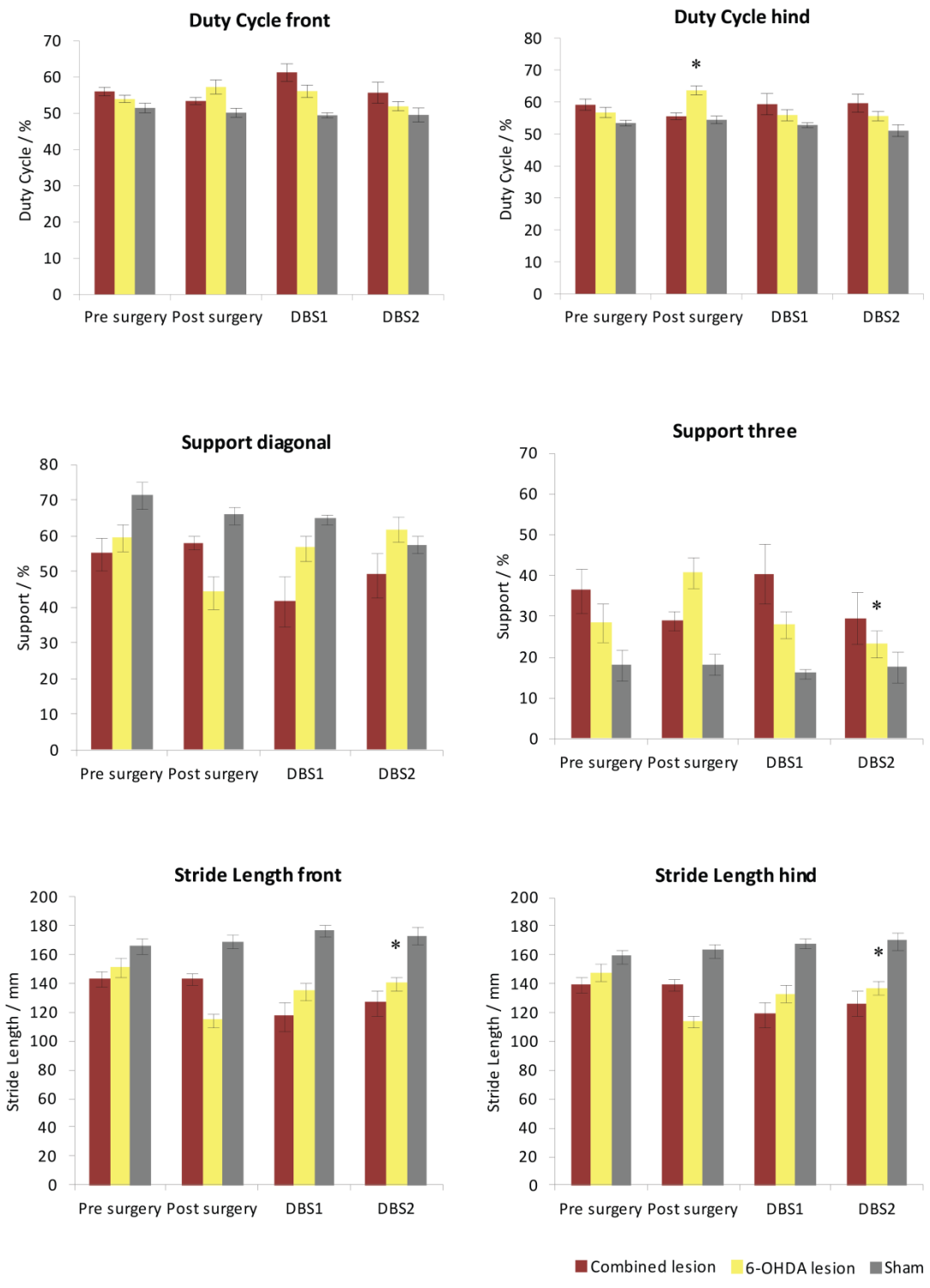

Figure 5.21. Posterior PPTg-DBS experiment: Duty Cycle, Support and Stride Length. Comparison between combined lesion group, 6-OHDA group and sham control group of pre and post surgery performance, and performance in DBS ON conditions DBS1 and DBS1 on the CW. Displayed are group means ± SEM. For statistical significances please refer to the text. *** indicates a significant difference compared to post surgery data with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$.

In contrast, the *Swing Speed* increased with stimulation with DBS1 but not DBS2 stimulation settings.

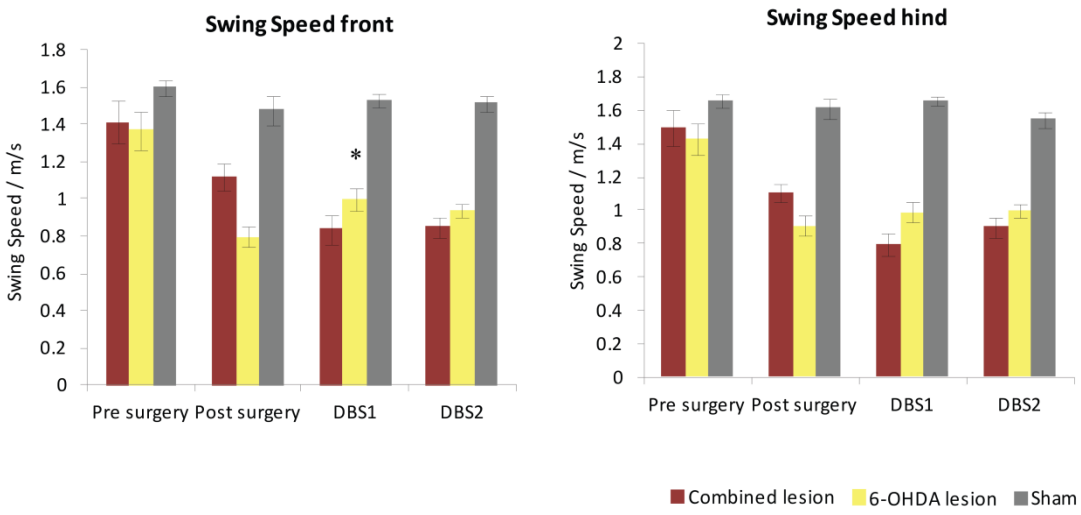

Figure 5.22. Posterior PPTg-DBS experiment: Swing Speed. Comparison between combined lesion group, 6-OHDA group and sham control group of pre and post surgery performance, and performance in DBS ON conditions DBS1 and DBS1 on the CW. Displayed are group means ± SEM. For statistical significances please refer to the text. *** indicates a significant difference compared to post surgery data with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$.

An increased inter-paw coordination judged by an increase of distance between ipsilateral paws with the hind paw placed behind the front paw could be observed with DBS2, which, however, did not reach significance. Stimulation of posterior PPTg had no effect on BOS.

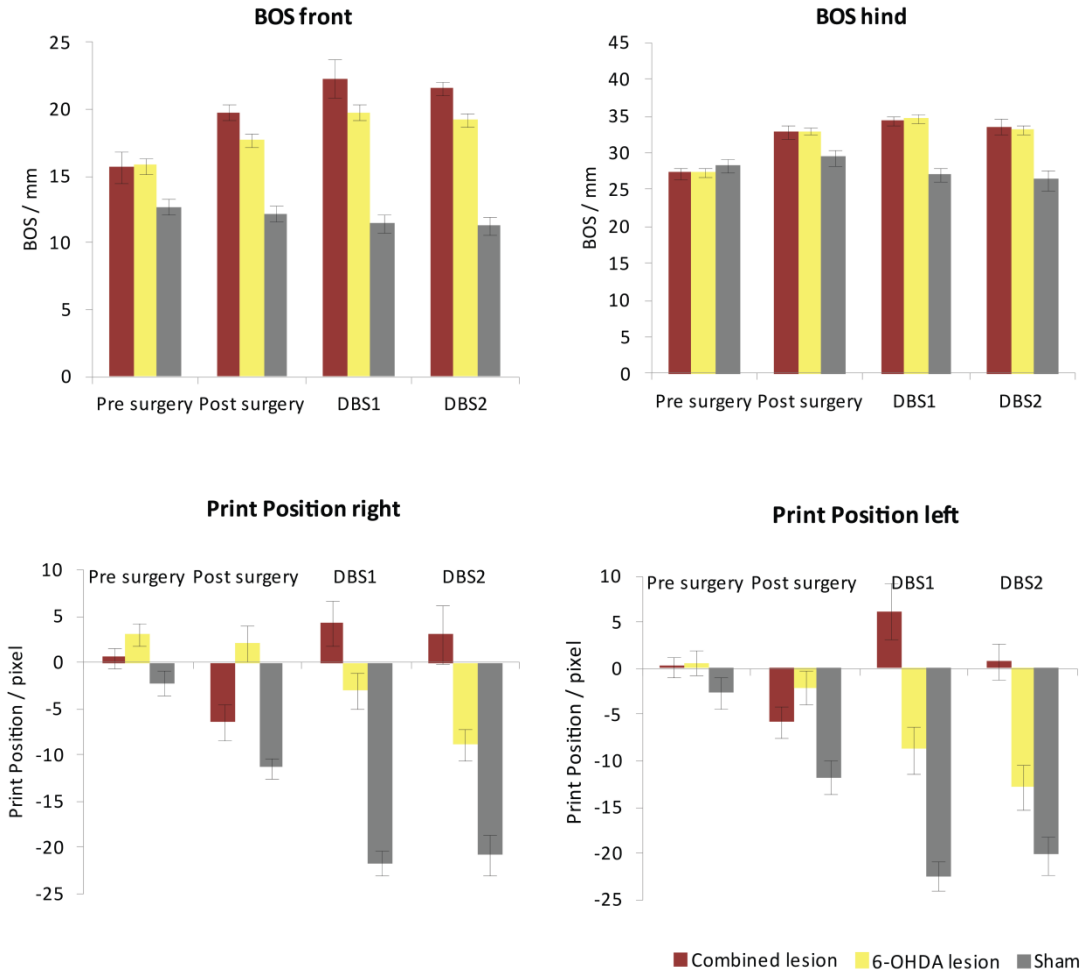

Figure 5.23. Posterior PPTg-DBS experiment: BOS and Print Position. Comparison between combined lesion group, 6-OHDA group and sham control group of pre and post surgery performance, and performance in DBS ON conditions DBS1 and DBS1 on the CW. Displayed are group means ± SEM. For statistical significances please refer to the text. *** indicates a significant difference compared to post surgery data with $p \leq 0.001$; ** indicates $p \leq 0.005$ and * indicates $p \leq 0.05$.

(Duty Cycle hind: $F_{3,9} = 8.380$ $p = 0.006$; $\eta^2_p = 0.736$; DBS2: $p = 0.020$; Support diagonal: $F_{3,9} = 4.951$ $p = 0.027$; $\eta^2_p = 0.623$; three: $F_{3,9} = 7.505$ $p = 0.008$; $\eta^2_p = 0.714$; diagonal: DBS2: $p =$

0.061; three: DBS2: p = 0.021; Stride Length front: $F_{3,9}$ = 13.555 p = 0.001; η^2_p = 0.819; hind: $F_{3,9}$ = 9.957 p = 0.003; η^2_p = 0.768; front: DBS2: p = 0.045; hind: DBS2: p = 0.044; Swing Speed front: $F_{3,9}$ = 10.633 p = 0.003; η^2_p = 0.780; hind: $F_{3,9}$ = 16.220 p = 0.001; η^2_p = 0.844; front: DBS1: p = 0.027).

Table 5.1. Summary of the data discussed above. The table answers the following questions: Is the 6-OHDA group different to sham controls? Is there a difference between the traditional PD model and the refined model (Does the additional PPTg lesion make a difference?)? Does the DBS ameliorate the lesion deficit? Are there differences between DBS1 and DBS2? Red indicates a deficit and/or worsening of gait parameters; green indicates improvements. Lighter shades represent trends, which are not statistically significant.

Anterior PPTg DBS		Is the 6-OHDA group different to shams?			Does the additional PPTg lesion make a difference? (combined vs 6-OHDA?)			Does DBS ameliorate the lesion deficit? (DBS compared to post surgery deficit)				Does DBS1 differ from DBS2?	
		Post surgery	DBS1	DBS2	Post surgery	DBS1 less data	DBS2 less data	combined DBS1 (less data)	combined DBS2 (less data)	6-OHDA DBS1	6-OHDA DBS2	combined	6-OHDA
BOS	front	bigger	bigger	bigger	No	No	No	No	No	bigger	bigger	No	No
	hind	bigger	bigger	bigger	No	No	No	No	No	No	No	No	No
Duty Cycle	front	longer contact	longer contact	longer contact	No	No	No	No	No	longer contact	longer contact	No	No
	hind	trend: longer contact	longer contact	longer contact	No	shorter contact because of DBS effect on 6-OHDA	shorter contact because of DBS effect on 6-OHDA	No	No	trend: longer contact	trend: longer contact	No	No
Support	diagonal	less	less	less	No	No	No	No	No	trend: less	less	No	No
	three	more	more	more	No	No	less	No	No	trend: more	more	No	No
Swing Speed	front	trend: slower	slower	slower	No	faster because of DBS effect on 6-OHDA	faster because of DBS effect on 6-OHDA	No	No	No	trend: slower	No	No
	hind	slower	slower	slower	No	faster because of DBS effect on 6-OHDA	faster because of DBS effect on 6-OHDA	No	No	No	trend: slower	No	No
Stride Length	front	shorter	shorter	shorter	No	longer because of DBS effect on 6-OHDA	longer because of DBS effect on 6-OHDA	No	No	No	trend: shorter	No	No
	hind	shorter	shorter	shorter	No	longer because of DBS effect on 6-OHDA	longer because of DBS effect on 6-OHDA	No	No	No	shorter	No	No
Print Position	right	No	No	No	No	No	No	No	No	No	No	No	No
	left	No	No	No	No	No	No	No	No	No	No	No	No

Posterior PPTg DBS		Is the 6-OHDA group different to shams?			Does the additional PPTg lesion make a difference? (combined vs 6-OHDA?)			Does DBS ameliorate the lesion deficit? (DBS compared to post surgery deficit)				Does DBS1 differ from DBS2?	
		Post surgery	DBS1	DBS2	Post surgery	DBS1	DBS2	combined DBS 1	combined DBS2	6-OHDA DBS1	6-OHDA DBS2	combined	6-OHDA
BOS	front	bigger	bigger	bigger	No	No	No	No	No	No	No	No	No
	hind	trend: bigger	bigger	bigger	No	No	No	No	No	No	No	No	narrow (but not different to post)
Duty Cycle	front	No	No	No	No	No	No	No	No	No	No	No	No
	hind	longer contact	No	No	less contact (=shams)	No	No	No	No	trend: less contact	less contact	No	No
Support	diagonal	less	No	No	No	No	No	No	No	trend: further	trend: further	No	No
	three	more	No	No	No	No	No	No	No	trend: further	less	No	No
Swing Speed	front	slower	slower	slower	No	No	No	No	No	faster	No (but same as DBS1)	No	No
	hind	slower	slower	slower	No	No	No	No	No	trend: faster	No (but same as DBS1)	No	No
Stride Length	front	shorter	No	No	Longer (=shams)	No	No	No	No	trend: longer	longer	No	No
	hind	shorter	No	No	Longer (=shams)	No	No	No	No	trend: longer	longer	No	No
Print Position	right	shorter	shorter	shorter	No	No	No	No	No	No	trend: further	No	No
	left	No	No	No	No	No	No	No	No	No	trend: further	No	No

4.3.3. L-DOPA induced abnormal involuntary movements

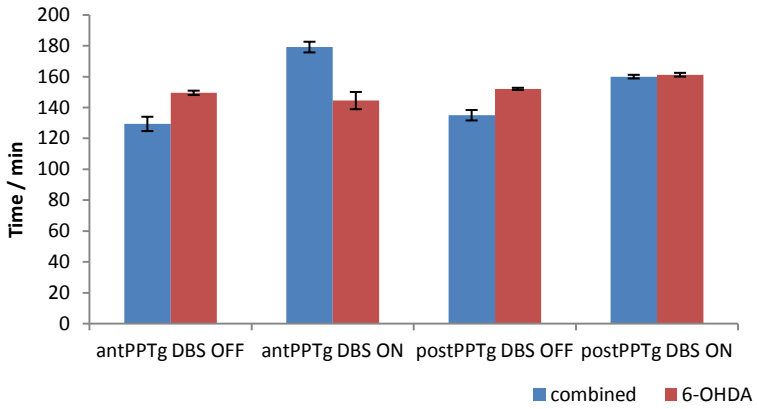

Figure 5.24. Comparison of the total duration of L-Dopa induced dyskinesias in combined lesion group and 6-OHDA group in DBS OFF and DBS ON condition with either anterior or posterior PPTg DBS electrodes. Displayed are group means ± SEM. For statistical significances, please refer to the text.

At an average time of 11.8 min (range 7 min to 20.8 min) after the administration of L-DOPA the parkinsonian rats showed the first AIMs which then developed into more severe dyskinesias over time. Sham controls did not show any signs of dyskinesias. The rate of progression and therefore the time the animals exhibited dyskinesias of different severity as categorised according to the Creese-Iversen scale (refer to the General Methods for details) differed between groups and testing condition. The total amount of time the animals showed AIMs however did not differ between groups in any of the 4 conditions (anterior DBS OFF: $F_{1,7}$ = 3.506 p = 0.103; DBS ON: $F_{1,6}$ = 3.241 p = 0.122; posterior DBS OFF: $F_{1,6}$ = 4.057 p = 0.091; DBS ON: $F_{1,5}$ = 0.086 p = 0.781) – it was the quality of dyskinesias that changed.

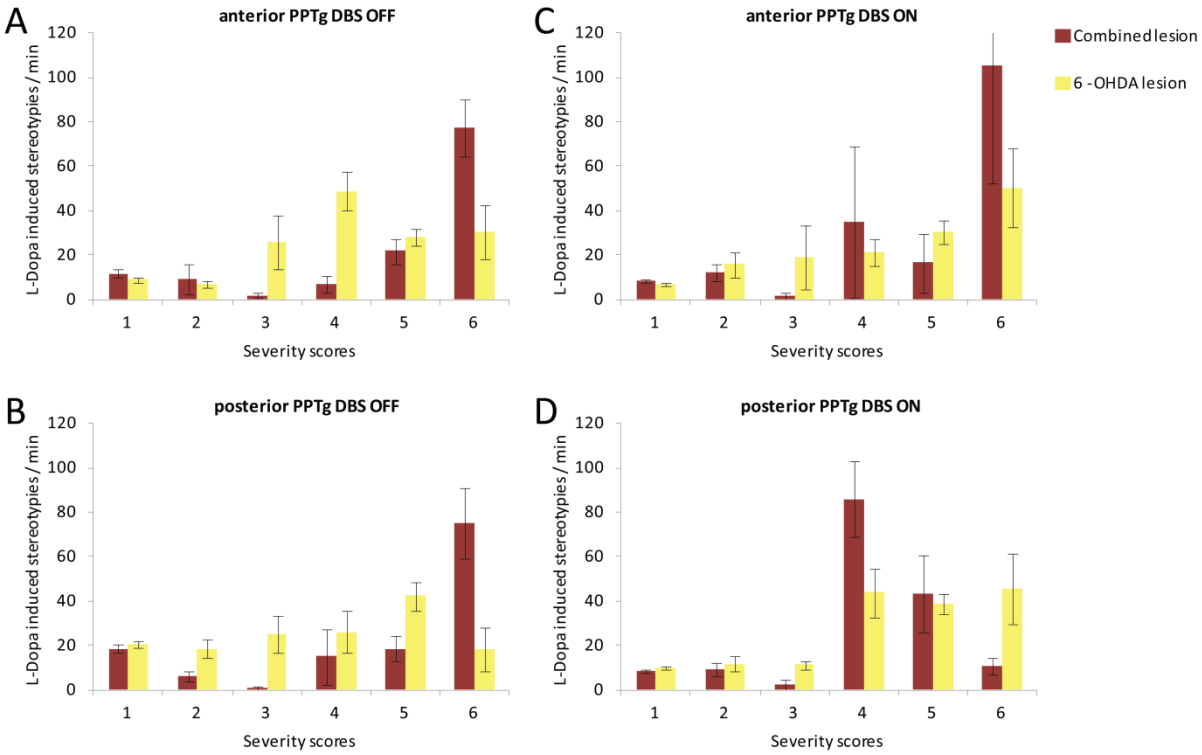

Figure 5.25. Comparison of the severity of L-Dopa induced dyskinesias in combined lesion group and 6-OHDA group. The duration of dyskinesias of each severity level were recorded in minutes. **Panels A-B** graphs compare the effect of L-Dopa in DBS OFF condition and **Panels C-D** show results of the DBS ON condition with either anterior or posterior PPTg DBS electrodes. Displayed are group means ± SEM. For statistical significances please refer to the text.

Figure 5.25. shows the time for which rats in the different groups and conditions were dyskinetic. Both parkinsonian groups showed AIMs after i.p. L-DOPA injections. The group with combined DA depletion and PPTg lesions showed more severe AIMs and mostly of a different quality (panels A and B) – irrespectively of the position of the stimulation electrode [licking and biting (scores 5-6) rather than stereotyped head and body movements (scores 2-4)] than the 6-OHDA group (electrode in anterior PPTg: score 6: $F_{1,7} = 7.151$ $p = 0.032$; score 4: $F_{1,7} = 15.890$ $p = 0.005$; electrode in posterior PPTg: score 6: $F_{1,6} = 9.220$ $p = 0.023$; score 2: $F_{1,6} = 7.377$ $p = 0.035$; score 3: $F_{1,6} = 8.828$ $p = 0.025$; score 5: $F_{1,6} = 7.573$ $p = 0.033$).

Focussing on these differences (dyskinesias scores 4 and 6) the analyses showed an amelioration of the severe AIMs in the combined lesion group with posterior DBS (panel D), but not with anterior DBS (panel C): Posterior PPTg-DBS (ON) caused a shift of the L-DOPA induced AIMs. The appearance of continuous licking and biting was reduced, dyskinesias instead taking the form of stereotyped up and down head movements, and crossing and lifting of the forelegs [anterior PPTg-DBS experiment: no *interaction of group and DBS condition* (ON /OFF): score 4: $F_{1,6}$ = 4.591 p = 0.076; score 6: $F_{1,6}$ = 0.005 p = 0.944; no effect of *condition*: score 4: $F_{1,6}$ = 0.000 p = 1.000; score 6: $F_{1,6}$ = 1.212 p = 0.313; posterior PPTg-DBS experiment: significant effect of *condition*: score 4: $F_{1,5}$ = 9.791 p = 0.026; score 6: significant *interaction of group and DBS condition*: $F_{1,5}$ = 8.504 p = 0.033].

4.4. Discussion

4.4.1. Summary

These two experiments aimed to answer a series of questions. The reason for the integration of many assessments into this experimental design is the innate complexity of the animal model and the need to have as many questions answered as possible. In the first place, materials and methods needed to be developed and tested to create a model of PPTg-DBS in a free-moving and refined rodent model of PD. The pilot surgeries and tests described above helped to refine coordinates, surgical techniques and the development of a headstage that allowed attaching the DBS device to the rats' head – a solution that was well tolerated by the animals and did not interfere with their behaviour. The work in this chapter was conducted to assess the effect of anterior and posterior PPTg LFS on gait deficits in parkinsonian rats – PD models that did (combined lesion model) or did not (traditional bilateral 6-OHDA model) include partial lesions of the PPTg.

Replicating the findings presented in the previous chapter, striatal infusions of 6-OHDA caused significant and consistent gait deficits which are by and large very similar. The only differences were in the Duty Cycle of the front limbs of the 6-OHDA group in the anterior PPTg-DBS experiment and the hind limbs in the posterior PPTg-DBS experiment, as well as in the Print Position of the right hand side of the same group showing deficits in comparison with sham control rats, which reached significance. Similar trends were observed in the combined lesion experiment (Chapter 4) but these did not reach significance. Further, as described in the combined lesion experiment, the additional PPTg lesion did not change the gait deficits already observed after DA depletion in the anterior PPTg-DBS experiment. Interestingly, in the posterior PPTg-DBS experiment the stride length of both front and hind limbs of the rats bearing lesions in SN and PPTg, was not only longer, but also did not differ from sham

operated rats – an effect that was also observed before. The absence of additional deficit with additional partial PPTg lesions is discussed in Chapter 4, as well as possible explanations for the differences in stride length between both PD models. Notably, this correction of stride length only occurs in the experiment where the PPTg lesion of the posterior portion was enlarged by the presence of electrodes, not with anterior PPTg electrodes. With regard to the proposed explanations in the literature, this might support the assumption of a decreased excitatory input to the hyperactive STN in the DA depleted BG circuit.

Interestingly, a significant and important effect just of the implantation of DBS electrodes differentiated rats implanted with anterior PPTg electrodes from groups with posterior PPTg electrode. In the DBS-OFF condition, rats of the combined lesion group with electrodes in anterior PPTg suffered from severe freezing episodes in form of start hesitation when they were sat into the CW for gait assessment. In some occasions, they were able to overcome it and initiate the run but the majority of these runs were not completed, because the rats' run down the glass walkway came to a halt when they approached the end of the CW – and they froze again (data not scored). Electrodes in the posterior part of the PPTg did not have this effect and neither did the 6-OHDA group suffer from significant freezing episodes, no matter where the electrode was implanted. Switching the DBS of anterior PPTg electrodes on, further increased the total occurrence of FOG in the combined lesion group (by increasing the occurrence of freezing episodes and/or decreasing the ability to overcome start hesitation). Stimulation of the posterior PPTg electrodes did cause FOG as well in this group; given the strong relation between anterior PPTg and FOG in the OFF-condition it is plausible to assume that this can be caused by a current spread from the posterior location of the electrode to the anterior portion. It was the effect of DBS on parkinsonian gait deficits that was of main interest conducting this work: Double blind studies and follow ups of PPTg-DBS studies in PD patients could not maintain the positive results reported after the first PPTg electrode implantations or

even show a decline of the transient gait amelioration (Moreau *et al.*, 2009; Moro *et al.*, 2010; Peppe *et al.*, 2010). The experiments presented here showed a clear differential effect of anterior and posterior PPTg-DBS: Anterior PPTg LFS worsened almost all gait parameter in the 6-OHDA group. The post-surgery deficit was enhanced with stimulation: observable trends in the DBS1 condition reached significance (compared to the post-surgery testing sessions) in the DBS2 condition (stimulation at a higher pulse duration). The increased pulse duration might be the cause for this effect, but the possibility of reaching significance at that point because of the overall duration of chronic stimulation cannot be excluded – the experiment did not offer the possibility of a counterbalanced order of testing conditions. (This will be discussed further below.) The combined lesion group did not benefit from the stimulation of anterior PPTg. However, this effect on gait could only be tested in very few occasions of very few animals, because, as described above, many times the rats were not able to complete the CW task due to the severe FOG episodes. The data describing the gait of the combined lesion group under PPTg-DBS is therefore very scarce and needs to be interpreted cautiously. The effect of posterior PPTg DSB was directly opposite: In the 6-OHDA group all gait parameters bar BOS improved in comparison with the deficit observed after the surgeries. In contrast to the worsening effect of anterior stimulation, amelioration with posterior LFS already appeared with DBS 1 settings (shorter pulse width) in some gait parameters, others only showed a trend which reached significance with DBS 2 stimulation. The parkinsonian group that had additional partial PPTg lesions did not benefit from stimulation of posterior PPTg.

In summary, the results suggest that the loss of PPTg neurons did further contribute to the chronic inhibition of the PPTg caused by the 6-OHDA lesion, which was not sufficient to induce FOG. The addition of the anterior, but not posterior, DBS electrode added further to the lesion and precipitated FOG already in the DBS OFF condition. Turning anterior PPTg-DBS on disrupted the remaining functioning structures further and made the FOG even worse. The

increase in freezing after posterior PPTg could be speculated to be due to current spread. In the 6-OHDA group stimulating anterior PPTg caused disruption of the BG circuitry leading to further impairment of gait and postural parameters; the stimulation of posterior PPTg however showed therapeutic potential.

4.4.2. Severe FOG after additional track lesions in anterior PPTg

The lesion caused by the anterior PPTg electrode was not big enough to cause FOG – the 6-OHDA group did not have difficulties. Neither was the partial lesion of anterior (in combination with posterior) PPTg caused by ibotenic acid big enough for eliciting FOG. However, the combination of both – anterior PPTg electrode in the combined lesion group – added to the dysfunction of the anterior PPTg group possibly caused by a dysfunctional BG circuitry in the parkinsonian DA depleted state.

Dysfunctional inhibitory afferents coming from the BG output nuclei GPi/SNr (Breit *et al.*, 2001; Carlson *et al.*, 1999; Mitchell *et al.*, 1989; Orieux *et al.*, 2000) target the anterior portion of the PPTg. It is plausible that this decreases PPTg activity and hinders adequate relay of BG information to downstream brainstem motor nuclei (or recurrent feedback into BG or thalamus) causing hypokinesia and the gait deficits observed in PD patients. The degeneration, mimicked here by partial PPTg lesions, might not have been sufficient to alter the already severe deficits observed in the rats of the 6-OHDA group – but further track damage might have augmented this problem of an "over-inhibited" PPTg. One observation, however, raises the question if this explanation is sufficient or, in fact, correct: The manipulation of anterior PPTg by LFS in the 6-OHDA group also causes deficits – not FOG but specific gait disturbances. This comparison would mean assuming that LFS causes a lesion-like effect on anterior PPTg. The possible mechanisms of the stimulation in the area will be discussed in Section 4.4.3. of this chapter. As discussed in the General Introduction, FOG constitutes a gait disturbance of its

own category – an observation supported by the extensive literature dedicated to this phenomenon. As in PD patients, FOG observed here was clearly contextual and it is likely that new sensory input, such as narrow walls of the CW and the approaching end of the glass walkway, might have triggered the FOG episodes just as obstacles and narrow doorways do in PD patients; that is, increasing task demand (having to judge the width of the walkway, distance to the end of the CW) or simply providing additional sensory input. It is also conceivable that the crossing of the CW in a way that needs to fulfil certain criteria, which the rats were trained to meet, posed some additional cognitive demand to the rats, creating a dual task situation – tasks that, in patients, also trigger freezing episodes. This contextuality strongly suggests that the PPTg is not just a gait control centre.

The key to this supposition is the position of the PPTg between the BG and brainstem and its ability to provide modulatory influence on BG activity via its strong, often reciprocal connections several BG nuclei – SNc, VTA, GPi, SNr, STN – or indirectly via extensive afferents into the thalamus. The PPTg receives fast polymodal sensory input from sites including the superior and inferior colliculi, the lemniscal nuclei, parabrachial nucleus and the trigeminal complex at a response latency of under 80 ms, and which can be as short as 4ms (Dormont *et al.*, 1998; Pan and Hyland, 2005). This is fast enough to supply DA neurons in the midbrain with relevant information about sensory events. It has been shown that these efferents are closely related and necessary for the phasic activity of DA neurons. DA neurons are essential for generating "reward prediction error" signals, phasic activity in response to reward related sensory input, which declines as an association is made with the reward predicting stimuli and re-occurs in the absence of a predicted reward (Schultz, 2006). Their response to stimuli is fast – too fast to consider the thalamus or cortical projections to be the source for information about the stimuli. Cortical responses related to object identification and discrimination do not occur before 80 – 160 ms after stimulus onset (Rousselet *et al.*, 2004; Schall, 2003) and

responses in extranigral subcortical structures – striatum and STN – also occur too late to be providing information before nigral phasic activity [(Matsumura *et al.*, 1992), reviewed in (Redgrave *et al.*, 2008)]. This short latency DA response must be dependent on an even shorter latency input. The response of the PPTg would be fast enough and anatomically there are direct connections to these DA neurons in VTA and SNc. In fact, the absence of nicotinic acetylcholine receptor β2 receptor subunit in genetically altered mice abolished the VTA's ability to switch to burst firing (Maskos, 2007; Maskos *et al.*, 2005), the electrical stimulation of the PPTg results in increased burst firing activity of midbrain DA neurons (Floresco *et al.*, 2003) and striatal DA efflux (Forster and Blaha, 2003). Conde and colleagues have shown that inactivation of the PPTg can interfere with processing of sensory information and disrupt ongoing conditioned motor performance (Conde *et al.*, 1998; Dormont *et al.*, 1998). DA neurons need therefore more information than just about the physical attributes of the stimuli. Indeed, the PPTg is able to provide these neurons with information about a stimulus regarding its salient aspects – the association of a stimulus with a reward, the prediction of the reward value, the reward itself, and the actual value of the reward (Kobayashi and Okada, 2007; Okada and Kobayashi, 2013; Okada *et al.*, 2009; Thompson and Felsen, 2013).

Against this background, it is conceivable to assume that the damaged anterior PPTg is unable to convey information about incoming sensory stimuli of the environment ("there are walls but I fit through", "there is the edge of the glass, but behind that I find my home cage") to the SNc. At this point the PPTg can normally mediate outflow from the BG output nuclei and assess whether to send this forward or if incoming sensory information pose a reason for giving preference to a different action ("yes, the way is clear, go ahead" or "no, there are obstacles/ danger of falling off the walkway"). The lack of sensory information to the SNc puts a brake on the system because the decision on competing actions cannot be made and the rat freezes.

4.4.3. The effect of low frequency stimulation

Before a tentative interpretation of the effect of LFS of anterior and posterior PPTg can be made, the possible mechanisms that stimulation of brain structures can have need to be considered.

The ability to ameliorate tremor by means of HFS of the thalamus and thereby producing the same effect as thalamotomies (Benabid *et al.*, 1991) and the similarity of motor effects in animal models of PD caused by lesions of the STN and the behavioural effects of HFS of the STN (Benazzouz *et al.*, 1993; Guridi *et al.*, 1996) has led to the assumption that high frequency-DBS causes functional inactivation of the structure (by means of a local depolarization block, inactivation of voltage-dependent channels, and functional deafferentation) reducing the tonic excitatory drive onto the BG output nuclei [reviewed in (Deniau *et al.*, 2010)]. Thanks to a large number of studies investigating the mechanisms behind HFS it is now clear that the effects cannot be generalized across the structures targeted by stimulation electrodes. The effect depends on the stimulation parameters, the distance of the neurons to the electrode contacts, the orientation of axons in relation to the electrode, the characteristics of soma and axons (type of neurotransmitter, diameter of axons, properties of the synaptic transmission) (Deniau *et al.*, 2010; McIntyre *et al.*, 2004; Zhang and Grill, 2010). In fact, STN-HFS evoked synaptic effects in other parts of the BG (Benazzouz *et al.*, 2000; Florio *et al.*, 2007; Potter-Nerger *et al.*, 2008), antidromic activations (Hammond *et al.*, 2008) and changes in neurotransmitter release (He *et al.*, 2014; Pazo *et al.*, 2010). Apart from inhibition, HFS therefore clearly also generates excitation and antidromic activation and the activations do not only cause changes in firing rates but also in firing patterns and oscillatory properties. Garcia and colleagues describe a replacement of spontaneous STN spikes by spikes evoked by the HFS and locked to that stimulus (Garcia *et al.*, 2003).

Whether soma or axons are activated also depends on a row of different factors (see Section 3.2.2. of this chapter). By using different pulse widths, the intention was to control for one of these factors and introduce a possible differential effect of the stimulation. In direct comparison, LFS of the PPTg at a shorter and longer pulse width did not cause significantly different effects on motor performance. When compared to the post-surgery deficit in some parameters the stimulation with 500 µs had a stronger effect (worsening with anterior PPTg stimulation and benefitting with posterior PPTg stimulation) compared to stimulation with pulses of a length of 160 µs: Where 160 µs caused trends in the same direction, the effect with 500 µs was significant. However, as mentioned above, the condition with the longer pulse width was always second (the order of conditions was not counterbalanced) so it is possible, that the effect increased with a longer duration of chronic stimulation.

The mechanisms behind LFS are not that well studied as HFS. The working hypothesis has been that, contrary to a lesion-like effect of HFS, LFS would cause an activation of the PPTg that shows decreased activity in the parkinsonian state because of over-inhibition from BG output nuclei. PPTg lesions have caused an increased firing rate of STN neurons in normal rats (Breit *et al.*, 2005), while lesions in parkinsonian rats have been reported to reduce STN firing (Breit *et al.*, 2006). Capozzo and colleagues reported an activating effect on STN neurons in PD rats and sham lesioned controls (Capozzo *et al.*, 2009) with acute LFS of the PPTg but an inhibitory effect with acute HFS (>50Hz). With chronic stimulation, Alam and colleagues observed a reduction of the enhanced activity in the STN of PD models, but no effect in sham lesioned controls (Alam *et al.*, 2012). In contrast, a further recent study only found markers for neuronal activity (c-fos expression) after LFS of the PPTg at stimulation site and in the central gray. In human, imaging studies revealed increased regional cerebral glucose metabolism in a series of cortical and subcortical areas (Stefani *et al.*, 2010), changes in regional cerebral blood flow in

different subcortical areas, including the thalamus (Ballanger *et al.*, 2009; Strafella *et al.*, 2008) and various cortical structures (Ballanger *et al.*, 2009).

What applies to HFS of the STN is also true for LFS of the PPTg: the actual effect depends on all those above-mentioned factors and without further studies assessing the effect of PPTg-DBS on its target structures at this point it is only possible to infer the mechanism of the stimulation from the behavioural outcome.

4.4.4. Gait worsening after anterior PPTg stimulation

Stimulation of the anterior PPTg worsened the already changed gait parameters of the 6-OHDA group significantly. Concerning the often-stated hypothetical assumption that PPTg-DBS would correct the effect of the over-inhibitory BG outflow in the parkinsonian brain, this seems surprising, considering that it is the anterior part of the PPTg that is receiving the efferents from SNr and GPi. Two explanations are possible: (i) similarly to the lesion effect of the electrodes in the combined lesion group, here the DBS is causing a disruption of sensory information transfer from the PPTg to the BG. However, if this was the case, observable deficits would be expected in the DBS-OFF condition in the 6-OHDA group as well due to the damage caused by the electrode. Further, FOG seems to have different pathological mechanisms compared to other gait deficits. These rats of the 6-OHDA group did not suffer from any significant FOG episodes, so it is likely that a different mechanism might be causing the gait deficits measured on the CW. (ii) It is plausible to believe that the stimulation in anterior PPTg is not affecting the neurons of the PPTg, but having an antidromic effect, driving the inhibitory afferents from the BG and therefore enhancing the already existing parkinsonian deficits.

4.4.5. Gait improvement after posterior PPTg stimulation

Interestingly, posterior PPTg stimulation had the opposite effect and improved several of the analysed gait parameters of the rats of the 6-OHDA group. On the assumption that LFS here is having an activating effect on the sensory component of the PPTg, two consequences are conceivable: (i) increased activity of GABAergic projections to the STN might amend its hyperactivity observed in PD (Nandi *et al.*, 2002; Obeso *et al.*, 2008). This modulatory influence has been shown before: PPTg lesions reversed hyperactivity in STN (Breit *et al.*, 2006) – likely by eliminating cholinergic efferents from the PPTg. This however does not match with the suggestion of an activating (rather than lesion) effect of LFS. (ii) Stimulating the sensory component of the PPTg might be increasing cholinergic excitatory input to thalamostriatal and thalamocortical pathways. The PPTg makes substantial innervations to the thalamus, in particular the ventral and intralaminar nuclei (Inglis and Winn, 1995) and cholinergic input has shown to have a role in the regulation of thalamic burst firing (McCormick and Prince, 1986). Ainge and colleagues have shown increased c-fos expression in thalamic nuclei after the stimulation of posterior PPTg (Ainge *et al.*, 2004). In PD patients, self-paced voluntary movements changed PPTg activity and functional interactions between the cortices and the PPTg (Tsang *et al.*, 2010). There were strong coherences between the PPTg and the cortical sensorimotor regions in the theta band on the ipsilateral side. On the basis of the known relation between the VT of the thalamus and the sensorimotor cortex by theta band coherences, the relations of oscillatory activity between these regions during the pre-movement period and during execution were interpreted by the authors as a possible evidence for a sensorimotor feedback loop: the information from the cortex may drive the PPTg during movement preparation while the PPTg is re-entering relevant sensory information for the execution of the movement (Tsang *et al.*, 2010). The beneficial effect of the stimulation

of posterior PPTg observed in the 6-OHDA group might hence stem from a modulation of this thalamicocortical circuit.

4.4.6. L-DOPA induced AIMs and improvements by PPTg-DBS

Initially the experimental design included the administration of systemic L-DOPA to both parkinsonian groups to be able to compare the therapeutic effect of L-DOPA on gait deficits to the effect of DBS. The dose of 12 mg/kg L-DOPA combined with 15 mg/kg Benserazide hydrochloride was chosen based on the dosages described in the literature used to induce LID in rodent models of PD. The intent was to find a dose that would be high enough for a therapeutic benefit but sufficiently low to avoid AIMs. Winkler and colleagues have shown that 6mg/kg, given chronically over 5 weeks was not enough to improve performance in the staircase test (Winkler *et al.*, 2002). Others used injections between 8 – 15 mg/kg twice daily over several weeks to induce LIDs (Mahmoudi *et al.*, 2011; Monville *et al.*, 2005; Picconi *et al.*, 2003). On this basis the dose of 12 mg/kg was chosen, considering that it was only to be given on 3 consecutive days (later reduced to 2 days): no severe LIDs were expected. The result was a different one. This, however, allowed for the exploration of the effect of PPTg lesions on LID and the therapeutic effect of PPTg-DBS on those motor complications. The reason for this unexpected reaction might lie in the difference of PD models used. The rats used in the studies referred to above were hemiparkinsonian rats caused by different 6-OHDA dosages, injections sites and targets. The rats in the experiments of this project presented considerable degrees of bilateral DA-degeneration. This degeneration might have been higher than the ones referred to which might be constituting the reason for the more readily reaction to L-DOPA with AIMs. Time did not permit the establishment of a dose-response-curve.

With the appearance of AIMs by the PD rats after administration of L-DOPA the intention was to take advantage of this effect to compare the severity and nature of AIMs in both

parkinsonian groups and assess whether the additional PPTg lesion changed the L-DOPA effect. Further, the potential of PPTg-DBS in reducing those side effects was tested. The results showed clearly that, in the DBS-OFF condition, the additional PPTg lesion in the combined lesion group shifted the severity of the L-DOPA induced AIMs to more severe dyskinetic movements, namely dyskinesias scoring a 5 and 6 on the Creese-Iversen scale, which involve repetitive orofacial movements: Rats of the combined lesion group showed those severe dyskinesias of continuous licking and biting for longer compared to the traditional 6-OHDA lesion group. This effect was observed in both, the anterior DBS and posterior DBS experiment.

Winkler and colleagues pointed out the necessity of having a model for LIDs that better mimics the physiological state considering the heterogeneous pattern of DA denervation in patients, which affects primarily the putamen and consequently they chose striatal injections sites over injections of 6-OHDA into the MFB (Winkler *et al.*, 2002). These experiments here take this one step further by adding extranigral degeneration to the existing model, just as it was done in the experiment described in Chapter 4. Abnormal involuntary movements in form of limb, trunk and orolingual movements as observed in these rats after L-DOPA injection provide a valid rat analogue of human LIDs (Lundblad *et al.*, 2002), especially considering that LIDs in patients often involve orofacial movements (Guridi *et al.*, 2008). Those AIMs are often rated by frequency counts according to a dyskinesia scale [for example (Cenci *et al.*, 1998)] during pre-determined monitoring periods of a short interval. By combining severity using a modified version of the Creese-Iversen scale and duration recording the total time of AIM exhibition the assessment was given a dynamic dimension and allowed for a precise assessment of the L-DOPA response.

The difference in the response to L-DOPA between both PD models was observable and significant. The PPTg innervates the facial, hypoglossus and the trigeminal motor nucleus (Allen

and Winn, 1995; Fay and Norgren, 1997a, 1997b, 1997c), all brainstem nuclei involved in the control of facial musculature. The PPTg being in receipt of BG output and directly connected to those nuclei may explain the enhanced AIMs in response to L-DOPA administration, in particular the increased severity of oromotor movements of licking and biting in the combined lesion group compared to the 6-OHDA lesion group. Since L-DOPA had not been given chronically the reason for AIMs has most likely been DA receptor supersensitivity in the denervated striatum in combination with chronic inhibition of the PPTg (Mitchell *et al.*, 1989). The additional PPTg lesion may have caused a further disinhibition of the oral motor nuclei. In fact, Allen and colleagues showed a shift in the response to intrastriatal injections of d-amphetamine in PPTg lesioned rats compared to sham-operated controls to more severe stereotypy levels; predominantly with respect to orofacial movements including continuous bouts of licking and biting, corresponding to scores 5 and 6 on the Creese-Iversen scale and comparable to the observations made in these experiments (Allen and Winn, 1995). D-amphetamine stimulates DA release and inhibits the reuptake of DA, therefore increasing the concentration of DA in the synaptic cleft, prolonging and increasing the effect of DA on postsynaptic receptors. This is comparable to the action of L-DOPA, which also increases DA concentrations, but by decarboxylation to DA by the lyase enzyme aromatic L-amino acid decarboxylase; the stimulant and AIMs inducing effects of d-amphetamine and L-DOPA can therefore be considered comparable.

In the combined lesion group, DBS of the posterior part of the PPTg had a beneficial effect on the AIMs, shifting the severity from the highest score to less severe and non-oromotor AIMs. There are two possible explanations for it: (i) In PD patients, STN-DBS is a very potent option for patients who suffer from LIDs after years of positive response to L-DOPA medication. It leads to a significant reduction of LIDs. Since this closely correlates with a reduction of L-DOPA dosage for ameliorating the parkinsonian symptoms this is most likely the main cause for the

reductions in LIDs. Nevertheless, it is also suggested that the HFS of the STN has anti-dyskinetic effects on its own, probably due to an effect on the dorsal border of the STN, reaching the lenticularis fasciculus and the zona incerta (Voges *et al.*, 2002). After showing enhanced theta band activity in the dorsolateral striatum of hemiparkinsonian rats that displayed LID after L-DOPA administration in comparison with non-LID hemiparkinsonian rats Alam and colleagues suggested that maladaptive synaptic plasticity changes within the BG circuitry after L-DOPA treatment lead to enhanced theta band activity in the 'off' state which is further pronounced in the 'on' state (Alam *et al.*, 2014). Enhanced theta band activity had already been shown in the SNr of L-DOPA primed PD rats (Meissner *et al.*, 2006) and in the STN of PD patients after L-DOPA administration (Alonso-Frech *et al.*, 2006). It is possible that DBS of the STN not only causes a reduction of LIDs by reducing the L-DOPA dosage necessary for symptomatic relief, but also by correcting the abnormal oscillatory activities in the BG circuitry. The effect of posterior PPTg-DBS might have similar reasons. Posterior PPTg has cholinergic, GABAergic and glutamatergic projections to the STN. By possibly acting on these PPTg efferents by means of the LFS altered neuronal activities in the STN and further related nuclei might be changed and relieve the BG circuit from those altered oscillatory activities that have been associated with LIDs. (ii) The PPTg has downstream control over cranial nerve nuclei involved in oromotor function such as the facial nucleus, the trigeminal motor nucleus and the hypoglossal nucleus. The disinhibiting effect on striatal outflow due to the loss of PPTg neurons, which in the condition of L-DOPA induced AIMs in rats, may have been partially compensated for by the DBS of the posterior PPTg, re-establishing downstream control.

4.4.7. Methodological considerations

Naturally, the DBS device could always be improved by miniaturization. However, the pilot studies showed that rats tolerate the devices attached to the headstages very well and that

carrying the device itself does not interfere with their general behaviour or gait as measured on the CW. Nevertheless, a few methodological points could be improved to increase the accuracy and ultimately the power of the experiments: (i) The electrodes were as small as they could possibly be made with the available means. Compared to the scale of the rat brain the track lesions caused by them appeared however very big. Especially considering the effect of the lesion caused on the behavioural performance it would be preferable to show that the same results would still be reached with smaller sized electrodes. (ii) One concern that arose during the first electrode implants was the fact, that using the same burr hole previously drilled for microinjections of ibotenic acid meant the absence of an intact dura to measure the dorsal-ventral coordinates from. In occasions when the burr hole could not be sufficiently cleaned up to then measure from the brain surface and/or estimate the variance caused by the loss of dura, the burr hole was extended until a clearer measurement was possible. This, however, needed to be avoided to not further compromise the stability of the already heavily "used" skull. Given the accuracy of electrode placement confirmed by the histological assessment, the concerns were not as serious as previously apprehended. Most of the electrodes hit the target well. (iii) In order to increase the power of the results of the experiments it would have been ideal to have bigger experimental groups. The care that bilateral PD models require and the time it takes to complete the necessary training and surgeries limited substantially the possible number of rats used in these experiments. For future experiments using the same model, it would require more assistance for the execution of the experiment or a re-design of the experimental plan.

Chapter 6

General Discussion

1. Aims and summary of results

There has been increasing interest in the role of the PPTg in PD and the potential for PPTg-DBS, but there is no agreement on either of the two questions almost a decade after the first implant of electrodes into the PPTg of patients. The first observations were interpreted enthusiastically and optimistically (Mazzone *et al.*, 2005; Plaha and Gill, 2005; Stefani *et al.*, 2007). However, a convincing therapeutic effect has yet to be proven and the stimulation of the PPTg in PD patients has failed to follow the same success story as the development of STN-DBS and other established targets (Ferraye *et al.*, 2010; Moro *et al.*, 2010; Stefani *et al.*, 2013). Subjective evaluations of complex symptoms, small numbers of patients and a great variety of factors such as patients' diagnosis, exact target site of the electrode, and stimulation parameters contribute to the differences reported in the literature, which shows the clear need for objective and controlled experiments.

The aim of this project was to expand on the existing literature on the role of the PPTg in locomotion in general and on movement in PD; specifically by assessing (i) the effect of full PPTg lesions on gait and (ii) the effect of the addition of partial PPTg lesions to the widely used 6-OHDA rodent model of PD. Further, (iii) the core of the project, the effect of PPTg-DBS on gait and L-DOPA induced AIMs was examined – more specifically, the effect of stimulation of the anterior and posterior PPTg.

It was shown that the PPTg itself is not a crucial structure for the generation of normal gait. Rats bearing partial or full PPTg lesions did not present differences in the individual gait parameters reflecting stability, speed, stride and coordination compared to sham lesioned rats. In Chapter 3 results were presented which compared the gait of rats that received bilateral infusions of ibotenic acid into anterior and posterior PPTg, using a concentration that causes a complete or near to complete lesion of the structure. Based on the extensive

literature that consistently showed a lack of locomotor deficits in rodents with PPTg lesions the hypothesis was, that assessing individual gait parameters would not indicate movement disturbances either. The results stand in contrast to PPTg-lesion studies in non-human primates the results of which, however, need to be interpreted with caution. This is discussed broadly in Chapter 3. The addition of a partial PPTg lesion did not cause any of the analysed gait parameters to differ significantly from the traditional Parkinson's model (using 6-OHDA to destroy the dopaminergic innervation of the dorsal striatum) but in comparison with the sham lesioned control group one specific difference was shown: The shortened stride length in the combined lesion model was not so much shortened as to reach significance in comparison with shams. Two possible explanations are discussed in Chapter 4: (i) a decrease of excitatory input into the STN might have corrected its hyperactive state in the PD model (ii) or the lesion might have caused a compensatory increase of activity in the remaining PPTg neurons counteracting the over-inhibitory outflow from the BG. Independently whether upstream or downstream alteration caused this selective behavioural change, this shows two things: (i) Additional lesions to DA depletion do not change the phenotype of the PD model substantially. Once again, this is difficult to reconcile with the assumption that the PPTg operates as a gait control centre. However, this subtle difference in stride length makes the close relationship with the BG eminent and is hence not surprising. The question is, whether this allows drawing the conclusion that the PPTg lesions can cause motor impairment per se, or if changes of behaviour can be explained differently. (ii) Secondly, it confirms the necessity of employing a PD model that takes into account more of the actual changes in the PD condition. This is most strongly confirmed by the results presented in Chapter 5: The effect of DBS of anterior and posterior PPTg differs significantly between the two models (6-OHDA lesion model and combined lesion model). Stefani and colleagues pointed out themselves "…how is it possible

that DBS of a degenerated area is promoting any effect?" [(Stefani *et al.*, 2013) p.7]. In order to investigate this, it was crucial to refine the traditional PD model as presented in Chapter 4.

One striking difference between the groups was in the response to the implanted electrodes. Differential effects of anterior and posterior PPTg on FOG were found: FOG was not seen in rats with just 6-OHDA lesions but was present in rats with a 6-OHDA lesion plus a partial PPTg lesion and an electrode implanted in anterior PPTg even in the 'OFF' condition. FOG was then further exaggerated when DBS was activated. Posterior electrodes did not cause freezing episodes in either of the models and it is conceivable that the occurrence of FOG in the combined lesion group after switching the posterior PPTg electrode 'ON' is due to current spread to the anterior part. FOG is a symptom that is more prominent in advanced stages of PD (Macht *et al.*, 2007) – in this stage it occurs in about 53% of patients (Giladi, 2001) – and FOG that occurs during a patient's 'ON'-period (on medication) is in general not responsive to DA treatment (Bloem *et al.*, 1996; Krause *et al.*, 2004), some PD patients never experience FOG and furthermore FOG is also present in other movement disorders (see General Introduction). There are, therefore, strong arguments for a cause of FOG lying outside the dopaminergic system, which stands confirmed by the results presented in Chapter 5.

Further, differential effects regarding the effect of PPTg-DBS in both parkinsonian models were found: DBS in posterior but not anterior PPTg ameliorated gait deficits in the 6-OHDA lesioned rats but not in rats with additional partial PPTg lesions – anterior DBS made gait deficits worse. Both, stimulation effects of ascending and descending projections are likely to be involved, as well as antidromic activation of afferent fibres. The proposed mechanisms for these effects are discussed in Chapter 5. The interpretation of the findings in sight of the role of PPTg in movement disorders is summarised below.

Finally, it was shown that posterior but not anterior PPTg stimulation attenuated strong AIMs which the parkinsonian models showed in response to L-DOPA injections. Rats from the combined lesion group developed significantly stronger AIMs than 6-OHDA lesioned rats. PPTg lesioned rats exhibited orofacial activities in response to systemic injections of amphetamine (Inglis, Allen*, et al.*, 1994); after microinjections of d-amphetamine directly into the ventrolateral caudate-putamen rats bearing ibotenate lesions of the PPTg showed more stereotypies classified as 5 and 6 on the Creese-Iversen scale compared to control rats – just like in this study (Allen and Winn, 1995). The observations presented in Chapter 5 confirm the role of the PPTg in mediating striatal outflow and controlling its impact on, here, orofacial behaviour, which is disinhibited due to the PPTg lesion. In advanced stages and after chronic treatment with L-DOPA parkinsonian patients develop L-DOPA-induced dyskinesias (Obeso *et al.*, 2000). Chronic inhibition of the PPTg and PPTg degeneration could be mediating and/or worsening those L-DOPA induced oral dyskinesias (Inglis, Allen*, et al.*, 1994). Here, posterior PPTg-DBS might have restored some of this control over the oral motor nuclei via its descending projections causing an attenuation of the AIMs observed in this study.

Table 6.1. Summary of findings presented in this thesis.

	Various gait parameters
Full PPTg lesions	No deficits
6-OHDA lesion alone	Deficits
6-OHDA lesion + PPTg lesion	Comparable deficits to 6-OHDA alone

			anterior PPTg-DBS	posterior PPTg-DBS
6-OHDA lesion alone	Gait	DBS on	gait gets worse	gait gets better
	FOG	DBS off	no FOG	no FOG
		DBS on	no FOG	no FOG
	L-DOPA	DBS off	L-DOPA induced AIMs	L-DOPA induced AIMs
		DBS on	L-DOPA induced AIMs	L-DOPA induced AIMs

			Anterior PPTg-DBS	posterior PPTg-DBS
6-OHDA lesion + PPTg lesion	Gait	DBS on	no effect on gait	no effect on gait
	FOG	DBS off	FOG	no FOG
		DBS on	FOG +++	FOG
	L-DOPA induced AIMs	DBS off	More severe AIMs than 6-OHDA group	More severe AIMs than 6-OHDA group
		DBS on	More severe AIMs than 6-OHDA group	Shift to less severe scores of AIMs

2. Why did PPTg-DBS not have the expected result?

The majority of articles published on PPTg-DBS refer to the PPTg as the major component of the MLR, a motor controlling structure. The assumption is often made that the excessive inhibitory BG output from the SNr and GPi onto the PPTg puts a brake on this motor control centre – leading to reduced glutamatergic output to the spinal cord, which could then be corrected by LFS of PPTg neurons, assuming LFS drives the inhibited cells. In the General Introduction the problems of clinical lesion reports, lesion studies in non-human primates, and stimulation studies in decerebrate rats and cats were discussed, naming the major reasons for an – at best – cautious conclusion regarding the role of PPTg in movement control: extensive lesions in case of stroke patients and monkeys treated with kainic acid, thermal lesions which cannot exclude the possibility of damage to the passing fibres, and post-surgical testing regimes that do not allow for sufficient recovery from the operation itself. The most recent non-human primate study assessing the role of the PPTg in movement and movement disorders – and the only experiment to study the effect of a combination of DA depletion and PPTg lesions so far published – does not show significant differences between MPTP lesions plus PPTg lesion and non-PPTg-lesioned MPTP-treated macaques (Grabli *et al.*, 2013).

The rodent literature is unambiguous and more extensive. Bilateral lesions and inactivation of PPTg have no effect on general motor behaviour as observed in the homecage, in locomotor test boxes or when performing tasks on the radial maze or operant boxes (Alderson *et al.*, 2003; Dellu *et al.*, 1991; Homs-Ormo *et al.*, 2003; Inglis, Allen*, et al.*, 1994; Inglis, Dunbar*, et al.*, 1994; Keating and Winn, 2002; Olmstead and Franklin, 1994; Steiniger and Kretschmer, 2004; Swerdlow and Koob, 1987; Taylor *et al.*, 2004; D. I. Wilson *et al.*, 2009). The findings presented here add to this by showing no gait deficits as measured on the CW after PPTg lesions.

Differences between species are at that point often drawn upon to explain the differences between the findings resulting from experiments with monkeys and with rodents (Alam *et al.*, 2011). Reasons for believing that this argument is not sufficient to explain the differences are detailed in the General Introduction and it seems rather likely that the above points of criticism constitute the main reasons for the differing conclusions regarding the functional role of the PPTg.

3. The role of the PPTg in motor disturbances – separating 'motor' and 'cognition'?

The involvement of PPTg in movement disorders is however not negligible and therefore an appropriate target in studying motor impairments in PD. But in view of conclusions drawn from all animal studies, the PPTg cannot be regarded as a motor structure in the sense of a control centre. This raises the question of the nature of movement *per se* and if it can be separated from cognitive processes?

From an expanding animal literature discussing PPTg functioning we know that the PPTg has a significant role in cognitive functions, such as learning and reinforcement processes, the updating of action-outcome associations and decision making (Alderson *et al.*, 2004; Alderson *et al.*, 2006; Corrigall *et al.*, 2001; Diederich and Koch, 2005; Kobayashi and Okada, 2007; Olmstead *et al.*, 1998; Samson and Chappell, 2001; D. I. Wilson *et al.*, 2009). The lack of motor impairments after PPTg lesions does not exclude the possibility that motorically expressed behaviour can be altered. In a delayed spatial win-shift task PPTg lesioned rats actually entered the arms of a maze faster than sham lesioned control rats. This was not due to the direct effect of the lesions on movement control, but the rats were impaired in the selection of the appropriate arm in order to receive an award and it is likely that the rats' undirected and disorganised completion of the task made them run quickly from arm to arm (Keating and

Winn, 2002). So how does the PPTg express its role in motor behaviour and how can the gait dysfunctions observed in these experiments be explained?

4. The differential role of anterior and posterior PPTg

Cholinergic, GABAergic and glutamatergic neurons within the PPTg are intermingled but distributed differentially along a gradient on the rostro-caudal axis (Charara *et al.*, 1996; Lavoie and Parent, 1994; Mesulam *et al.*, 1983; Wang and Morales, 2009); their intimate reciprocal connections with the BG, important projections to the thalamus, projections to sites of non-specific cortical input and connections with the medullary reticular formation, cerebellum and spinal cord are complex and widespread. Its anatomical/ neurochemical and electrophysiological heterogeneity is of great significance because it explains the structure's functional diversity (Martinez-Gonzalez *et al.*, 2013; Martinez-Gonzalez *et al.*, 2012; Ros *et al.*, 2010). Understanding this diversity is crucial because it suggests that the effect of PPTg-DBS will depend on exact location within the PPTg. In fact, of the few cases that reported benefits from PPTg-DBS the greatest beneficial effects were achieved with more posterior stimulation (Ferraye *et al.*, 2010; Stefani *et al.*, 2013). Finding different activity patterns of anterior and posterior PPTg in response to movement in PD patients Thevathasan and colleagues also pointed out the probability of differential clinical effects from anterior and posterior PPTg-DBS (Thevathasan, Pogosyan*, et al.*, 2012).

The posterior part of the PPTg receives very fast sensory input and it does not simply relay it: it responds to its meaning and value and passes this information on to thalamocortical and corticostriatal systems via direct connections with the thalamus and midbrain DA neurons of SN and VTA [(Kobayashi and Okada, 2007; Okada and Kobayashi, 2013; Okada *et al.*, 2009; Pan and Hyland, 2005), also see (Maskos, 2008; Mena-Segovia, Winn*, et al.*, 2008)]. But at the same time the PPTg can influence directly downstream structures such as the reticular

formation and spinal cord, initiating action if necessary. Via the anterior PPTg the BG can keep a brake on the PPTg controlling it by GABA mediated efferents from BG output nuclei. Anterior PPTg has descending connections but also ascending connections to DA neurons, mainly in the SN. Anterior PPTg electrodes (and stimulation) proved in this study to have rather devastating results: It caused marked FOG in the combined lesion group. It seems however that the damage of neurons caused by electrode implantation added to the ibotenate induced lesion causing a loss that led to FOG and was further too big to be corrected by any stimulation (in fact stimulation made it even worse).

The literature that examines FOG in patients with movement disorders clearly demonstrates that this is not a pure motor problem. FOG and falls in PD patients correlate with cognitive decline, particularly executive dysfunction. As in patients FOG experienced by the rats as described in Chapter 5 was clearly contextual: it did not affect homecage behaviour, but on the CW, within its confined and narrow space some animals showed pronounced start hesitation. FOG can be a sequence effect of gradually decreasing steps, an increased stance phase and an increased stride to stride variability and impaired coordination (Chee *et al.*, 2009; Hausdorff, Schaafsma*, et al.*, 2003). Given that the type of FOG observed in these rats did not occur during on-going gait or in form of destination hesitation (Schaafsma *et al.*, 2003) these type of gait changes seem unlikely to have caused the freezing (Plotnik *et al.*, 2008). Freezing episodes are largely triggered by external stimuli and when patients need to negotiate tight spaces and obstacles (Schaafsma *et al.*, 2003). As discussed in Chapter 5 the narrow walkway on the CW bounded by high black walls could be compared to narrow spaces or doorways which often trigger FOG in patients. Another possibility is to consider the CW a situation of increased cognitive demand for the rats where they have to perform a previously learned task, comparable to dual task situations which can also trigger freezing in patients (Hausdorff, Schaafsma*, et al.*, 2003).

It is conceivable to understand the freezing episodes as an enhanced problem of disrupted automatic or habitual control of behavioural output, which has been described as a consequence of disrupted normal BG functioning, particularly of the sensorimotor circuitry of that system (Redgrave *et al.*, 2010). Many anatomical and behavioural findings allow the conclusion that goal-directed and habitual control is mediated independently by two circuits: one that connects with the associative and one that connects with the sensorimotor functional territories of the striatum: the dorsomedial and the dorsolateral striatum (in humans corresponding to anterior caudate and posterior lateral putamen), respectively (Balleine and O'Doherty, 2010; Lehericy *et al.*, 2005). In an outcome devaluation task, for example, when the subjects' response during extensive training shifted from goal-directed to habit-based control, an increased activation of the dorsolateral posterior putamen in response of the cue was measured (Tricomi *et al.*, 2009). In PD patients a greater loss of DA is apparent in the posterior putamen (Kish *et al.*, 1988). In rodents, lesions to the dorsolateral striatum blocked stimulus-response learning (Yin *et al.*, 2004). Instead, inactivation of the dorsolateral striatum enhanced the rats' sensitivity to changes in action-outcome contingencies, while rats without this inactivation maintained the same rate of lever presses, not changing to goal-directed behaviour after a session of omission contingency (Yin *et al.*, 2006). So it was suggested that PD patients replace automatic control by goal-directed processes, which would explain the slower execution of movements and the susceptibility to interferences from other goal-directed tasks (Redgrave *et al.*, 2010) and executive dysfunction. In a dual task situation, when goal-directed and habitual control clearly have to act "in concert" (Redgrave *et al.*, 2010), and when several tasks compete for goal-directed control, it is likely that a cessation of ongoing gait or of the processing of gait initiation can ensue – and FOG occurs.

Not all PD patients suffer from FOG. Further, it was shown that patients that belong to the category of "freezers" show a more marked PPTg degeneration and decreased cholinergic

afferents to the thalamus (Bohnen *et al.*, 2009; Karachi *et al.*, 2010). The PPTg degeneration, especially damage to the anterior PPTg as observed in the experiment described in Chapter 5, might additionally cause two problems:

(i) Redgrave and colleagues hypothesise that the output from the goal-directed system has to overcome distorted, 'noisy' output from stimulus-response habitual control circuits at a point where those two systems must converge to give rise to one behavioural output (Redgrave *et al.*, 2010). Given that the BG outflow targets directly the anterior PPTg, it might be the case that the lack of a functioning target structure – the anterior PPTg – further hinders the goal-directed information to overcome the distorting inhibitory signals.

(ii) The PPTg is in an ideal position to mediate outflow from the BG output nuclei and assess whether to send this forward or if incoming sensory information poses a reason for giving preference to a different action. The ability of the PPTg to make such a decision lies in the fact that it is able to analyse sensory information about a stimulus regarding its salient aspects – the association of a stimulus with a reward, the prediction of the reward value, the reward itself, and the actual value of the reward (Kobayashi and Okada, 2007; Okada and Kobayashi, 2013; Okada *et al.*, 2009; Thompson and Felsen, 2013). PPTg neurons showed task-related tonic activity, increasing or decreasing during the task execution period correlated with the response magnitude to cues predicting large or small rewards, and reward-related phasic responses (Okada and Kobayashi, 2013; Okada *et al.*, 2009). Sensory input into the dorsolateral striatum from the anterior PPTg via remaining and functioning midbrain DA neurons of the SNc might support the maintaining of some functioning of the sensorimotor circuitry. The lack of sensory information due to a lesioned anterior PPTg might increase the chance of the system to freeze, especially during a dual task or any other situation that requires goal-directed control, so that these resources cannot be used to compensate for lack of habitual control.

Improvement in motor performance when guided by sensory stimuli supports this assumption (Kadivar *et al.*, 2011; Spaulding *et al.*, 2013): it might cause an increase of sensory output from the PPTg, increasing the chances that the dorsolateral circuit receives this input to support its processes.

Further, the damaged anterior PPTg is unable to convey important information about possibly conflicting, incoming sensory stimuli of the environment ("there are walls but I fit through", "there is the edge of the glass, but behind that I find my home cage") to the SNc. At this point, where the PPTg would assess whether to send the BG output further or if the sensory input carries information that needs to be fed back into the BG system to make a decision on a different motor output ("yes, the way is clear, go ahead" or "no, there are obstacles/ danger of falling off the walkway"), the lack of sensory information to the SNc might put a brake on the system because the decision on competing actions cannot be made and the rat freezes.

Whether anterior PPTg stimulation in the combined lesion group had no effect on gait because there were simply not enough neurons left to have an effect on, or if the number of actual completed CW runs was too small (reduced by the substantial amount of FOG episodes that impeded the completion of CW runs) is not clear. The information, however, coming from the results obtained from the 6-OHDA group allow interesting assumptions about the possible mechanism behind the stimulation (in detail discussed in Chapter 5). Theoretically, driving anterior PPTg neurons that are under pathological over-inhibition from the BG could be expected to have a beneficial effect, implying that it is an activating, stimulating effect LFS has on those neurons. However, given the further aggravation of already observed deficits, it is more likely that the LFS is enhancing the already existing problem, which would imply that the stimulation in anterior PPTg is not affecting the neurons of the PPTg, but having an antidromic effect, driving the inhibitory afferents from the BG. These results suggest two things: (i) the

mechanism behind PPTg LFS might be a different one than expected, which makes it (ii) even more important to consider the exact stimulation site, as the differential effects presented here show.

Both the lesion caused by the electrode and LFS in the anterior PPTg had negative behavioural effects: severe FOG in the combined lesion group and worsening in the 6-OHDA group respectively. Posterior stimulation on the contrary improved gait parameters in the 6-OHDA lesion group. Given its strong connection with the thalamus the PPTg has an important influence on cortical activity. Assessing the functioning of postural sensory integration in PD patients using a balance platform Mueller and colleagues related the results to cholinergic terminal loss measured by means of PET imaging. In a regression analysis controlling for cognitive capacity and PD motor impairments they found an association of decreased thalamic cholinergic innervation and decreased sensory integration measured as an increased centre of pressure sway speed on the balance platform. This led to the suggestion that the disrupted integration of sensory information from the PPTg to the thalamus is involved in postural disturbances (Muller and Bohnen, 2013). Ballanger and colleagues showed increased activity in several cortical areas with PPTg stimulation, amongst these changes in the medial sensorimotor area (Ballanger *et al.*, 2009). Underactivity in this area has been suggested an important factor in parkinsonian motor symptoms (DeLong and Wichmann, 2009) and increased activation has been associated with motor improvement (Brooks and Samuel, 2000; Jenkins *et al.*, 1992). Another study found increased activity in several prefrontal areas with PPTg-DBS, amongst others the dorsolateral prefrontal cortex, orbitofrontal cortex, anterior cingulate (Stefani *et al.*, 2010). It is therefore likely that the benefits seen here after posterior PPTg-DBS can be attributed to influences on cortical structures. For the clinical application it needs to be highlighted that this effect was absent in the model that takes into account the PPTg degeneration that Parkinson's patients manifest. It is however patients at later stages of

the disease –effected by more advanced dopaminergic and extranigral degeneration – who are in most need for surgical treatment and would likely be considered candidates for PPTg-DBS.

It is clear that the PPTg is more than an BG outflow target: (i) it has influence over BG systems; (ii) it constitutes an interface between BG and other brainstem structures and the spinal cord; and (iii) it has extensive connections with brainstem structure involved in motor control such as the medial reticular formation: Humphries and colleagues suggested this to be a potential action selection system in the brainstem (Humphries *et al.*, 2007). In addition, the very short latency of response to sensory input of the PPTg and its role in the startle response and prepulse inhibition (Diederich and Koch, 2005; Fendt *et al.*, 2001; Koch *et al.*, 1993) suggest that it has the capacity to act upon the brainstem without involving the BG. It is not only in receipt of sensory data but can also analyse the input. This way it can control and gate the information before it gets processed further in the BG: sensory information that requires quick action might be prioritised over incoming BG output, which gets integrated with ongoing sensory input. Otherwise, the sensory information gets integrated into the BG and thalamus, exerting influence on the cortico-basal ganglia-cortical circuits.

The findings of this project support this view: lesions of the PPTg disturbed the system in different ways: interrupting the extraction of specific salient aspects of sensory input and the crucial and necessary interface of this information into the corticostriatal system and interrupting the integration of BG output activity at the level of the PPTg before it can be relayed to the reticular formation and other brainstem motor output sites. Severe freezing was the consequence. Further, the inability to integrate oral motor control and to control oral motor systems caused more severe AIMs in combined lesion rats than in DA depleted animals alone. The presumably stimulating effect of DBS on the sensory component of the PPTg improved gait parameters of PD rats, driving sensorimotor integration by acting on ascending

projections to the thalamus and midbrain DA system, but also regaining control over oral motor nuclei downstream, easing L-DOPA induced AIMs.

5. Future experiments

The new refined PD model, unresolved questions and hypotheses made regarding the observed behavioural results present a source for subsequent experiments.

The lack of further deficit or worsening of existing deficits after the addition of a partial PPTg lesion to the 6-OHDA induced DA depletion was discordant with a study conducted on non-human primates assessing also the behavioural effect of the combination of PPTg lesions and DA depletion. Furthermore, the degree of cholinergic cell loss is reported to correlate with disease severity according to the Hoehn and Yahr stages (Braak *et al.*, 2003) and patients who suffer more frequently from falls than other PD patients were reported to show a higher cell loss in the PPTg (Karachi *et al.*, 2010) and a decreased thalamic innervation of cholinergic afferents (Bohnen *et al.*, 2009). The CW might not be able to detect such differences. 1) It would be interesting to test the combined lesion model, in comparison to the simple DA depleted model, in tests more focused on balance. The beam-walking test assesses the rat's interlimb coordination and ability to balance on a narrow beam. Another option is the rotarod test: Monville and colleagues compared different testing protocols and described that testing the rats on different fixed speeds showed maximum sensitivity to detect small changes (Monville 2006). An accelerating protocol might test the animal's ability to detect and adapt to the changing speed, assessing their ability to integrate constantly changing sensory input with their ongoing behaviour.

2) Further, the deficits caused by PPTg lesions in rodents appear in operant tasks or other tasks that require the rats' ability to form associations between action and outcome and the

contingencies. The deficits are of cognitive nature and have been compared to frontal lobe syndrome, a condition that includes cognitive deficits such as executive dysfunction and disorganised behaviour (Winn, 1998). FOG has been repeatedly been associated with executive dysfunction (Giladi and Hausdorff, 2006) and given, again, that it is suggested that patients who suffer from FOG have a higher loss of PPTg neurons, it sounds reasonable to ask the question, whether rats with combined lesions of PPTg and SNc perform worse in cognitive tasks than just DA depleted rats. The difficulty in designing cognitive tasks for parkinsonian rats lies in their severe motor impairment. Faure and colleagues successfully tested bilaterally 6-OHDA lesioned rats in operant boxes, where the rats had to either operate levers or chains. The DA loss, however, was inferior to the DA degeneration caused in the experiments presented here (Faure *et al.*, 2005). It would remain to be tested, whether the rats modelling a more severe stage of PD are able to complete the tasks in an operant box. Radial maze tasks would offer an alternative that does not require the skilled movement of the front limbs. With an adequate test, in the case of behavioural differences between the models, the effect of PPTg-DBS could be assessed, as well. In fact, as described in the General Introduction, neurosurgeons have turned to assessing the effect of PPTg-DBS on cognitive deficits in PD patients, as well [see (Stefani *et al.*, 2013)].

3) It remains the question of the mechanism behind the effects of PPTg-DBS, or, in fact, DBS – LFS and HFS – in general. The mechanisms of DBS on the STN have been studied extensively and many different mechanisms have been described [for detailed reviews please refer to (Benabid *et al.*, 2009; Nowak *et al.*, 2011)]. Benabid and colleagues suggest a function of several interacting mechanisms, which all might have a different weight within this function (Benabid *et al.*, 2009). Furthermore, as discussed earlier, many different factors determine on what and how DBS acts. Regarding the PPTg, it also needs to be considered that the ascending and descending motor connections of the PPTg arise largely from separate, but intermingled,

neurons of different neurochemical compositions (Martinez-Gonzalez *et al.*, 2013). Most likely, it is inadvisable to speak about a gross effect in terms of activation or inhibition of DBS. However, precise tests might be able to isolate possible pathways and test their involvement in DBS mechanisms. a) Electrophysiological imaging of neuronal activity during PPTg-DBS could give first insights of its effect. To date, this has only been done in anaesthetised, but not in freely behaving rats. b) To test whether the improvement of gait after posterior PPTg stimulation was due to an activating effect of posterior ascending projections the effect of chemical stimulation by means of for example the GABA antagonist bicuculline or a L-glutamatate re-uptake inhibitor instead of electrical stimulation would allow reducing the variability that DBS entails (electrode orientation, positioning and distance of nerves and fibres to the contact, current spread). Nandi and colleagues reported that in two MPTP-treated monkeys microinjections of bicuculline into the PPTg significantly improved akinesia. The crucial factor here would be to control the spread of the compound and restrict it to the posterior portion of the PPTg by either tagging the compound and later verifying the spread or by using control groups of different placements of the compound and to compare different effects. c) It would be ideal if it was possible to interrupt the pedunculopontine-thalamic pathway to examine if stimulation of posterior PPTg would still be able to improve gait in the absence of cholinergic input from the PPTg into the thalamus. The fusion toxin Dtx-UII causes selective cholinergic lesions when injected into the PPTg. At the moment ongoing work in this laboratory is examining if this toxin can also lesion cholinergic terminals. Should this be successful, it would offer a possibility to produce selective cholinergic denervation of thalamic nuclei. d) The use of optogenetic techniques would allow for further control of selectively activating or inhibiting pathways of interest. In order to investigate whether the gait improvement with posterior PPTg-DBS is due to the activations of cholinergic projections to the thalamus this pathway can be manipulated in Chat::Cre rats (Witten *et al.*, 2011). The

expression of channelrhodopsin in cholinergic neurons of the PPTg can be achived by targeting cholinergic terminals in the thalamus by injecting a Cre-dependent AAV virus carrying the gene for channelrhodopsin directly into the terminal region. Channelrhodopsin will then be retrogradely expressed in cholinergic neurons of the PPTg, which can then be activated using blue light on the the PPTg. The problem with this approach is that not only the axons directed at the thalamus but also collateral to other targets will be activated. Further, cholinergic neurons in the LDTg that project to the thalamus will equally be light sensitive and might be activated should the light spread to this structure. Alternatively, the entire cholinergic population of the PPTg can be rendered light sensitive (or as much of the population as this technique allows) by injection of the virus directly into the PPTg. By delivering the blue light to the thalamus the cholinergic terminals can be activated. The limitation of this approach might be a retrograde activation of the cholinergic neurons in the PPTg and subsequent activation of collateral projections to other regions.

4) The administered dose of L-DOPA was too high for the here employed PD models to avoid the development of L-DOPA induced AIMs. However, it was observed that during the first 10 to 15 minutes L-DOPA reduced the rats' rigidity notably. It would be interesting to assess the ability of L-DOPA to correct gait deficits in the combined lesion model. Westin and colleagues showed improvement of stride length but not swing speed or duty cycle of bilaterally 6-OHDA lesioned rats tested on the CW (Westin *et al.*, 2012). Given that the additional PPTg lesion did not result in worsening of gait it is likely that the gait deficits might be improved by L-DOPA in the same way as they could improve gait deficits of DA depleted rats alone – provided a dose of L-DOPA is found that can provide benefit without causing L-DOPA induced side-effects.

6. Conclusion

Our understanding of the role of the PPTg has been broadened over the years: Anatomical, physiological and behavioural findings in rodents, non-human primates and humans have changed the focus from traditionally a motor structure and master switch for sleep regulation to the recognition of the PPTg as a heterogeneous, widely connected structure with integrative and complex functions that involve formation and updating of action–outcome associations and decision making. This work has confirmed this view and proven that it is important that this understanding is now also being reflected in the PPTg-DBS literature to (i) better understand the variable outcomes of PPTg-DBS in PD patients so far and (ii) to consider crucial factors concerning the effect of PPTg-DBS: the effect depends on the exact location within the PPTg and on the extent of survival of PPTg neurons. The actual therapeutic potential of this target site for DBS needs to be considered carefully, especially in consideration of PPTg degeneration in PD and balanced with potential adverse effects of PPTg-DBS.

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Appendix

Supplementary material

Supplementary Table 3.1.: Pre-surgical data presented as means ± SEM. Univariate Anovas comparing the PPTg lesion and the sham lesion group proved that for all analysed parameters a pre-existing difference between groups could be ruled out. Corresponding p-values are given.

	BOS		Duty Cycle		Support		Swing Speed		Stride Length		Print Position	
	front	hind	front	hind	diagonal	three	front	hind	front	hind	right	left
PPTg lesion (N = 6)	11.90 ± 0.44	26.27 ± 0.44	47.62 ± 0.91	52.19 ± 0.82	64.22 ± 1.15	14.25 ± 1.79	1.39 ± 0.05	1.52 ± 0.06	154.26 ± 3.58	149.93 ± 3.86	-2.52 ± 1.4	-3.59 ± 1.51
Sham lesion (N = 4)	12.78 ± 1.05	25.8 ± 0.55	46.62 ± 1.04	50.15 ± 1.33	62.61 ± 2.18	12.66 ± 2.31	1.38 ± 0.05	1.5 ± 0.06	159.94 ± 4.62	152.99 ± 3.94	-3.35 ± 2.61	-2.95 ± 1.78
p value	0.615	0.595	0.632	0.252	0.567	0.685	0.949	0.825	0.453	0.675	0.824	0.850

Supplementary Table 4.1.: Data from the two individual sets presented as means together with the range. The group numbers of the individual sets were considered too small for statistical testing. Inspection of the raw numbers comparing set 1 and set 2 of each experimental group considering the given range led to the conclusion that no systematic differences were detected that would prevent the data of both sets to be merged.

	BOS front			BOS hind			Duty Cycle front			Duty Cycle hind		
Pre surgery	mean	min	max	mean	min	max	mean	min	max	mean	min	max
PPTg 1st set (N = 3)	13.13	10.40	16.42	26.43	23.27	28.83	43.56	36.81	48.68	50.71	45.89	57.97
PPTg 2nd set (N = 4)	12.91	10.16	15.86	25.38	21.06	28.03	47.04	39.27	54.76	51.17	44.13	59.97
combined 1st set (N = 2)	12.57	8.99	15.99	27.55	25.30	29.64	43.40	39.20	48.15	50.76	46.86	54.26
combined 2nd set (N = 5)	13.50	8.37	17.30	24.29	19.94	31.87	46.51	35.03	53.20	50.30	40.89	59.63
shams 1st set (N = 6)	12.29	6.50	16.45	27.35	23.02	31.75	44.08	32.35	51.78	50.74	40.83	57.21
shams 2nd set (N = 2)	11.44	8.74	14.56	26.79	25.07	28.67	48.13	40.73	53.73	51.79	40.15	58.06
Post surgery	mean	min	max	mean	min	max	mean	min	max	mean	min	max
PPTg 1st set (N = 3)	13.22	8.67	16.99	23.99	15.39	30.69	45.46	35.40	52.47	50.50	40.75	60.54
PPTg 2nd set (N = 4)	12.68	6.18	17.19	24.09	18.82	28.19	45.87	37.22	53.67	49.87	38.22	61.91
combined 1st set (N = 2)	17.21	12.58	20.41	33.33	29.20	37.67	43.91	29.51	55.34	52.40	39.51	64.32
combined 2nd set (N = 5)	18.91	13.52	22.59	29.79	25.49	36.15	49.22	41.04	57.29	52.48	37.92	69.93
shams 1st set (N = 6)	10.68	4.80	14.65	21.94	13.42	28.82	43.16	26.55	55.21	49.86	38.46	62.93
shams 2nd set (N = 2)	9.33	6.72	14.27	22.43	18.55	25.28	45.85	37.96	56.08	51.49	45.97	60.17

	Support diagonal			Support three			Swing Speed front			Swing Speed hind		
Pre surgery	mean	min	max	mean	min	max	mean	min	max	mean	min	max
PPTg 1st set (N = 3)	64.29	40.97	76.50	8.51	3.03	20.75	1.33	0.97	1.55	1.52	1.19	1.70
PPTg 2nd set (N = 4)	62.93	45.55	72.94	14.23	0.00	39.83	1.16	0.85	1.35	1.29	0.92	1.65
combined 1st set (N = 2)	64.23	48.46	73.58	8.96	3.25	14.31	1.33	1.13	1.45	1.53	1.32	1.78
combined 2nd set (N = 5)	61.86	45.39	81.39	13.11	0.00	31.11	1.21	0.89	1.67	1.31	0.85	1.77
shams 1st set (N = 6)	64.92	43.76	82.22	9.12	0.00	25.51	1.39	1.08	1.70	1.57	1.21	1.89
shams 2nd set (N = 2)	66.67	55.72	82.04	14.11	1.19	33.09	1.17	0.94	1.34	1.27	0.98	1.55
Post surgery	mean	min	max	mean	min	max	mean	min	max	mean	min	max
PPTg 1st set (N = 3)	63.14	50.16	81.38	9.42	0.00	34.24	1.21	1.01	1.41	1.33	1.11	1.58
PPTg 2nd set (N = 4)	66.86	52.18	78.03	9.67	0.00	28.02	1.18	0.86	1.42	1.29	0.94	1.53
combined 1st set (N = 2)	52.14	27.42	67.83	16.50	0.00	41.66	0.86	0.58	1.13	1.02	0.71	1.24
combined 2nd set (N = 5)	58.57	34.66	77.14	18.46	0.00	44.49	0.94	0.52	1.42	1.01	0.77	1.46
shams 1st set (N = 6)	61.50	37.79	81.31	8.85	0.00	36.18	1.25	0.89	1.68	1.40	1.11	1.92
shams 2nd set (N = 2)	62.11	49.62	74.13	13.16	1.59	35.98	1.32	1.08	1.76	1.48	1.19	2.00

	Stride Length front			Stride Length hind			Print Position right			Print Position left		
Pre surgery	mean	min	max	mean	min	max	mean	min	max	mean	min	max
PPTg 1st set (N = 3)	158.85	137.98	170.50	158.07	146.82	174.74	-4.61	-28.46	11.54	-6.21	-28.40	4.03
PPTg 2nd set (N = 4)	145.20	116.53	166.97	142.20	114.31	162.02	-2.03	-12.81	6.36	-3.71	-9.39	2.54
combined 1st set (N = 2)	155.70	142.90	167.24	152.91	139.96	164.56	-2.56	-12.05	2.48	0.32	-2.75	5.68
combined 2nd set (N = 5)	147.91	114.79	177.66	145.08	117.45	175.79	-4.91	-14.35	10.25	-4.82	-16.74	6.45
shams 1st set (N = 6)	159.12	137.96	183.18	155.44	136.62	176.82	-0.33	-9.69	12.93	-2.14	-12.05	6.63
shams 2nd set (N = 2)	143.67	131.60	161.20	140.88	126.88	160.29	5.71	-1.24	12.35	-0.98	-7.36	5.34

Post surgery	mean	min	max	mean	min	max	mean	min	max	mean	min	max
PPTg 1st set (N = 3)	157.31	130.90	169.19	153.26	131.17	165.59	-13.78	-21.89	9.45	-13.43	-24.35	4.36
PPTg 2nd set (N = 4)	160.39	125.27	185.18	156.95	123.37	186.12	-9.02	-19.98	4.49	-10.98	-22.04	-0.58
combined 1st set (N = 2)	140.98	120.96	159.01	138.13	118.59	159.79	-3.62	-14.47	15.93	-5.60	-12.20	1.67
combined 2nd set (N = 5)	141.55	94.12	201.14	140.15	92.79	201.98	-7.41	-23.74	3.53	-9.97	-24.86	3.38
shams 1st set (N = 6)	161.25	137.31	197.56	155.67	125.96	188.88	-8.21	-22.29	10.78	-7.60	-27.31	6.97
shams 2nd set (N = 2)	159.55	129.21	189.84	151.94	124.80	182.63	-6.05	-18.17	1.83	-9.47	-21.68	-3.18

Supplementary Table 4.2.: Pre-surgical data presented as means ± SEM. Univariate Anovas comparing the PPTg lesion and the sham lesion group proved that for all analysed parameters a pre-existing difference between groups could be ruled out. Corresponding p-values are given.

	BOS		Duty Cycle		Support		Swing Speed		Stride Length		Print Position	
	front	hind	front	hind	diagonal	three	front	hind	front	hind	right	left
partial PPTg lesion (N = 7)	13.00 ± 0.35	25.83 ± 0.40	45.55 ± 0.8	50.97 ± 0.85	63.51 ± 1.72	11.78 ± 1.65	1.23 ± 0.03	1.39 ± 0.04	151.05 ± 2.67	149.00 ± 2.57	-3.13 ± 1.48	-4.78 ± 1.09
Combined lesion (N = 7)	13.24 ± 0.5	25.22 ± 0.61	45.62 ± 0.82	50.43 ± 0.85	62.53 ± 1.67	11.92 ± 1.67	1.24 ± 0.03	1.38 ± 0.04	150.13 ± 3.20	147.32 ± 3.06	-4.24 ± 1.19	-3.35 ± 1.09
6-OHDA lesion (N = 7)	12.51 ± 0.36	26.94 ± 0.51	44.46 ± 1.04	51.6 ± 1.05	59.83 ± 1.88	12.47 ± 1.92	1.30 ± 0.04	1.48 ± 0.04	152.71 ± 2.71	149.29 ± 2.80	-5.07 ± 1.44	-5.65 ± 1.77
Sham lesion (N = 8)	12.08 ± 0.43	27.21 ± 0.35	45.09 ± 0.9	51.00 ± 0.76	65.35 ± 1.76	10.37 ± 1.4	1.33 ± 0.03	1.5 ± 0.04	155.26 ± 2.33	151.80 ± 2.18	1.18 ± 1.09	-1.85 ± 0.79
p value	0.549	0.289	0.918	0.923	0.482	0.91	0.451	0.414	0.855	0.906	0.186	0.509

Supplementary Table 5.1.: Pre-surgical data presented as means ± SEM. Univariate Anovas comparing the combined lesion, 6-OHDA lesion and the sham lesion group proved that for all analysed parameters a pre-existing difference between groups could be ruled out. Corresponding p-values are given.

anterior PPTg-DBS	BOS		Duty Cycle		Support		Swing Speed		Stride Length		Print Position	
	front	hind	front	hind	diagonal	three	front	hind	front	hind	right	left
combined lesion (N = 4)	13.56 ± 0.91	32.48 ± 0.69	55.06 ± 0.86	59.68 ± 1.28	57.10 ± 2.72	34.66 ± 3.00	1.43 ± 0.08	1.57 ± 0.08	144.91 ± 4.73	141.12 ± 4.43	4.14 ± 1.36	1.62 ± 2.10
6-OHDA lesion (N = 5)	12.94 ± 0.69	29.68 ± 0.98	53.27 ± 0.90	58.84 ± 0.98	61.03 ± 2.63	30.51 ± 2.65	1.42 ± 0.06	1.60 ± 0.06	149.33 ± 3.76	145.27 ± 3.73	-0.82 ± 1.42	0.17 ± 1.25
Sham lesion (N = 5)	12.98 ± 0.58	29.51 ± 0.73	52.27 ± 0.77	57.51 ± 1.09	66.22 ± 2.95	24.64 ± 3.07	1.61 ± 0.07	1.78 ± 0.07	160.26 ± 4.71	156.54 ± 4.78	-2.77 ± 1.34	-1.59 ± 1.08
p value	0.908	0.309	0.269	0.65	0.346	0.297	0.383	0.371	0.336	0.33	0.117	0.654

posterior PPTg-DBS	BOS		Duty Cycle		Support		Swing Speed		Stride Length		Print Position	
	front	hind	front	hind	diagonal	three	front	hind	front	hind	right	left
combined lesion (N = 4)	15.64 ± 1.16	27.41 ± 0.76	55.98 ± 1.21	59.17 ± 1.76	54.99 ± 4.50	36.34 ± 5.53	1.41 ± 0.11	1.49 ± 0.11	143.29 ± 5.69	139.77 ± 5.68	0.57 ± 1.05	0.21 ± 1.05
6-OHDA lesion (N = 4)	15.79 ± 0.67	27.50 ± 0.60	53.94 ± 1.02	56.74 ± 1.61	59.63 ± 3.96	28.39 ± 4.65	1.37 ± 0.10	1.43 ± 0.09	151.83 ± 6.97	148.28 ± 6.49	3.10 ± 1.29	0.63 ± 1.33
Sham lesion (N = 4)	12.75 ± 0.58	28.35 ± 0.80	51.48 ± 1.32	53.40 ± 0.92	71.46 ± 3.98	18.02 ± 3.79	1.60 ± 0.04	1.66 ± 0.04	165.78 ± 5.11	159.41 ± 4.65	-2.21 ± 1.32	-2.52 ± 1.67
p value	0.235	0.697	0.221	0.232	0.254	0.254	0.484	0.448	0.25	0.316	0.179	0.490