Deep Brain Stimulation of the Subthalamic Nucleus: c-Fos expression in the Lateral Habenula and Dorsal Raphe Nucleus of Parkinsonian Rats

Dorien MERKEN

promotor :
dr. Y. TEMEL

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List of abbreviations

AADC: aromatic amino acid decarboxylase
ACh: acetylcholine
AD: autosomal dominant
AR: autosomal recessive
BG: basal ganglia
BSA: bovine serum albumine
c-Fos ir: c-Fos immunoreactivity
COMT: catecholamine-o-methyl transferase
CP: cerebral peduncle
CRF: corticotropin-releasing factor
DA: dopamine
DAB: 3,3-diaminobenzidine
DBS: deep brain stimulation
DPX: Depex
DRN: dorsal raphe nucleus
EP: entopeduncular nucleus
GABA: γ-amino butyric acid
GFAP: glial fibrillary acidic protein
GLU: glutamate
GPe: globus pallidus pars externa
GPi: globus pallidus pars interna
HE: hematoxylin-eosin
HF: high-frequency
IEG: immediate early gene
L-dopa: levodopa
LF: low-frequency
LH: lateral habenula
MAO: monoamine oxidase
MC: (primary and secondary) motor cortex
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTN: mean total number
NeuN: neuronal nuclei
NMDA: N-methyl-D-aspartate
OT: optical tract
PD: Parkinson's disease
ROS: reactive oxygen species
SN: substantia nigra
SNpc: substantia nigra pars compacta
SNpl: substantia nigra pars lateralis
SNpr: substantia nigra pars reticulata
STN: subthalamic nucleus
TBS: Tris-buffered saline
TBS-T: Tris-buffered saline with Triton X-100
TH: tyrosine hydroxylase
THir: TH immunoreactivity
UPS: ubiquitin-proteasome system
VTA: ventral tegmental area
5-HT: 5-hydroxytryptamine; serotonin
6-OHDA: 6-hydroxydopamine

Preface

The human brain has always been a mysterious and fascinating organ to me, controlling all functions of the human body but also holding one's essence and soul. This project on Deep Brain Stimulation and Parkinson's disease has given me a great opportunity to study the sometimes unexpected workings of the brain in an interesting preclinical setting.
In a society that continues to grow older, Parkinson's disease is affecting more and more people every day, not only the patients themselves, but also their families, physicians and health carers. The fact that medicational treatment is not effective in the long-term only enlarges the impact of the disorder on the daily lives of these people. Deep Brain Stimulation has changed that, and has become a desired treatment option. But the unexpected behavioural side effects that accompany the procedure pose new questions that need to be resolved and show us that some important pieces of the puzzle are still missing. This is why this project drew my attention and why it has been so interesting to work on it the past six months.

Many people have helped and guided me throughout this project. I am very grateful to my promoter, Dr. Yasin Temel, for his teaching, advice and guidance and for showing me that you can always achieve more than you initially held possible. I would also like to thank Prof. Dr. Harry Steinbusch and Dr. Veerle Visser-Vandewalle for their help and support during the last six months. For their excellent assistance in the lab, I am very grateful to Hellen Steinbusch and Marjanne Markerink, I could not have done it without them.
Of course, one cannot succeed without the required daily dose of moral support, help, pep talk and energy that only friends can give, and for this I want to thank Rinske Vlamings, Rob Hameleers, Marijke Lemmens, Silvie Timmers, Sofie Lemmens, and Eveline Strackx. A very special thank you goes out to Marlies Celis, Lies Gelders, Kristien Hebbrecht and Veerle Nicolaers, for all their friendship, love and support that could never be replaced.
I am forever grateful to my parents, Nicole Schoefs and Martin Merken, for giving me the freedom to spread my wings and see the world, for their support during my studies and for always loving me. I also want to thank my brother Maarten, for being my friend and help in need. And last but not least, I want to thank Jeroen Van der Velden for all his love.

Dorien Merken
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Abstract

Parkinson's disease (PD) is characterized by the progressive and extensive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). As a result of this, the subthalamic nucleus (STN) becomes hyperactive and motor control by the basal ganglia (BG) is impaired, leading to PD symptoms. Chronic stereotactic Deep Brain Stimulation (DBS) is used to "silence" the hyperactive STN. However, the occurrence of stimulation-dependent behavioural side effects such as depression and impulsivity still imposes a large restriction on the common use of this surgical technique.

Because mood disorder symptoms have long been linked with the midbrain 5-hydroxytryptamine (5-HT; serotonin) system, this study aimed to test the hypothesis that high-frequency DBS of the STN alters neuronal activity in these limbic brain regions.
Specifically, the 6-OHDA rat model for PD was subjected to STN DBS using clinically relevant stimulation parameters (frequency 130 Hz; pulse width 60 µs; amplitude 150 µA). Brain sections were stained immunohistochemically for c-Fos, a commonly used marker for neuronal activity. Consequently, immunoreactivity was measured in distinct limbic brain regions.

Increased c-Fos expression was observed in neurons of the dorsal raphe nucleus (DRN) and lateral habenula (LH) of STN-stimulated rats when compared to non-stimulated animals. These results show for the first time an effect of chronic bilateral STN DBS on specific mood-related brain regions, suggesting the existence of a novel signalling pathway connecting the STN to the dorsal raphe and lateral habenula.

1 Introduction

Parkinson's disease (PD) is one of the major neurodegenerative disorders our present-day society is faced with. The first description of PD dates from 1817, when James Parkinson published his *"Essay on the Shaking Palsy"* and described the clinical features of the disorder [1]. During the next 150 years, the pathological hallmarks of the disease were discovered. It is now known that PD involves the progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), accompanied by depigmentation of the SNpc and resulting in DA depletion in the striatum. Additionally, the remaining nigral DA neurons show the presence of Lewy bodies, pathogenic intraneuronal inclusions composed of protein aggregates (Figure 1) [2].

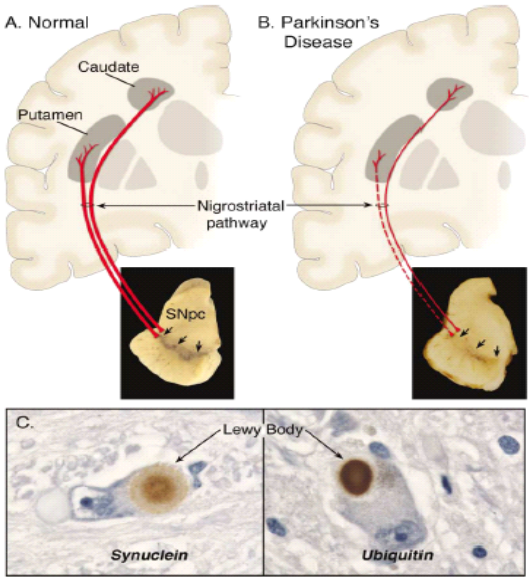


Figure 1: Pathological hallmarks of PD. (A and B) Loss of DA neurons in the SNpc leads to nigral depigmentation and striatal DA depletion. (C) The remaining DA neurons show Lewy bodies.

Striatal DA depletion leads to the main clinical manifestations of PD: (1) bradykinesia, a slowness in initiating and executing movements; (2) muscular rigidity that leads to difficulties in walking, writing, speaking and masking of facial expressions; (3) resting tremor; and (4) impaired postural reflexes and instability [3].

From an epidemiological point of view, PD is difficult to define. Estimating incidence and prevalence rates is problematic because of the lack of an 'in-life' marker for (idiopathic) PD. Across Europe, prevalence rate estimates range from 65.6 per 100,000 to 12,500 per 100,000 and annual incidence estimates range from 5 per 100,000 to 346 per 100,000 as a result of this [4]. Nevertheless, it is known that PD incidence and prevalence generally increase with age, but that younger people can also be affected [5, 6].

1.1 Etiology and pathogenesis of Parkinson's disease

PD, like other common (neurodegenerative) disorders, displays a dichotomy between familial (rare) and nonfamilial (sporadic) forms. While several PD-related gene mutations have been identified (see Table 2 for an overview), the cause of the more common, sporadic form of the disease still remains unknown. One popular theory is the so-called *"Environmental hypothesis"*, which states that exposure to endo- or exogenous (neuro)toxins triggers one or more signalling cascades that eventually lead to neuronal death in the SNpc [2]. Examples of exogenous neurotoxins that cause PD-like disease include MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), rotenone and their metabolites; substances that can be found in herbicides and pesticides. Epidemiological studies have described an association between PD and exposure to these compounds, but no specific causal relationship has yet been shown [7]. As an extension of the environmental hypothesis, it is also thought that environmental exposure to toxic substances can lead to the formation of endogenous neurotoxins through distortion of normal cellular metabolic processes [2].

Whether it is a toxic environmental insult or an inherited gene defect that is considered to be the cause of PD, the deleterious events set in motion by the etiological hit have been classified under two major theories: (1) *Mitochondrial dysfunction and oxidative stress*; and (2) *Protein misfolding and aggregation*.
First, exposure to environmental toxins is thought to suppress the activity of the mitochondrial respiratory chain complex I, leading to mitochondrial dysfunction, generation of reactive oxygen species (ROS), and oxidative stress [2]. This may create a vicious cycle of neurotoxic cell damage. Consistent with an excess of oxygen-free radicals, several biomarkers of oxidative stress are elevated in the SNpc of PD brains, while the antioxidant glutathione is reduced [8, 9]. In addition, several PD-related gene mutations increase the cells' vulnerability to oxidative stress and mitochondrial dysfunction (Table 2).
Second, it has been shown *in vitro* and *in vivo* that oxidative stress promotes the formation of Lewy bodies by mediating the aggregation of α-synuclein, a key protein in PD pathology (Table 2) [10]. α-Synuclein is a molecular chaperone, and its aggregation with other proteins into Lewy bodies impairs the activity of the ubiquitin-proteasome system (UPS), a system essential for the non-lysosomal degradation and removal of damaged, mutated and misfolded proteins [11]. When it is suppressed, the accumulation of damaged proteins can initiate various neurotoxic pathways [2].

Overall, it is thought that mitochondrial dysfunction, the generation of ROS, and the impaired UPS activity (independently) activate apoptotic cascades, which – in PD – eventually culminate in the death of DA neurons in the SNpc. Moreover, DA neurons seem to possess a specific vulnerability for neuronal death in PD, due to the fact that DA metabolism in itself produces hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\circ -}$) radicals [2].

Table 1: Genes involved in familial Parkinson's disease (AD: autosomal dominant; AR: autosomal recessive).

Locus name	Chromosomal localization	Gene	Protein	Protein function	Mutation(s)	Inheritance pattern	Properties of the mutated protein(s)	Role in PD neurodegeneration	References
PARK1	4q21-22	*SNCA*	α-synuclein	Presynaptic vesicular function and/or transport modulation; Molecular chaperone	A30P A53T Locus duplication Locus triplication	AD	↓ degradation; ↑ tendency to form soluble oligomeric intermediates (toxic protofibrils)	Component of Lewy bodies; Interaction with pro-apoptotic proteins	[2, 12-15]
PARK2	6q25-27	*PRKN*	Parkin	E3-ubiquitin-protein ligase	Exon deletion Thr240Arg Gln311Stop	AR	Loss of protein function Elimination of a CKII phosphorylation site Formation of a truncated protein	Incomplete degradation of Lewy body-associated proteins; Accumulation of misfolded parkin substrates	[16-19]
PARK5	4p14	*UCH-L1*	Ubiquitin C-terminal hydrolase L1	Ubiquitin recycling	I93M S18Y	AD	Loss of protein function Increased UCH-L1 activity	Accumulation of building blocks for neurotoxic fibrils **Protection against PD development**	[2, 19, 20]
PARK6	1p35-1p36	*PINK1*	PTEN-induced putative kinase 1	Protection against stress-induced mitochondrial dysfunction and apoptosis	G309D W347OPA	AR	Loss of protein function Formation of a truncated protein	↑ susceptibility to stress-induced damage and/or apoptosis	[21]
PARK7	1p36	*DJ1*	DJ-1	Participant in oxidative stress response?	14 kb deletion L166P	AR	Loss of gene expression Loss of protein function	↓ oxidative stress response	[20, 22]
PARK8	12p11.2-q13.1	*LRRK2*	Leucine-rich repeat kinase 2	Several functional domains, but its function(s) are currently unknown	splice site mutation Y1699C R1441C I1122V I2020T L1114L	AD	Mutations are found in different functional domains → which one(s) related to neurodegeneration?	Currently unknown	[23, 24]

1.2 Basal Ganglia-Thalamocortical Circuitry

The nigrostriatal DA pathways which are selectively affected in PD are part of the so-called 'Basal Ganglia (BG) Circuitry' of the brain. The BG are a group of interconnected nuclei located in the extrapyramidal motor system, just below the cerebral cortex [25]. They consist of the striatum (nucleus caudate and putamen), the globus pallidus pars interna (also called the entopeduncular nucleus or EP) and pars externa (GPi and GPe), the substantia nigra pars reticulata, compacta and lateralis (SNpr, SNpc, SNpl) and the subthalamic nucleus (STN) [26].
Functionally, the BG work together with cortical areas and thalamic nuclei in the execution of goal-directed behaviours. This involves not only movement, but also cognitive and emotional processes. The BG-thalamocortical motor, associative, and limbic circuits are schematically represented in Figure 2 [27].

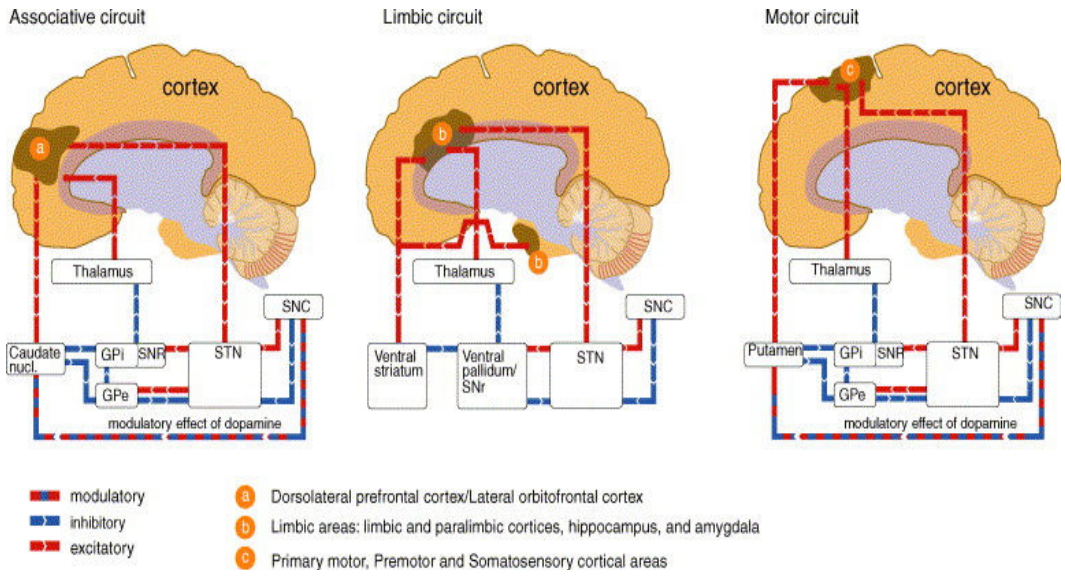


Figure 2: The BG-thalamocortical associative, limbic, and motor circuits. (inhibitory signalling: GABA-mediated; excitatory signalling: GLU-mediated)

The structure and working mechanism of these loops is generally the same. When the body is in rest (eg. when no goal-directed behaviours need to be performed), the STN exerts a physiological tonic firing activity. The targets of its excitatory glutamatergic (GLU) signalling are the two main BG output nuclei, the SNpr and the GPi. These nuclei, in turn, inhibit specific thalamic (and brainstem) target nuclei in order to prevent unwanted stimulation of cortical areas. However, to prevent its own hyperactivity and, consequently, thalamocortical hypoactivity, the STN also stimulates the GPe, which then suppresses STN and GPi activity in an inhibitory γ-amino butyric acid (GABA)-mediated feedback loop.

On the other hand, when the body needs to act or move, the cortex initiates the proper BG-thalamocortical signalling by stimulating the striatum. This activates inhibitory striatal fibres projecting to the SNpr and GPi, leading to a transient interruption of the BG output signal and activation of thalamocortical projections. The SNpc plays an important role in strengthening this thalamocortical disinhibition via (1) excitatory fibres that stimulate the inhibitory striatal neurons projecting to the GPi and SNpr; and (2) inhibitory fibres that suppress GABA-ergic striatal neurons projecting to the GPe [28].

Loss of DA neurons in the SNpc – as happens in PD – therefore has profound effects on the functional activity of the BG circuits. Due to the loss of inhibitory input from the SNpc and the GPe, the STN becomes hyperactive. It has been reported that in patients with limb tremor, many STN neurons display high-frequency synchronized oscillatory activity. Additionally, variations in firing pattern have also been reported [29]. It is now thought that STN hyperactivity due to DA depletion is the main cause of PD motor symptoms, disrupting normal BG physiology via modification of the electro-physiological and metabolic activity in the SNpr and GPi [30]. Furthermore, STN overdrive is predicted to enhance DA release in the SNpc as an initial compensatory mechanism after DA depletion. The excessive release of GLU following STN hyperactivity can, however, lead to excitotoxic damage and further loss of DA neurons. This would accelerate the progression of the disease [31].

In conclusion, hyperactivity of the STN does not only play a major part in causing PD symptoms, but can also worsen the disease through GLU-mediated excitotoxicity. The identification of the STN as a key component in PD-associated BG pathology has made this nucleus an interesting target for new and alternative therapeutic interventions. An overview of PD therapies is given in the next section.

1.3 Treatment of Parkinson's disease

As yet, the treatment of PD remains symptomatic, and a lasting improvement of the patients' motor functions is the main focus of most treatment strategies. The different categories of therapeutic interventions for PD are summarized in Table 3 [32]. Of relevance here are the DA drugs and the surgical therapies. They are briefly described below.

Table 3: Current therapeutic interventions to treat the motor features of PD.

Drugs
Dopaminergic medications
L-dopa
Dopamine agonists (apomorphine, bromocriptine, cabergoline, dihydroergocriptine, lisuride, pergolide, piribedil, pramipexole, ropinirole)
MAO-B inhibitors (selegiline)
COMT inhibitors (entacapone, tolcapone)
Nondopaminergic medications
Antiglutamate (amantadine)
Anticholinergic (benztropine, biperiden, orybenadrin, procyclidine, trihexyphenidyl)
Surgery
Lesion (thalamotomy, pallidotomy, subthalamotomy)
Deep brain stimulation (thalamus, pallidum, subthalamic nucleus)
Rehabilitation
Physical therapy
Occupational therapy
Speech therapy

1.3.1 Dopamine replacement therapies

L-dopa, or levodopa, is probably the most effective drug in the (symptomatic) treatment of PD. It was first described as an antiparkinsonian drug by Cotzias *et al.* in 1967 [33]. Levodopa is a metabolite in the synthesis of DA. It is formed out of tyrosine by the enzyme tyrosine hydroxylase (TH) and further converted to DA via aromatic amino acid decarboxylase (AADC).

Exogenous L-dopa crosses the blood-brain barrier and is taken up by the nigral DA neurons, where it is integrated into the endogenous DA synthesis. In this way, the DA stores of the nigral neurons can be refilled. Other drugs that act on DA metabolism include catecholamine-o-methyl transferase (COMT) inhibitors and monoamine oxidase (MAO)-B inhibitors, which prevent conversion of L-dopa into metabolites other than DA and thus increase L-dopa bioavailability. Finally, DA agonists exist that act on postsynaptic DA receptors (for examples, see Table 3) [34].

Initially, these drugs markedly improve PD symptoms and greatly enhance the patients' quality of life. Unfortunately, long-term L-dopa use leads to "dopa-resistant" motor symptoms such as speech impairment, abnormal posture, and gait and balance problems; "dopa-resistant" nonmotor symptoms like autonomic dysfunction, mood and cognitive impairments, sleep problems, and pain; and/or drug-related side effects such as psychosis, motor fluctuations, and dyskinesias [32].

It appears that as the disease progresses, the beneficial effect of each dose of levodopa progressively shortens. This is called *"end-of-dose deterioration"* or *"off-phase"*, and is due to the progressive loss of DA neurons in the SNpc. Increasing dose frequency will alleviate these complications, but not for long. Eventually, the patient enters a state where his responses to L-dopa become unpredictable, and his motor capabilities frequently switch from akinesia to dyskinesia. L-dopa treatment is therefore not the ideal long-term solution for PD patients.

1.3.2 Surgical therapies

The detrimental motor complications associated with long-term L-dopa use, together with the identification of the hyperactive STN as a key-player in the pathology of PD, have increased the interest in surgical therapies that modulate STN activity.
It was first described by Pollak *et al.* in 1993 that electrical High-Frequency Deep Brain Stimulation (HF DBS) of the STN significantly improves the motor symptoms of PD patients [35]. Previously, others had demonstrated that STN lesions reversed MPTP-induced parkinsonism in primates [36, 37].

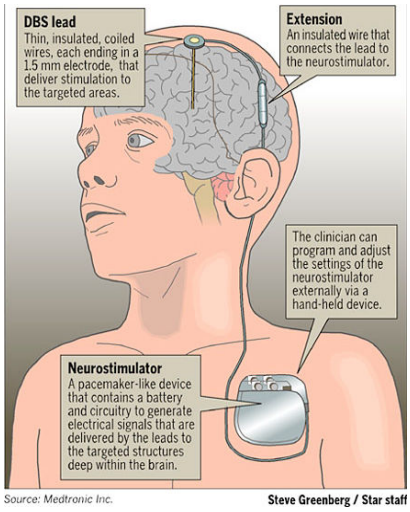


Figure 3: Electrode implantation for Deep Brain Stimulation.

The DBS procedure is as follows: using stereotaxy, stimulating depth electrodes are placed in the STN. The wires emerging from the skull are initially attached to an external stimulator to allow empirical adjusting of the stimulation parameters. Usually, pulse duration lies between 60 and 200 µs, amplitude ranges from 1 to 5 V and frequency is set between 90 and 180 Hz. Frequency is the most important parameter in this procedure, because low-frequency stimulation (LFS; <50 Hz) has no effect or even worsens PD symptoms [38]. Several days postoperatively, the external stimulator is replaced by an internal device that is inserted subcutaneously below the clavicle. It is connected to the electrodes via a wire tunnelled subcutaneously from the scalp. The stimulater can then be adjusted by an external programmer (Figure 3).

Since its introduction, many studies have shown beneficial effects of STN DBS on PD symptoms and long-term follow-up studies indicate a lasting improvement on motor symptoms that cannot be reached by drug-treatment alone [39, 40]. However, some serious adverse effects have also been reported [27]. These involve mainly stimulation-dependent behavioural effects such as depression and impulsivity, affecting approximately 1 in 400 patients [41]. Moreover, despite its rising popularity and the recognition of HF STN DBS as an effective treatment for advanced PD, little is still known about the exact working mechanism of the procedure. Based on seemingly conflicting experimental data – pointing out that STN HFS can inhibit as well as excitate subthalamic neurons and STN target neurons in the GPi and SNpr – Garcia *et al.* have hypothesized that STN HFS switches off a pathological disrupted activity in the STN and imposes a new type of discharge on the STN neurons [38]. While this hypothesis might explain the beneficial effects of STN DBS on PD motor symptoms, it does not cover the above-mentioned side effects.

1.4 Aim of the study

HF stimulation of the STN has proven to be an effective symptomatic treatment option for patients with advanced forms of PD who have reached the stage where they can no longer be helped by drug treatment alone. However, the behavioural side effects that can be the consequence of STN stimulation still impose a large restriction on the common use of this surgical technique.
Furthermore, the effects of STN HFS on regions in the brain other than the BG output nuclei SNpr and GPi are still poorly understood. Given the nature of the behavioural side effects, especially limbic structures seem vulnerable to the effects of STN DBS. Therefore, it is the aim of this study to investigate the possible effects of STN HFS on limbic regions outside the BG of the brain. To do so, a rat model for PD will be subjected to STN DBS and neuronal activity will be investigated in distinct mood-related regions by measuring immunoreactivity for c-Fos, an immediate early gene (IEG) which is a commonly used marker for neuronal activity. Stimulation will be performed at parameters known to have clinical effects. It is hypothesized that following STN HFS, neuronal activity in specific limbic areas of the rat brain will be altered.

2 Materials and methods

2.1 Subjects

All subjects were male Lewis rats (*n* = 20) with an average body weight of 300 g. The animals were bred and housed at the Central Animal Facility of Maastricht University (Maastricht, The Netherlands), where they were kept individually in standard Makrolon™ cages on sawdust bedding under a 12/12-h reversed light/dark cycle in an airconditioned room (± 20°C). Animals had ad libitum access to food and water. All experiments were approved by the Animal Experiments and Ethics Committee of Maastricht University.

2.1.1 6-Hydroxydopamine rat model of Parkinson's disease

The 6-hydroxydopamine (6-OHDA) model was the first animal model of PD associated with selective DA neuronal death in the SNpc. It was originally described by Ungerstedt *et al.* in 1968 that targeting the nigrostriatal DA pathway through injection of 6-OHDA into the striatum or substantia nigra (SN) of rat brains resulted in depletion of transmitter content of DA nerve terminals and cell bodies [42]. Because 6-OHDA cannot cross the blood-brain barrier, intracerebral injection is required. 6-OHDA-induced toxicity is selective for monoaminergic neurons due to its preferential uptake by dopaminergic and noradrenergic transporters. Once inside neurons, 6-OHDA accumulates in the cytosol and causes cell death through oxidative stress. In this study, stereotaxic injection of 6-OHDA was done in the striatum. This specifically results in a retrograde degeneration of nigrostriatal neurons, which lasts for at least 3 months [2, 42, 43].

2.2 Surgical procedure

To investigate the effects of HF STN DBS, rats were randomly assigned to one of three groups: (A) Sham-operation (*n* = 6); (B) 6-OHDA administration (*n* = 6); and (C) 6-OHDA administration + STN stimulation (*n* = 8). Rats in the latter group were further randomly divided in two equally sized groups (*n* = 4): (a) STN stimulation at 0 µA; and (b) STN stimulation at 150 µA. Before starting the surgical procedure, the rats were anesthetized using a combination of intraperitoneally administered ketamine (10 mg/ml) and xylazine (1.6 mg/ml), and they were kept under anesthaesia during the entire procedure. To prevent corneal dehydration the eyes of the rats were covered with sterile Vasilinum album. Next, the animals were placed in a stereotactic apparatus (Stoelting, Wood Dale, USA; model 51653) and at four sites burr holes were made in the skull (two per hemisphere; coordinates from Bregma: AP 0.7 and –0.4, ML 2.8 and 3.4, and V –5.0 and –5.0, based on the Stereotactic Atlas of the

Rat Brain, 4th edition by Paxinos and Watson). At these sites, rats of groups B and C received stereotactic striatal injections of 2 µl 6-OHDA (5 µg/µl in 0.9% saline and 0.2% ascorbic acid) while rats of group A received stereotactic injections of vehicle solution (0.9% saline and 0.2% ascorbic acid) at the same coordinates. To prevent any unwanted effect of 6-OHDA on noradrenergic neurons, desimipramine (20 mg/kg) was administered intraperitoneally one hour before surgery. Finally, rats of group C underwent implantation of the DBS electrodes (Figure 4). To this end, two burr holes were made in the skull immediately above the STN (coordinates from Bregma: AP –3.8, ML 2.5 and V –8.0). A construction of two gold-plated needle-like electrodes with an inner wire of a platinum-iridium combination (Technomed, Beek, The Netherlands), a tip diameter of 50 µm and a shaft diameter of 350 µm was used. The exposed tips of the electrodes (length 75 µm and interpole distance 50 µm) were inserted through the burr holes and the construction was fixed using dental cement (Heraeus Kulzer, Hanau, Germany).

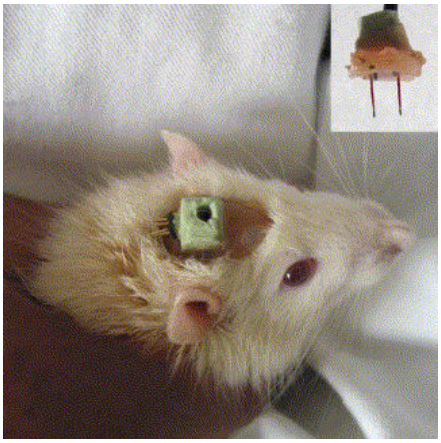


Figure 4: A picture of the bilateral electrode construction (inset) which is implanted in the brain and attached to the skull. A connected cable allows the stimulation of freely moving rats.

2.3 Deep Brain Stimulation

After surgery, rats were left two weeks to recover. Next, the rats in group C underwent STN stimulation for 2 hours. The following parameters were used: 130 Hz (frequency), 60 µs (pulse width) and varying amplitudes (0 and 150 µA). A World Precision Instrument accupulser (A310, WPI, Berlin, Germany) and a stimulus isolator (A306, WPI, Berlin, Germany) were used to deliver the stimuli.

2.4 Immunohistochemistry

Immediately after STN stimulation, all subjects were killed through transcardial perfusion with 0.1 M Tyrode and fixative containing 4% paraformaldehyde, 15% picric acid and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.6). Brains were removed, postfixed for 2 h and immersed overnight in 15% sucrose at 4°C. Next, the brain tissues were quickly frozen with CO_2 and stored at -80°C. Finally, the entire brains were cut serially on a cryostat into 30 µm frontal sections that were again stored at -80°C. For immunohistochemistry, free floating sections were taken out of -80°C, rinsed immediately with Tris-buffered saline (TBS, pH 7.6) and TBS with 3% Triton X-100 (TBS-T, pH 7.6). Primary antibodies used were polyclonal rabbit anti-c-Fos (1:10,000 in 0.1% Bovine Serum Albumin (BSA) in TBS-T; Santa Cruz Biotechnology Inc., Santa Cruz, CA); a mixture of anti-c-Fos with monoclonal mouse anti-glial fibrillary acidic protein (GFAP; 1:15,000 in 0.1% BSA TBS-T; Sigma, St Louis, MO); or a mixture of anti-c-Fos with monoclonal anti-neuronal nuclei (NeuN) primary antibody (1:50,000 in 0.1% BSA TBS-T; Chemicon, Temecula, CA). All primary antibody incubations occurred overnight at 4°C on a shaker. Next, the sections were incubated for one hour with biotinylated donkey anti-rabbit secondary antibody (1:400 in 0.1% BSA TBS-T; Jackson Immunoresearch Laboratories Inc., Westgrove, PA) and subsequently exposed to an avidin-biotin-peroxidase complex (Elite ABC kit, Vectastain; Vector Laboratories, Burlingame, CA) for 2 hours. In between steps, sections were washed with TBS and TBS-T. The immune complexes were then visualized using DAB/Ni solution (3,3-diaminobenzidine; 5 ml Tris/HCl + 5 ml DAB + 50 µl $NiCl_2$ 8% + 3,35 µl H_2O_2). After approximately 10 minutes this reaction was stopped by rinsing all sections thoroughly with TBS. The sections used for double staining with GFAP or NeuN were consequently incubated with biotinylated donkey anti-mouse secondary antibody (1:400 in 0.1% BSA TBS-T; Jackson Immunoresearch Laboratories Inc., Westgrove, PA). Formation of the coloured end product was also performed using the ABC-method, but visualization was done with DAB instead of DAB/Ni. All sections were then mounted on gelatin-coated slides and left to dry. Double stained sections were dehydrated and coveslipped with Pertex (Histolab Products ab, Göteborg, Sweden). Finally, sections exposed only to c-Fos primary antibody were counterstained with cresyl-violet (Sigma, St Louis, MO) for 30 seconds, dehydrated and coverslipped with DPX (Klinipath, Geel, Belgium).

Additionally, TH immunohistochemistry was performed using mouse anti-TH primary antibody (1:2,000 in 0.1% BSA TBS-T; Chemicon, Temecula, CA) and biotinylated donkey anti-mouse secondary antibody (1:400 in 0.1% BSA TBS-T, Jackson Immunoresearch Laboratories Inc., Westgrove, PA) following the same staining procedure as described above, again using the ABC-method. Visualization of the reaction product was done with DAB.

Electrode placements were evaluated in every tenth section using a hematoxylin-eosin (HE; Merck, Germany) staining.

2.5 Analysis of c-Fos immunohistochemistry

To identify the brain regions affected by HF STN stimulation, serial frontal sections of prefrontal cortex, striatum, SN and dorsal raphe nucleus (DRN) of Sham-operated, 0 µA- and 150 µA-stimulated rats were examined under a light microscope and c-Fos immunoreactive areas were identified based on the Stereotactic Atlas of the Rat Brain by Paxinos and Watson, 4th edition. Areas were listed when they showed an increased number of c-Fos reactive cells when compared to nearby non-immunoreactive regions. Consequently, specific (limbic) areas of interest were selected for further analysis. Photographs of the areas of interest were taken at 4x or 2x magnification, using an Olympus DP70 camera connected to an Olympus AX70 microscope. The areas were then delineated based on the Sterotactic Atlas of the Rat Brain and the number of c-Fos positive nuclei was counted. Cell counting was done using the Olympus Cell^{P} program. A c-Fos immunoreactive cell was counted as positive when its intensity was significantly higher than the background intensity of a nearby non-immunoreactive region.

2.6 Analysis of TH immunohistochemistry

Quantitative analysis of TH positive cells in the SNpc was performed at Samsun University, Turkey, using high-precision design-based stereology. All stereological investigations were carried out with a stereological computer microscopy system. On all sections showing the SNpc, the region comprising the TH immunoreactive (THir) cells within the SNpc was delineated and total numbers of THir cells were estimated with the optional fractionator [44, 45].

2.7 Statistical Analysis

Oneway Analysis of Variance (ANOVA) was used to test the TH immunohistochemistry data. The c-Fos immunohistochemistry data were evaluated qualitatively and quantitatively by nonparametric statistical tests due to small sample sizes. All statistical analyses were performed with SPSS 11.5 for Windows. Results were considered to be significant when the corresponding P-value remained below 0.05, while a P-value <0.10 was considered to point out a trend towards significance.

3 Results

3.1 Evaluation of electrode placement

In order to evaluate the location of the electrode tips in the rat brains, HE histological processing was performed (Figure 5). In one animal of the 150 µA group, the electrode tips where not situated in the STN. Therefore, this rat was excluded from further analysis. Additionally, one rat of the 0 µA group was excluded due to extremely deviating values. This resulted in the following group sizes: Sham: *n* = 6; 6-OHDA: *n* = 6; 0 µA: *n* = 3; and 150 µA: *n* = 3.

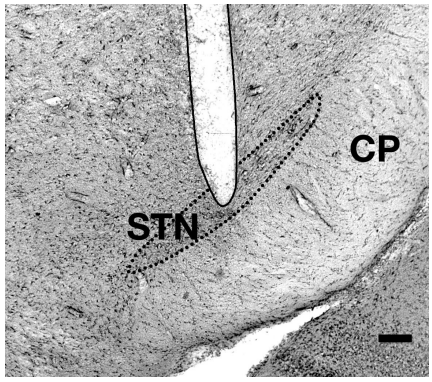


Figure 5: Localization of the stimulation electrode within the STN (CP: cerebral peduncle).

3.2 TH immunohistochemistry

TH immunohistochemistry was performed to evaluate the 6-OHDA lesion in the rat model used. Because TH catalyzes the rate-limiting step in the synthesis of DA, its presence is a valuable criterion for identifying DA neurons (Figure 6). Bilateral striatal 6-OHDA injection significantly decreased the mean total number (MTN) of THir neurons in the SNpc of the 6-OHDA-lesioned and STN-stimulated group compared to the Sham group (Figure 6). High-precision design-based stereological analysis revealed a significant 6-OHDA-induced reduction in TH-positive neurons of about 60% on both sides. (Oneway ANOVA: $F(2, 14) = 140.470$, $P<0.05$) Specifically, in the right SNpc 20,544.93 ± 933.66 THir cells were counted in group A, as compared to 8,317.52 ± 340.87 cells in group B and 9,205.87 ± 251.84 cells in group C. Similarly, in the left SNpc 20,880.09 ± 709.91 THir neurons were counted in group A, while only 8,099.29 ± 368.95 THir neurons were counted in group B and 8,892.96 ± 732.34 THir neurons were counted in group C. Post hoc analysis showed that group B and C did not differ significantly from each other, but both groups were significantly different from group A ($P<0.05$, Fig. 6).

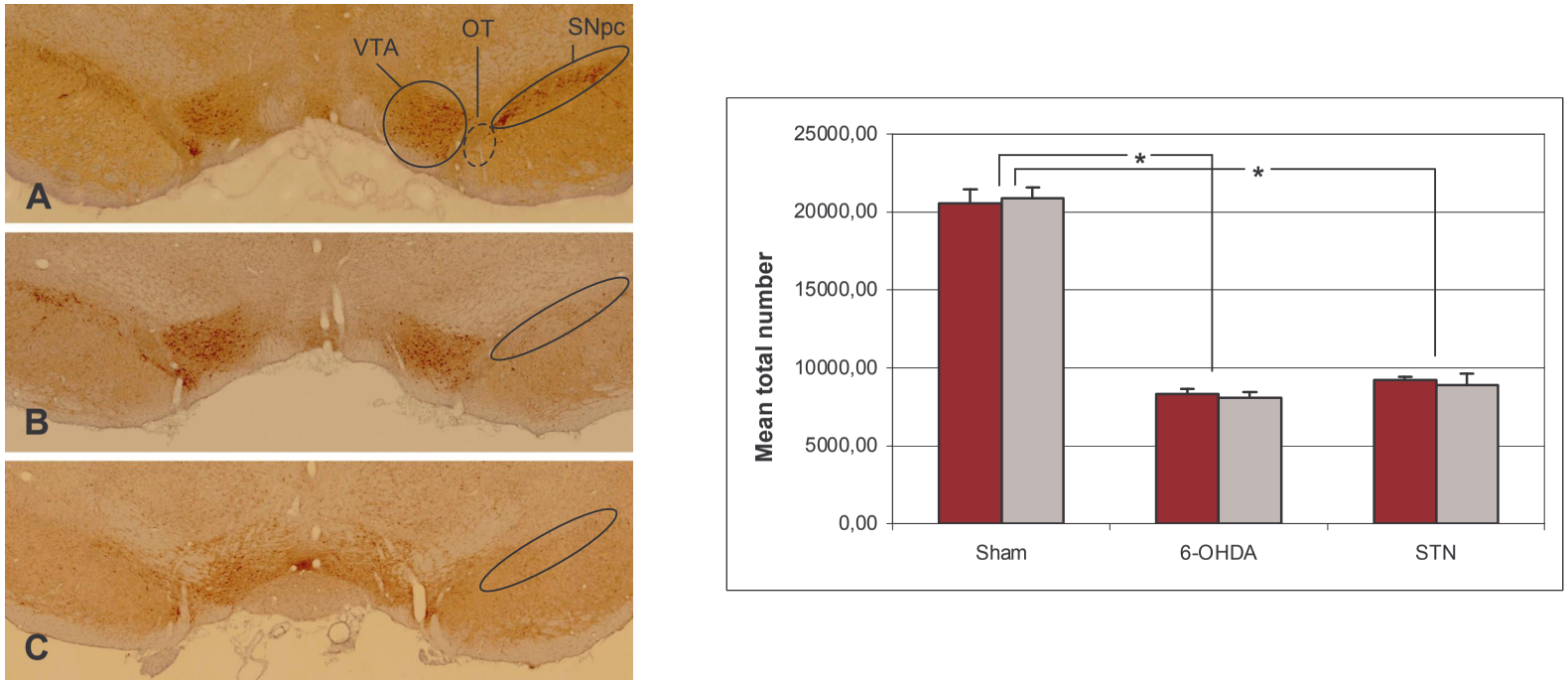


Figure 6: TH immunohistochemistry in the SNpc of Sham-operated (A), 6-OHDA-lesioned (B), and STN-stimulated (C) rats. Right: Bars show the mean total number of THir neurons ± sem; *: P<0.05. ■ : right SNpc; ■ : left SNpc. VTA: ventral tegmental area; OT: optical tract.

3.3 c-Fos immunohistochemistry

HF DBS of the STN is known to increase c-Fos immunoreactivity in the STN itself [46]. However, little is known about the effects of bilateral STN DBS on c-Fos expression in other brain regions. A light microscope was used to identify regions in the rat brain that showed c-Fos expression after HF STN stimulation. They are listed in Table 4. Of these, the following areas were selected for further quantification of c-Fos positive cells: DRN, lateral habenula (LH), and primary and secondary motor cortex (MC). The DRN and LH were selected because of their involvement in emotional behaviour, while the MC was included as a control region. The results of this analysis are described below.

Table 4: c-Fos immunoreactive areas in brains of STN-stimulated and sham-operated rats ('+' indicates an increased number of c-Fos positive cells when compared to nearby non-immunoreactive regions).

c-Fos immunoreactive areas	150 µA	0 µA	Sham	c-Fos immunoreactive areas	150 µA	0 µA	Sham
Prefrontal cortex				**Striatum**			
cingulate gyrus	+	+	+	cingulate gyrus	+	+	+
frontal cortex	+			*primary motor cortex*	+		
infralimbic cortex	+			*secondary motor cortex*	+	+	
claustrum	+			retrosplenial agranular cortex	+		
ventrolateral orbital cortex	+	+		retrosplenial granular cortex	+		
lateral orbital cortex	+	+		claustrum	+	+	
				caudate - putamen complex	+	+	
Dorsal raphe nucleus				nucleus accumbens	+	+	
dorsal raphe nucleus	+	+		ventral pallidum	+		
paradorsal raphe nucleus	+			globus pallidus pars interna	+	+	+
lateral wings	+			lateral septal nuclei	+	+	
laterodorsal tegmental area		+		*lateral habenula*	+		
				paraventricular thalamic nucleus	+	+	
Substantia nigra				central medial thalamic nucleus	+	+	
peri-aquaductal grey	+	+	+	mediodorsal thalamic nucleus	+		
substantia nigra	+	+	+	hypothalamic nuclei	+		
red nuclei	+	+	+	amygdala	+	+	+
mammillary nuclei	+	+	+				

3.3.1 Dorsal Raphe Nucleus

To investigate the possible functional effects of STN stimulation on limbic structures in the rat brain, c-Fos expression was first measured in the DRN. Specifically, c-Fos immunoreactivity (ir) was evaluated in two regions: the midline and lateral wings of the DRN (Figure 7). Per rat, an average of three sections was counted. Results are given as Mean Total Number (MTN) of c-Fos ir neurons ± sem and MTN per 0.1 mm^2 ± sem (Figure 8).

The midline DRN showed fewer c-Fos ir neurons when compared to the lateral wings in all groups. Nonparametric statistical analysis revealed a significant difference between the two subregions in the Sham and 6-OHDA group (Wilcoxon Signed Ranks Test, Z = -2.201, P<0.05), but not in the 0 µA and 150 µA group (Z = -1.604, P>0.10; Figure 8 A, B, H). c-Fos ir in the midline DRN of Sham versus 6-OHDA animals, Sham versus 0 µA animals and 6-OHDA versus 0 µA animals did not differ significantly (Mann-Whitney Test, P>1.00; Figure 8 E, F, H). The same results were found for the lateral wings, except for a significant increase in the MTN of c-Fos ir neurons in the lateral wings of 6-OHDA versus Sham animals (Figure 8 C, D, H). After STN stimulation at 150 µA the lateral wings and midline DRN showed significantly higher numbers of c-Fos ir neurons when compared to the Sham animals (Mann-Whitney Test, P<0.05; Figure 8 C, E, H). This was repeated for the MTN per 0.1 mm^2 in the midline DRN in these groups (P<0.05; Figure 8 F, H), while a trend towards significance could be detected for the lateral wings (P<0.10; Figure 8 D, H). When compared to the 6-OHDA group, the 150 µA-stimulated group showed a significantly higher MTN of c-Fos ir neurons per 0.1 mm^2 in the lateral wings and midline DRN (P<0.05, Figure 8 D, F, H) as well as a significantly higher MTN of c-Fos ir cells in the central DRN (P<0.05, Figure 8 E, H). No significance could be detected when comparing the 0 µA and 150 µA group. The surface area counted in the lateral wings and midline DRN did not differ between the groups (Kruskal-Wallis Test, X^2 = 2.193 and 0.626 resp., P>0.05, Figure 8 G).

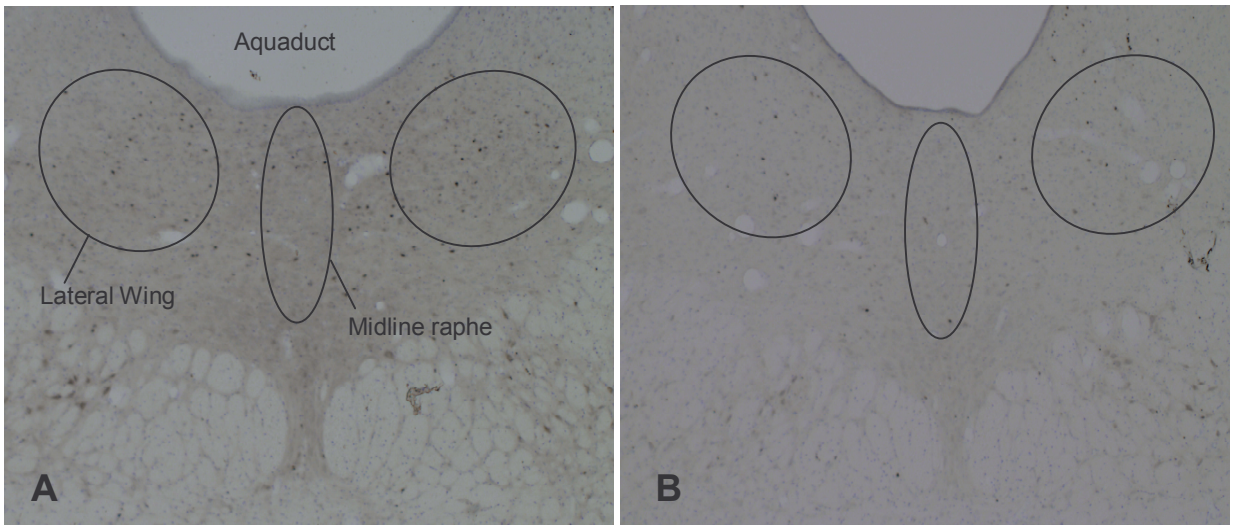
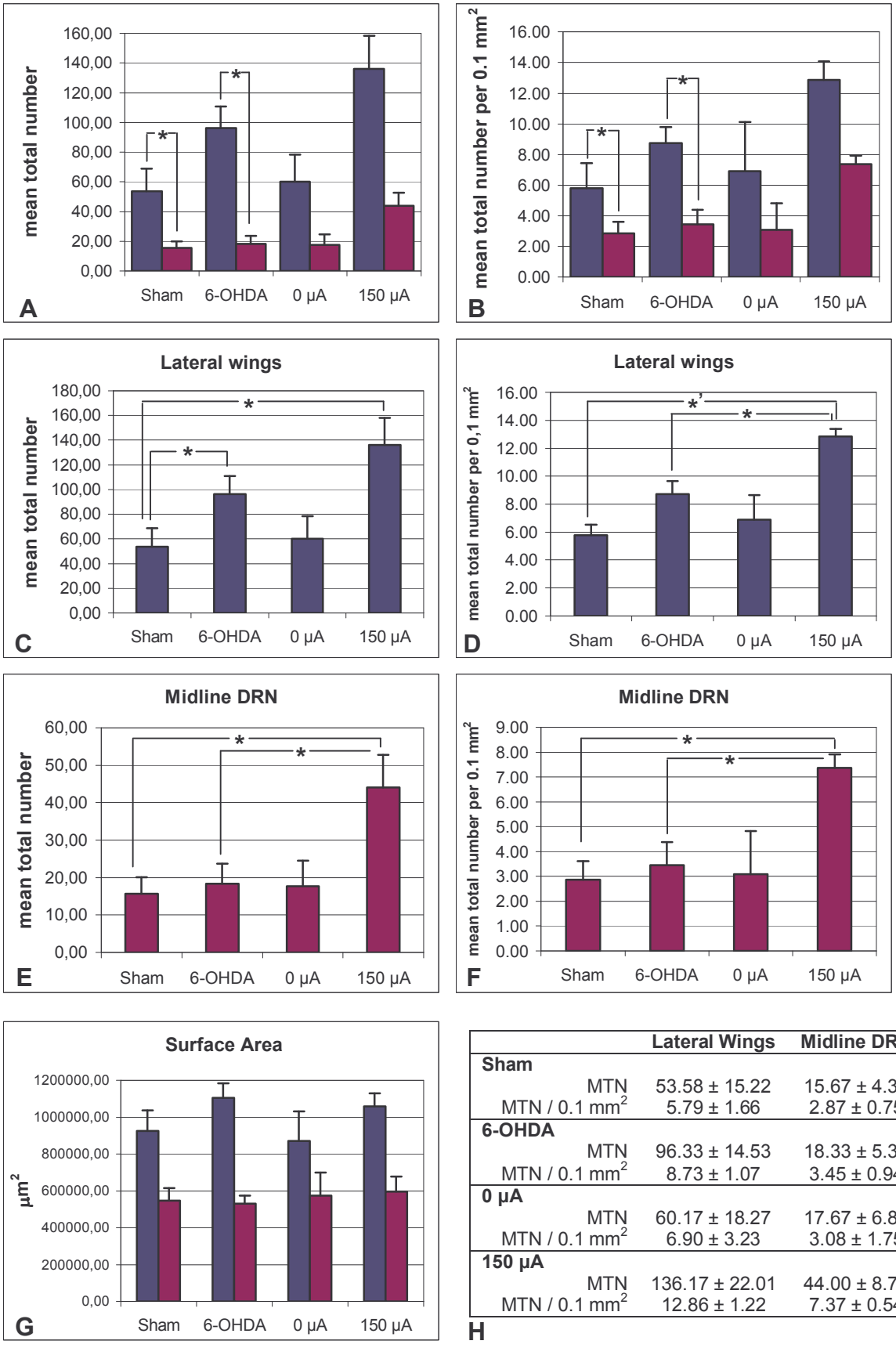


Figure 7: c-Fos expression in the DRN of a 150 µA-stimulated (A) and a Sham-operated (B) rat at 4x magnification.



	Lateral Wings	Midline DRN
Sham		
MTN	53.58 ± 15.22	15.67 ± 4.39
MTN / 0.1 mm^2	5.79 ± 1.66	2.87 ± 0.75
6-OHDA		
MTN	96.33 ± 14.53	18.33 ± 5.35
MTN / 0.1 mm^2	8.73 ± 1.07	3.45 ± 0.94
0 µA		
MTN	60.17 ± 18.27	17.67 ± 6.84
MTN / 0.1 mm^2	6.90 ± 3.23	3.08 ± 1.75
150 µA		
MTN	136.17 ± 22.01	44.00 ± 8.74
MTN / 0.1 mm^2	12.86 ± 1.22	7.37 ± 0.54

H

Figure 8: c-Fos expression in the DRN of Sham, 6-OHDA-lesioned and STN-stimulated rats. (A, C, E) absolute values; (B, D, F) per 0.1 mm^2; (G) counted surface area; (H) immunohistochemical data; : lateral wings; : midline raphe. Bars show mean values ± sem; *: P<0.05; *': P<0.10.

3.3.2 Lateral Habenula

The LH was investigated as a second limbic region of interest (Figure 9). Per rat, an average of two sections was counted. Results are given as MTN of c-Fos ir neurons ± sem and MTN per 0.1 mm^2 ± sem (Table 5; Figure 10). Because nonparametric statistical analysis revealed no significant differences between c-Fos ir in the left and right LH (Wilcoxon Signed Ranks Test, P>0.10, Figure 10 C), these data were pooled for further analysis.

c-Fos ir did not differ significantly in the Sham versus 6-OHDA group, the Sham versus 0 µA group or the 6-OHDA versus 0 µA group (Mann-Whitney Test, P>0.10; Table 5; Figure 10 A and B). A significant difference in MTN and MTN per 0.1 mm^2 of c-Fos ir neurons could be detected when comparing the Sham versus the 150 µA group (Mann-Whitney Test, P<0.05; Table 5; Figure 10 A and B), but this result was not repeated in the 6-OHDA versus 150 µA group or the 0 µA versus 150 µA group (Mann-Whitney Test, P>0.10; Table 5; Figure 10 A and B). A Kruskal-Wallis Test revealed no significant difference between the surface area counted in the LH for all groups (X^2 = 1.772, P>0.10).

Table 5: c-Fos immunohistochemical data in the LH of Sham, 6-OHDA and STN-stimulated rats.

Lateral Habenula			
Sham		**0 µA**	
MTN	10.67 ± 3.55	MTN	39.00 ± 25.94
MTN / 0.1 mm^2	3.32 ± 1.10	MTN / 0.1 mm^2	11.96 ± 7.68
6-OHDA		**150 µA**	
MTN	27.83 ± 8.58	MTN	38.33 ± 5.55
MTN / 0.1 mm^2	8.68 ± 2.68	MTN / 0.1 mm^2	11.78 ± 1.58

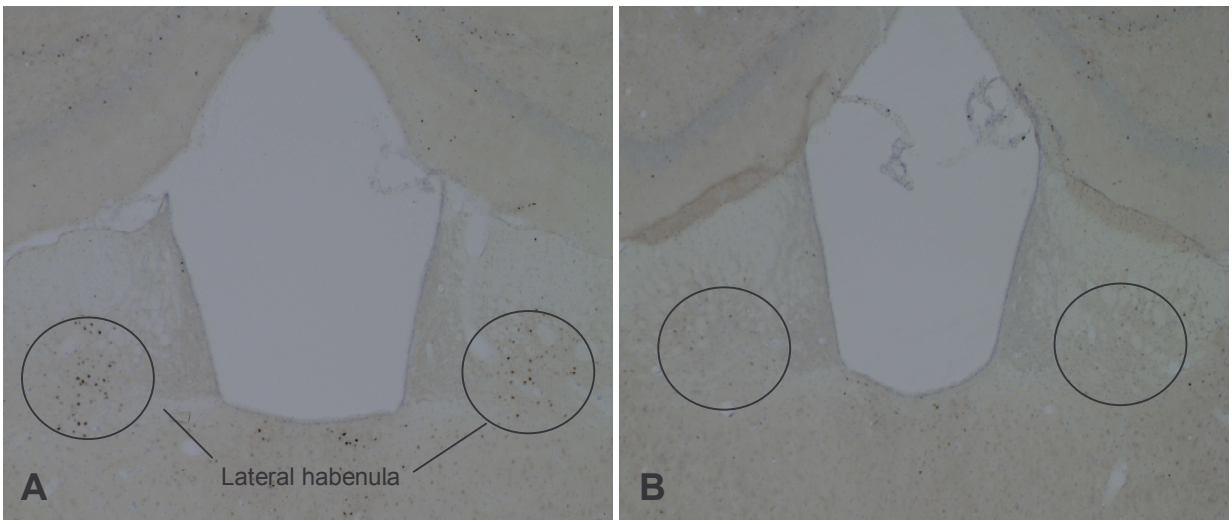


Figure 9: c-Fos expression in the LH of a 150 µA-stimulated (A) and a Sham-operated (B) rat at 4x magnification.

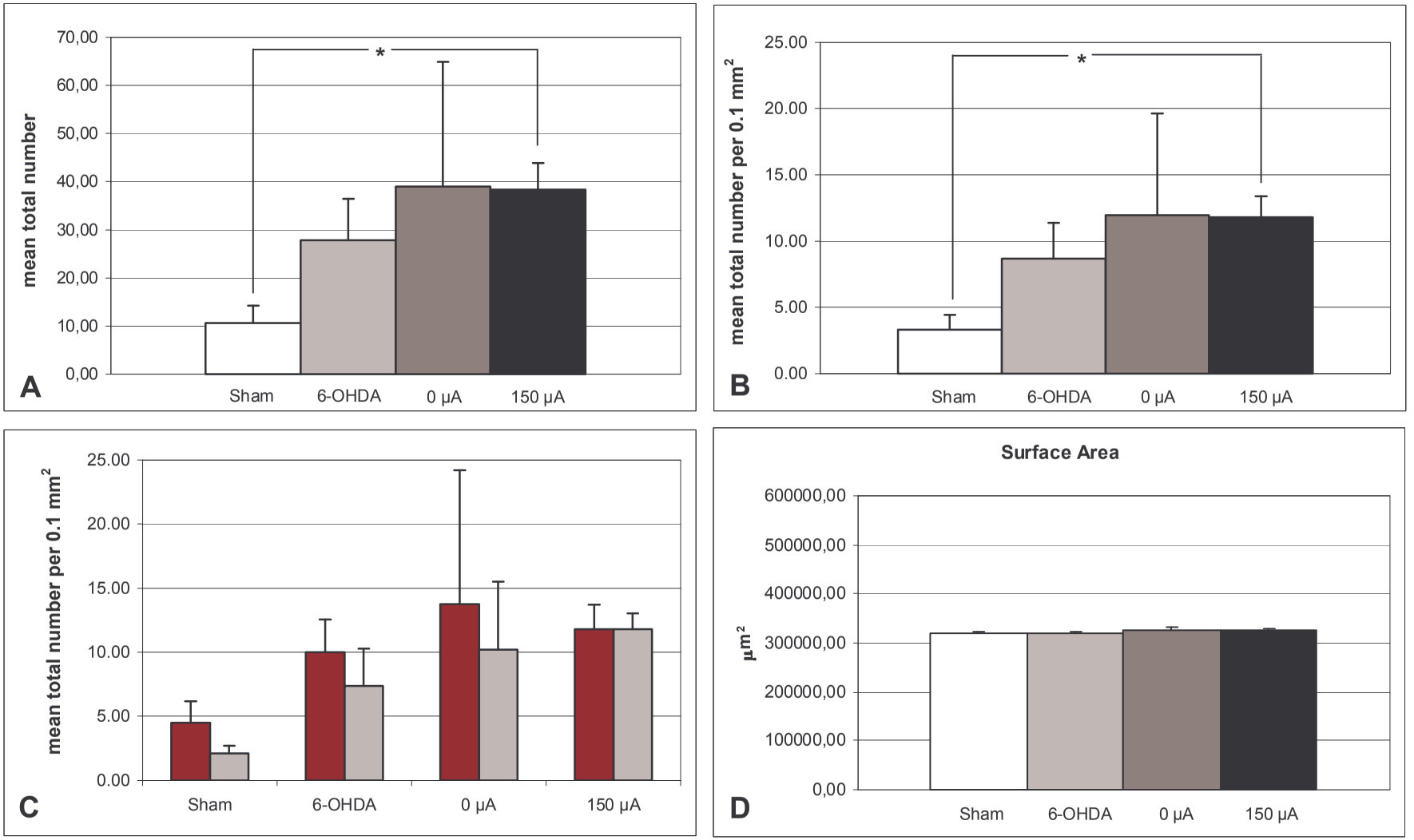


Figure 10: c-Fos expression in the LH of Sham, 6-OHDA-lesioned and STN-stimulated rats. (A) absolute values; (B) per 0.1 mm^2; (C) right (■) versus left (■) LH; (D) counted surface area. Bars show mean values ± sem; *: P<0.05.

3.3.3 Motor Cortex

The MC was investigated as a control region since it is known that neuronal activity in the motor cortex is closely related to STN activity [47] (Figure 11). Four sections were counted bilaterally per rat. Results are given as MTN of c-Fos ir neurons ± sem and MTN per 0.1 mm^2 ± sem (Table 6; Figure 12). Because nonparametric statistical analysis revealed no significant difference between c-Fos ir in the left and right hemisphere (Wilcoxon Signed Ranks Test, P>0.10; Table 6; Fig. 12 C), these data were pooled for further analysis.

There was no significant difference in MTN and MTN per 0.1 mm^2 of c-Fos ir neurons between the Sham and 6-OHDA group (Mann-Whitney Test, P>0.10; Table 6; Fig. 12 A and B). However, the same test revealed a significant increase in MTN of c-Fos ir neurons in the 0 µA group when compared to the Sham and 6-OHDA group (P<0.05; Table 6; Fig. 12 A). This increase was also significant for the MTN per 0.1 mm^2 in the 0 µA versus 6-OHDA group (P<0.05; Table 6; Fig. 12 B). Further analysis showed that STN stimulation at 150 µA significantly increased the number of c-Fos ir neurons when compared to the Sham and 6-OHDA group (Mann-Whitney Test, P<0.05) and a trend towards significance was found when compared to the 0 µA group (P<0.10; Table 6; Fig. 12 A and B). No difference could be detected between the surface area counted in all groups (Kruskal-Wallis Test, X^2 = 0.509, P>0.10; Fig. 12 D).

Table 6: c-Fos immunohistochemical data in the MC of Sham, 6-OHDA and STN-stimulated rats.

Motor Cortex			
Sham		**0 µA**	
MTN	34.83 ± 13.85	MTN	106.67 ± 8.29
MTN / 0.1 mm^2	0.42 ± 0.22	MTN / 0.1 mm^2	1.24 ± 0.08
6-OHDA		**150 µA**	
MTN	20.00 ± 4.34	MTN	215.67 ± 32.11
MTN / 0.1 mm^2	0.23 ± 0.05	MTN / 0.1 mm^2	2.59 ± 0.44

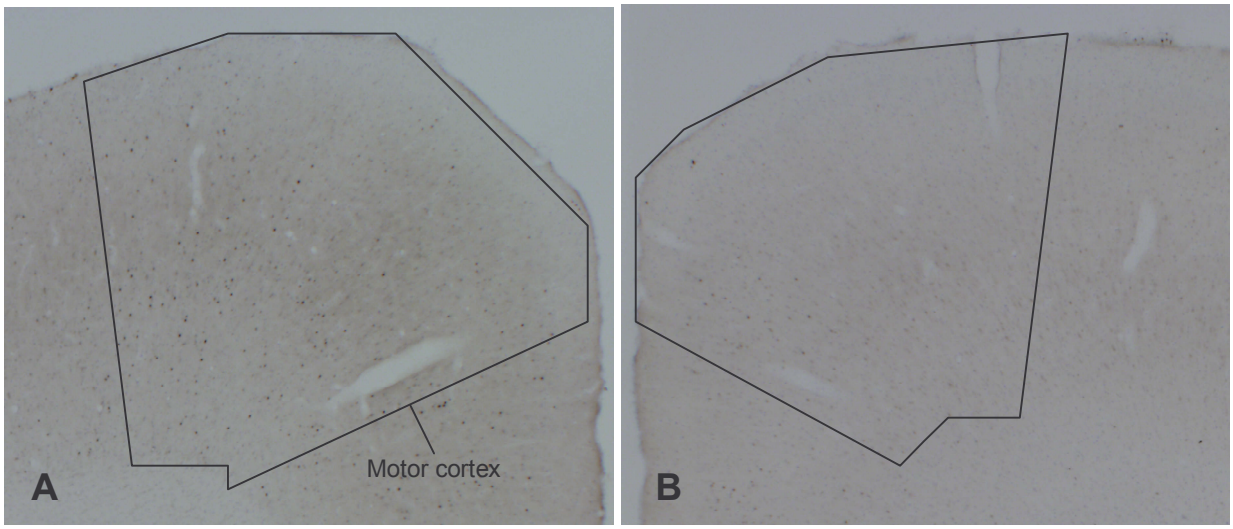


Figure 11: c-Fos expression in the MC of a 150 µA-stimulated (A) and a Sham-operated (B) rat at 2x magnification.

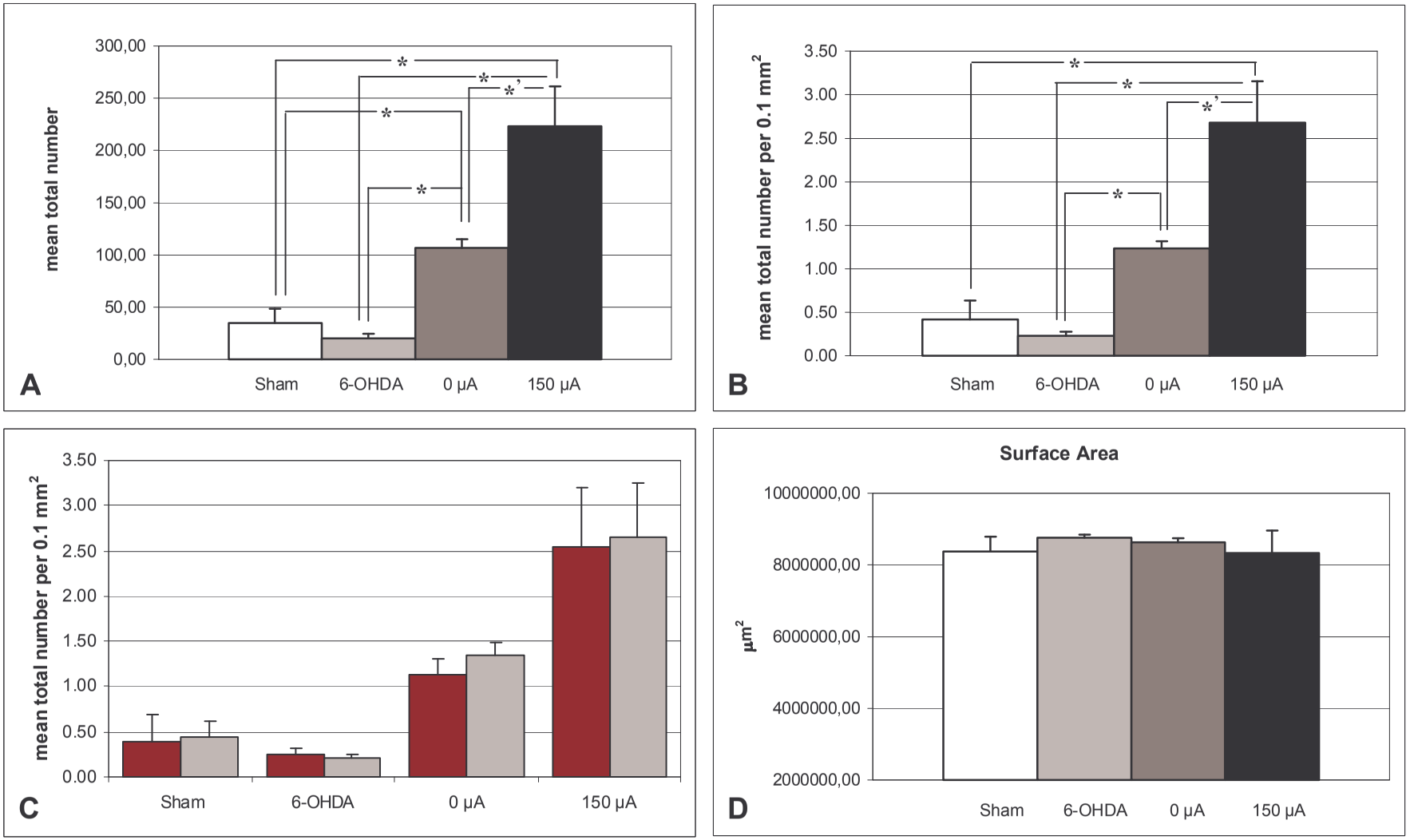


Figure 12: c-Fos expression in the MC of Sham, 6-OHDA-lesioned and STN-stimulated rats. (A) absolute values; (B) per 0.1 mm^2; (C) right ( ) versus left ( ) hemisphere; (D) counted surface area. Bars show mean values ± sem; *: P<0.05; *': P<0.10.

3.3.4 c-Fos double labelling

In order to identify the cellular origin of the c-Fos expression observed, an immunohistochemical double staining was performed on a small number of sections. This staining made use of a combination of c-Fos and the astrocyte marker GFAP, or a combination of c-Fos and the neuron marker Neun as primary antibodies. The results are shown in Figure 13. No co-localization can be detected between c-Fos and GFAP (Figure 13 A), while there is a clear co-expression of c-Fos and NeuN (Figure 13 B).

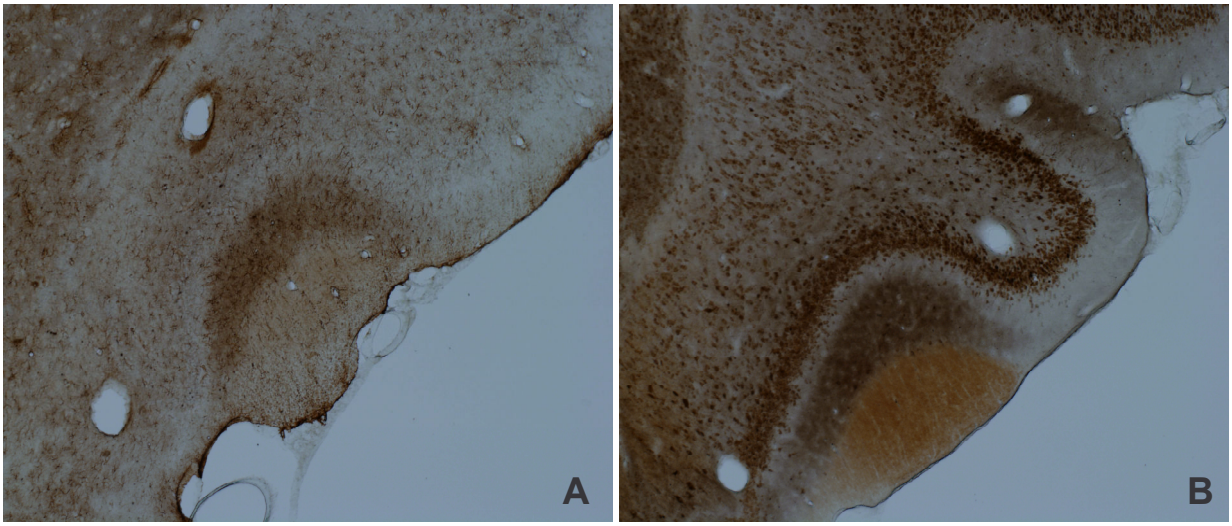


Figure 13: Double staining between c-Fos and GFAP (A) or NeuN (B). Pictures show piriform cortex at 2x magnification.

4 Discussion

Chronic HFS of the STN has become a popular technique to surgically treat patients with advanced forms of PD. DBS strongly improves the motor symptoms of PD patients, probably by changing the firing pattern of subthalamic neurons [38]. However, the stimulation-dependent behavioural side effects observed in patients suggest that STN DBS could affect neuronal activity in limbic brain regions as well [27, 41].

c-Fos as a marker of neuronal activity

In the present study, neuronal activity was assessed by measuring immunoreactivity for c-Fos, an immediate early gene. IEGs are rapidly synthesized in response to cellular stimulation and can then act as transcriptional activators to induce the synthesis of second-order genes. In this way, c-Fos regulates neuronal excitability and survival and can thus be considered a good marker of intracellular activation [48]. In this experiment, stimulation was performed using parameters known to elicit clinical effects in patients. An initial mapping study revealed that this clinically relevant bilateral HF STN stimulation induced Fos protein expression in the cingulate gyrus, frontal cortex, infralimbic cortex, claustrum, (ventro)lateral orbital cortex, (para)dorsal raphe nucleus, laterodorsal tegmental area, peri-aquaductal grey, substantia nigra, red nuclei, mammillary nuclei, primary and secondary motor cortex, retrosplenial (a)granular cortex, caudate-putamen complex, nucleus accumbens, ventral pallidum, globus pallidus pars interna, lateral septal nuclei, lateral habenula, other thalamic and hypothalamic nuclei and the amygdala. Of these, the DRN and LH were selected to measure c-Fos expression in order to test the hypothesis of altered neuronal activity in limbic brain regions. Additionally, the primary and secondary MC was included as a control region expected to show c-Fos immunoreactivity after STN DBS. Indeed, brains of 150 µA-stimulated rats showed increased expression of Fos protein in the MC when compared to Sham, 6-OHDA and 0 µA rats. This is consistent with a recent study showing c-Fos expression in the primary and secondary MC after unilateral STN DBS in naive rats [49] and with the finding of improved motor functioning in parkinsonian rats and patients after HF STN stimulation [40, 43].

The c-Fos induction observed in the present study is unlikely to be due to mechanical injury to the brain after electrode insertion. Although the difference in c-Fos expression between the 0 µA- and 150 µA-stimulated group was not significant in the two limbic brain regions examined, double labelling with c-Fos/GFAP revealed that c-Fos expression was absent in astrocytes, indicating that no tissue damage was present in the rat brains. The lack of a statistically significant difference is therefore more likely to be the result of the small size of the groups. In addition, evaluation of electrode localization showed correct placement of the electrodes in the STN and revealed no tissue damage.

6-OHDA model of Parkinson's disease

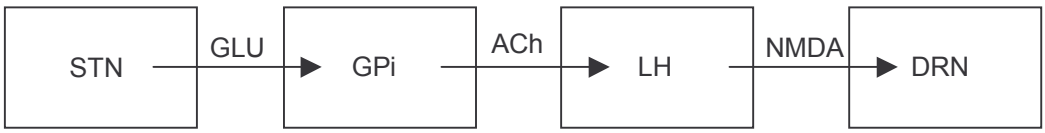
The animal model of PD used in this study (bilateral striatal 6-OHDA injection) showed significant DA depletion in the SNpc, as evaluated by TH immunohistochemistry, and can thus be considered an effective model in this experimental context. Application of the 6-OHDA lesion increased neuronal activity in the lateral wing subregion of the DRN. It is known that the lateral wing subregion contains mainly GABA-ergic neurons, while the midline DRN has a large population of serotonergic (5-hydroxytryptamine; 5-HT) neurons [50]. The finding of increased c-Fos expression in the lateral wing subregion suggests an effect of DA on GABA-ergic raphe neurons. No reports have been published describing such an effect in the DRN, but it is known that DA decreases GABA release in the nucleus accumbens via D_1-like DA receptors on GABA-ergic neurons [51]. If such a mechanism is also present in the DRN, this would explain the effect of DA depletion observed in the lateral wings. In contrast, no effects of the 6-OHDA lesion could be detected in the midline DRN in this experiment. However, it has been reported previously that DA stimulates 5-HT release in the hippocampus through the D_2-like DA receptor [52], and that D_2-like DA receptor activation excites rat dorsal raphe 5-HT neurons *in vitro* [53]. DA depletion would therefore be expected to decrease neuronal activity in the midline 5-HT neurons. The fact that multiple peptides (such as substance P and corticotropin-releasing factor – CRF) control 5-HT release in the DRN could entail a compensatory mechanism that might explain why this effect was not observed in the present study [54]. Furthermore, no significant 6-OHDA lesion effects could be observed in the LH and MC. Since it was shown that 6-OHDA injection decreased nigral DA levels, the lack of a significant lesion effect is probably due to low subject numbers.

Increased neuronal activity in limbic brain regions

The present study demonstrates for the first time an effect of chronic bilateral STN DBS on neuronal activity of limbic brain regions, especially the DRN. The DRN belongs to the midbrain reticular formation of the brainstem and is the largest serotonergic nucleus in the brain [55]. The midbrain 5-HT system has long been linked to mood disorder symptoms, suggesting a possible role for the DRN in the generation of the stimulation-dependent behavioural effects observed after HF STN DBS. In this study, neuronal activity increased in the DRN after STN stimulation at 150 µA, both in the lateral wing and midline subregions. It should be noted here that – independent of 6-OHDA lesioning or STN stimulation – neuronal activity was consistently higher in the lateral wings than in the midline DRN. Since it has been described that GABA-ergic neurons fire at higher frequencies than 5-HT neurons in naive rats [50], this could explain the increased neuronal activity observed in the lateral wing subregion. Furthermore, it is known that an elaborated network of reciprocal innervations exists between the GABA-ergic and 5-HT neuronal populations in the DRN. GABA-ergic neurons contain several types of 5-HT receptors on their surface, as well as GABA autoreceptors. Similarly, 5-HT neurons express different types of GABA receptors and 5-HT autoreceptors on their surface. In this way, a complex (auto)regulatory system is maintained, allowing a fine-tuned control of neurotransmitter release [56].

Impulsivity and depression – two of the main stimulation-dependent side effects seen in DBS-treated patients – are associated with low 5-HT levels [57]. PD itself is also associated with a higher comorbidity of mood (depression) and anxiety (panic attacks) disorders [58]. It is therefore tempting to speculate that STN DBS could aggravate an already existing disturbance of the 5-HT system in PD patients, possibly by altering the highly sensitive signalling network between GABA-ergic and 5-HT neurons. However, whether and how the increased activity of these neurons also leads to alterations in neurotransmitter release in this kind of experimental setting remains a topic for future research.

Furthermore, the projection pathways through which stimulation of STN neurons can influence DRN neurons, are currently still in the dark. Canteras *et al.* have described a modest afferent projection to the STN arising from the DRN [59], but no reciprocal efferent connection has been reported. Kita *et al.* have described sparse projections from the STN to the DRN by use of the axonal transport of phaseolus vulgaris-leucoagglutinin (PHA-L) [60], but later studies could not confirm this [61]. A direct pathway therefore seems unlikely to be responsible for the observed effects. It is more likely that HF STN stimulation affects the DRN through a multisynaptic route including at least one other nucleus. A strong possible candidate is the LH, a nucleus in the dorsal thalamus known to strongly project to the DRN [62]. It uses (amongst others) N-methyl-D-aspartate (NMDA) to affect 5-HT neurons in the DRN either directly, or indirectly through raphe GABA-ergic neurons [63, 64]. Furthermore, the LH receives afferent projections from the GPi, one of the BG output nuclei. A possible signalling pathway linking the STN to the DRN could thus be composed of the STN, GPi, LH, and DRN (see schematic overview below).



Schematic overview of proposed signalling pathway connecting STN and DRN. (GLU: glutamate; ACh: Acetylcholine; NMDA: N-methyl-D-aspartate)

The increased neuronal activity seen in the DRN in this experiment would then be the result of increased activity in the GPi and consequently the LH. Indeed, neuronal activity in the LH was found to be increased in the 150 µA-stimulated rats in this study. Furthermore, a recent study on unilateral STN stimulation in naive rats by Schulte *et al.* reported increased c-Fos expression in the GPi [49]. In the present study, neuronal activity in the GPi could not be evaluated and thus remains an important future research topic.

Confounding experimental artefacts

Takase *et al.* have showed that inescapable shock activates 5-HT neurons in the raphe nuclei of the rat [65]. In the current experimental setting, DBS was performed on freely moving animals to

minimize exposure to stress and to avoid effects of anaesthetics on stimulation-induced c-Fos expression [66]. Furthermore, extensive handling of the animals was carried out in the weeks prior to the surgical procedure and STN DBS to minimize confounding effects of experimental manipulation. Nevertheless, such experimental artefacts cannot be completely ruled out.

Conclusion and future plans

Induction of IEGs like c-Fos by DBS is consistent with previous studies in which (repetitive) electrical stimulation was employed to various structures in order to study the effects on related brain regions [67]. However, only a few reports have been published on HF STN DBS. Schulte *et al.* have described c-Fos expression in the STN and its projection areas after unilateral HF and LF STN stimulation in naive rats, while Salin *et al.* have published data on STN neuron metabolic activity and neurotransmitter-related gene expression in the BG after unilateral HF STN stimulation in naive and hemiparkinsonian rats [46, 49]. Their findings show increased c-Fos expression in the STN and its projection sites [49], decreased metabolic activity of STN neurons and antagonized 6-OHDA lesion-mediated cellular defects in the BG output structures [46]. However, both studies did not report c-Fos expression in the DRN or LH.

The present study describes for the first time the effects of bilateral STN stimulation on brain regions related to the limbic system in the 6-OHDA animal model of PD. It shows increased neuronal activity in these regions in response to HF stimulation and proposes a possible pathway through which these effects could be mediated. Future plans will aim to investigate the precise effects of STN DBS on neuron function in the DRN and LH in greater depth and will focus on identifying a possible signalling pathway responsible for the behavioural effects of STN DBS. It is believed that this holds great promise for a better surgical treatment option for patients suffering from Parkinson’s disease.

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