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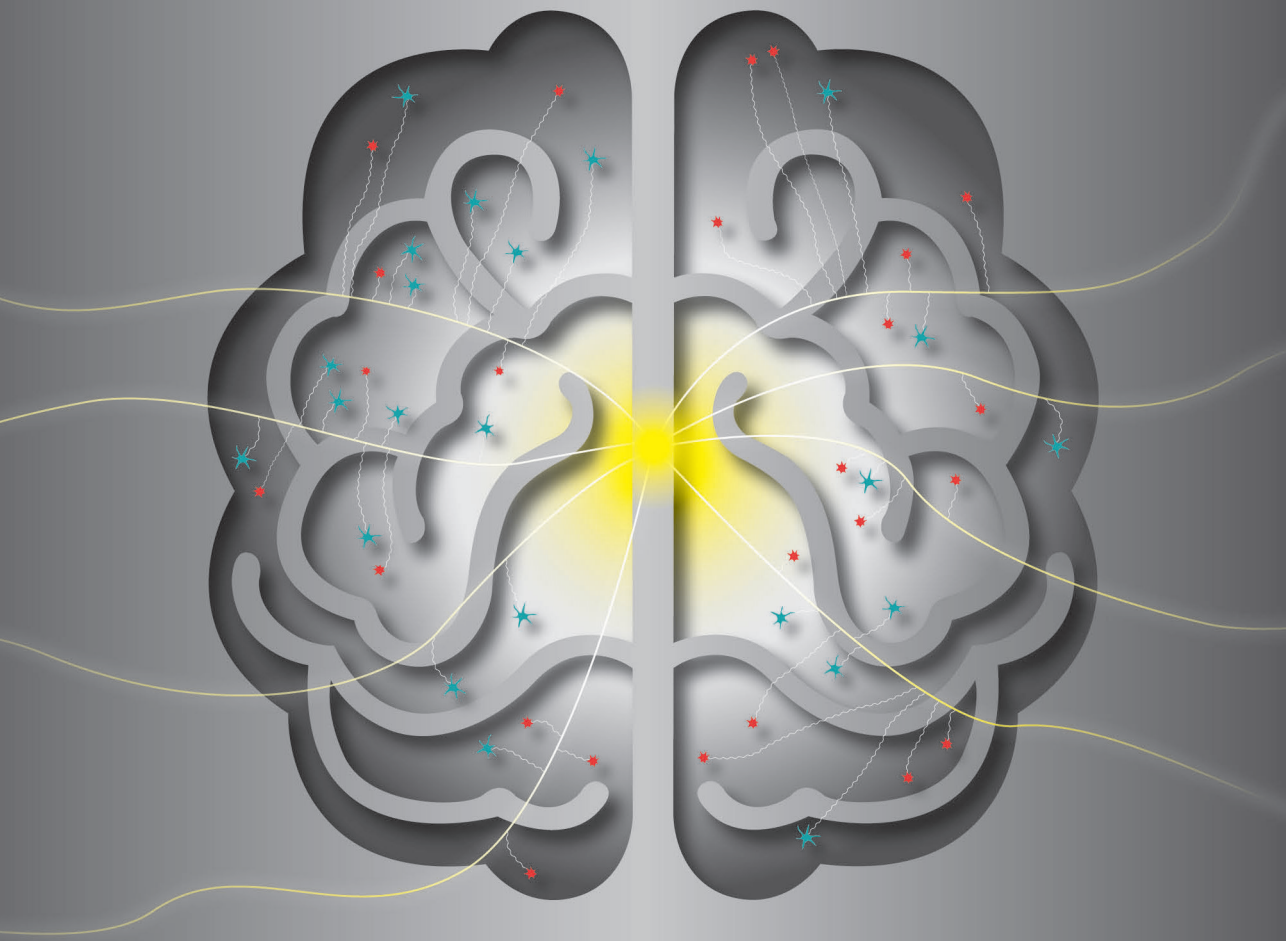

FAISAL MOHAMMED ALOSAIMI

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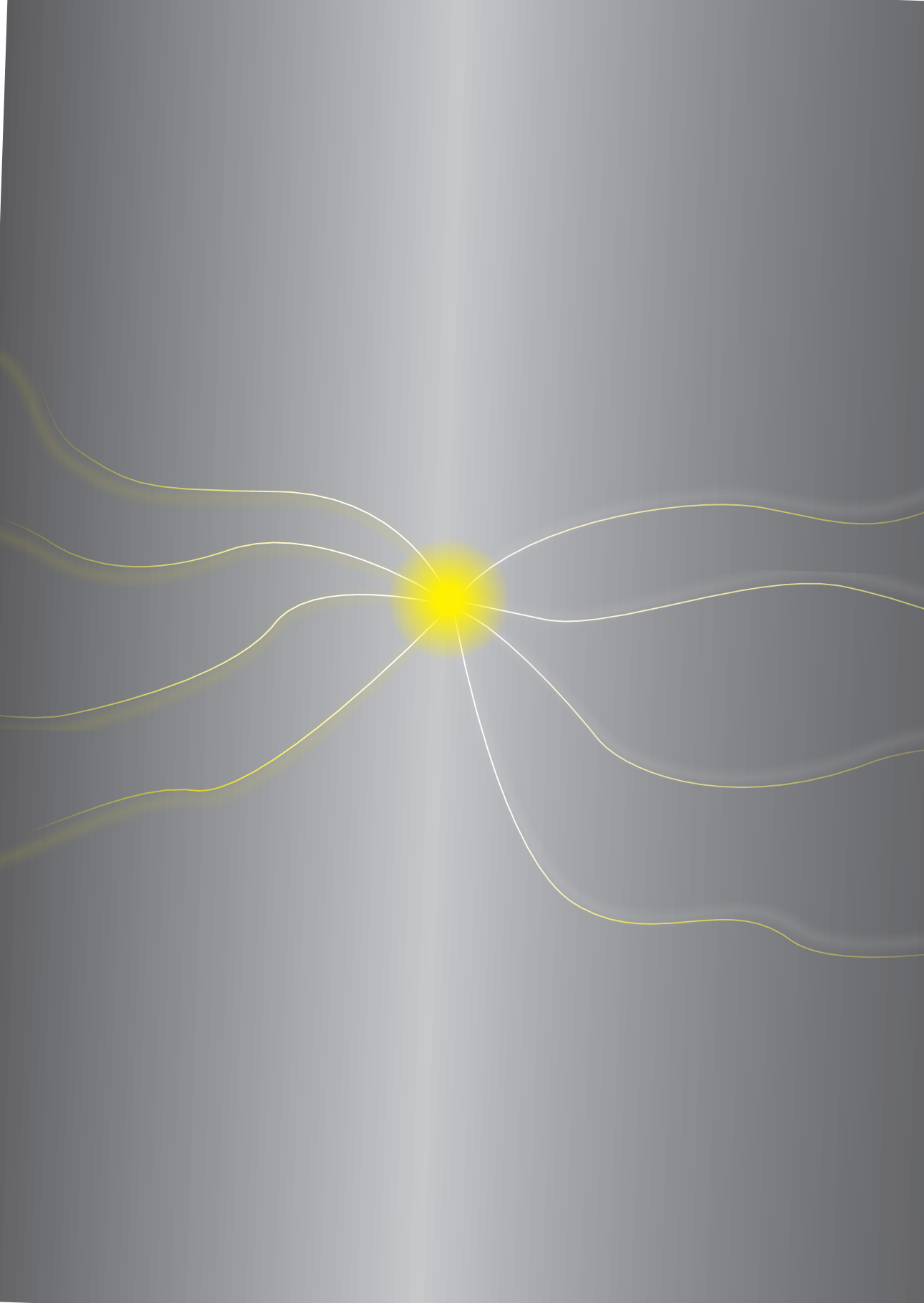

CHAPTER 1

General introduction

1. PARKINSON'S DISEASE

In 1817, a British physician called James Parkinson published an essay about six people with 'shaking palsy' [1]. This movement disorder would later be named after him. Today, more than 200 years later, an estimated ten million people worldwide and about 1.2 million people in the European Union suffer from Parkinson's disease (PD). The key motor symptoms are tremor, rigidity, bradykinesia, and postural instability [2]. Besides the motor symptoms, PD patients also suffer from non-motor symptoms such as cognitive impairments and mood changes [3]. The motor symptoms of the disease result from the death of neuromelanin-containing dopamine cells in the substantia nigra pars compacta (SNc) [4]. The cause of this cell death is poorly understood but involves the build-up of misfolded proteins into Lewy bodies inside neurons [5]. Up to date, there is no cure for PD. In the early stages, dopamine replacement therapy can adequately treat motor symptoms, mainly by the dopamine precursor levodopa (L-dopa). However, as the disease progresses, the beneficial effects of L-dopa wear off and are replaced by disabling side effects, such as 'on-off' fluctuations and L-dopa-induced dyskinesias [6]. Deep brain stimulation (DBS) can offer symptomatic relief for patients affected by long-term complications of L-dopa therapy [7].

2. DEEP BRAIN STIMULATION

DBS is a surgical procedure that requires the implantation of electrode leads in specific brain regions to apply an electrical impulse that modulates neural activities [8]. Interestingly, in the last decades, traditional neurosurgical approaches for movement disorders have been performed via radiofrequency thermocoagulation lesioning of the globus pallidus internus (GPi) or the thalamus has been shown to improve motor symptoms of PD [9, 10]. However, the breakthrough of L-dopa in 1967 causally led to a cessation of these techniques because of their unilateral, not bilateral, and irreversible nature. Pharmacological treatments also have several drawbacks, such as drug resistance and side effects including dyskinesia, and motor fluctuations [6].

Important developments in the neurosurgery field are related to improving stimulation equipment and expanding knowledge in neuroimaging, by which the magnetic resonance imaging (MRI) -scan and the fusion technique of the preoperative MRI-scan with computer tomography (CT) -scan improved and the calculations of the coordinates of the target structure became more reliable. Moreover, intraoperative electrophysiology techniques such as microelectrode recordings enabled more precise brain region targeting with a higher therapeutic yield by providing neurophysiologic support in determining the position of intracerebral structures, especially the subthalamic nucleus (STN) [11, 12]. Accordingly, high-frequency stimulation (HFS) of the STN, developed by Benazzouz and Colleagues in MPTP monkeys in 1993 [13], and transferred to patients by Benabid and Colleagues, became a standard treatment option for PD [11].

However, the exact mechanism behind its therapeutic benefits and adverse effects is not fully understood. Several theories have been discussed, such as the neuronal firing rate and pattern theories, which emphasize the effect of DBS on local circuits while providing less details from electrophysiological

readouts in remote areas [14-16]. These theories propose DBS modulates an overactive basal ganglia target in PD patients by normalizing the firing rate at the electrophysiological single-cell level [14], interferes with pathological patterns such as subcortical beta oscillations (13–30 Hz), promotes cortical gamma activity (40–200 Hz), and modulates local field potential readouts [15, 16]. Furthermore, several lines of evidence from preclinical and clinical data suggest that DBS affects distant monoaminergic neurotransmitters in PD, as discussed in **Chapter 2** of this thesis. For instance, STN-DBS has been shown to have an effect on the striatal dopaminergic and brainstem serotonergic system [17, 18], GPi-DBS on the striatal dopaminergic system [19, 20], and DBS of the pedunculopontine nucleus (PPN) on the cholinergic systems [21].

3. MONOAMINERGIC NEUROTRANSMITTER SYSTEMS

Monoaminergic neurotransmitter systems play a crucial role in regulating a wide range of central nervous system (CNS) functions, such as locomotion, mood, and behaviour [22]. The main monoaminergic neurotransmitters in the CNS, which are primarily related to these functions, are dopamine, noradrenaline and serotonin [23]. Dopaminergic neurons are located at different levels of the brain (from A8 to A16), but they are predominantly located in the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) of the midbrain. The SNc dopamine neurons are preferentially projecting to the striatum via the nigrostriatal pathway and VTA neurons to the ventral striatal areas such as nucleus accumbens and the prefrontal cortex via mesolimbic pathways [24, 25]. Noradrenaline is primarily produced in locus coeruleus (LC) located in the pons and projects to cortical, subcortical, and spinal structures [26]. Noradrenergic LC neurons have shown to be involved in a wide range of sensory-motor, behavioral, and cognitive functions [27, 28]. Serotonergic neurons are predominantly located and produced in the dorsal raphe nucleus (DRN) and project to several brain areas, including the basal ganglia and the prefrontal cortex [29]. The noradrenergic and serotonergic systems play a major role in regulating several CNS functions, such as mood, and their dysregulation could lead to psychiatric disorders such as depression and anxiety [30-32].

Other neurotransmitters, such as acetylcholine, may interact with monoaminergic systems. The cholinergic system is predominantly located in the nucleus basalis of Meynert, the PPN, and the striatum [33, 34]. Moreover, SNc dopaminergic neurons and PPN cholinergic interneurons project to the striatal GABAergic neurons where interaction between dopamine and acetylcholine receptors in the striatum influence the movement regulation in the basal ganglia and a misbalance between these neurotransmitters has been shown to be involved in PD pathology [35].

The monoaminergic neurotransmitter systems are modulated by many pharmacological agents that treat and manage several neurological and psychiatric disorders. For example, medications that increase dopamine are used to treat PD [9], while medications that decrease dopamine are used for schizophrenia [36]. Similarly, drugs that increase serotonin levels are used to treat depression and anxiety disorders [37], while medications that reduce serotonin help manage migraines [38]. Generally, the monoaminergic systems are essential for the proper functioning of the nervous system, and

dysregulation of these systems can lead to a wide range of neurological and psychiatric disorders and also interact with their medical treatment [22]. Consequently, understanding the involvement of monoaminergic neurotransmitter systems in many neurological and psychological disorders and also their responses to different medical treatments will help optimize the pharmacological and surgical intervention options to improve the overall quality of patient healthcare.

4. THE ROLE OF DBS ON MONOAMINERGIC NEUROTRANSMITTERS

The monoaminergic neurotransmitter systems play an essential role in the therapeutic effects of DBS and their side effects. DBS can modulate the activity of the monoaminergic systems, including the striatal and mesolimbic dopaminergic systems, noradrenergic LC system and the DRN serotoninergic system, as well as the PPN cholinergic system, thereby contributing to both therapeutic and side effects of DBS. For instance, in preclinical studies, STN-DBS has been shown to improve motor symptoms and increase dopamine in the striatum [39-41], probably via an increase of the firing rate of SNc dopamine neurons [42]. However, these data are less consistent in clinical research [43-46]. Nevertheless, STN-DBS improves the motor systems and decreases the need for dopaminergic medication [47]. A preclinical animal study showed that severe noradrenergic dysfunction reduces STN-DBS therapeutic efficiency within a PD rat model [48]. In addition, STN-DBS sometimes induces non-motor side effects, such as mood disorders which might be related to the effect of STN-DBS on the DRN serotonergic system [49]. Although there is no direct projection from the STN to the DRN., previous research suggests it could be indirectly relayed via the lateral habenula (LHb) [50]. The STN neural network also interacts with several other structures in the basal ganglia. For instance, there is a direct connection between STN and globus pallidus externa (GPe), and indeed STN-DBS has been shown to change the firing rate of GPe neurons [51].

The neurotransmitter identity of the neurons has been thought to be fixed throughout life. However, environmental stimuli can drive behaviorally relevant transmitter switching in the mature brain through a recently discovered phenomenon termed neurotransmitter respecification [35]. Recent studies have demonstrated neurotransmitter respecification in the adult brain, where external environmental stimuli lead to neurotransmitter phenotypic switching, neurotransmitter induction, or elimination, which is associated with behavioral alterations [52, 53]. Moreover, preclinical research has shown that DBS of the anterior nucleus of the thalamus increases the number of dopaminergic neurons in the VTA, which provides evidence for neurotransmitter switching [54]. Understanding the effects of DBS on the monoaminergic systems is essential for optimizing the therapeutic benefits and minimizing the adverse effects of DBS.

5. CHALLENGES

Despite the remarkable therapeutic benefits of DBS in alleviating motor symptoms of several neurological disorders, it still requires an invasive surgical intervention that could lead to complications such as infections, cerebral hemorrhage, and electrode lead dislocation [7]. In around 15-34% of patients, DBS surgery requires follow-up interventions for electrode replacement or removal due to hardware malfunctions or infection [55]. As a result, patients are hesitant to undertake DBS surgery which leads to the under-utilization of this technique [56]. Consequently, other approaches were investigated, such as designer receptors exclusively activated by designer drugs (DREADD), optogenetics, and DBS with magnetothermal nanoparticles (MTNPs) [57]. Recently, another alternative approach to deliver DBS wirelessly, namely with magneto-electric nanoparticles (MENPs), has been introduce [58]. The MENPs comprise a magnetostrictive (cobalt ferrite) core and a piezoelectric (barium titanate) shell. The paired strains can generate an electric field in response to an applied magnetic field. The generated electric field can then induce specific and local neurostimulation at the particular injection site. In addition, surface coating with barium titanate improves the biocompatibility of these nanoparticles. The major advantage of MENPs, when compared to other less invasive DBS approaches, is that no genetic modification is required. As this tool for wireless magnetic stimulation is still in its infancy, further research is needed to optimize the technique, such as designing the powering device and less invasive delivery routes to the target brain area. It is also limited in terms of the freedom to deliver neuromodulation effects to the targeted brain area in multiple contacts, similar to conventional DBS, and it still needs to be explored whether multiple MENPs can be placed and coordinated.

6. AIMS OF THE CURRENT THESIS

The current thesis consists of two parts. In part 1, I describe the long-term effects of conventional STN-DBS on the monoaminergic systems due to neuroplasticity. In part 2, I investigate nanoscale material for neuromodulation and its potential to be tested and developed into a less invasive DBS tool.

Part 1 explores conventional DBS and its impact on monoaminergic neurotransmitter systems. In **Chapter 2**, I start with a literature review of general DBS effects on the main related neurotransmitters, including the monoaminergic systems, such as dopamine and serotonin, and also the cholinergic system in PD. **Chapter 3** discusses the effect of long-term high-frequency STN stimulation on serotonin neuronal cell activity and phenotype. **Chapter 4** reports how the GPe affects DRN serotonergic neurons after long-term STN-DBS using DREADD. **Chapter 5** assesses whether there is an effect of STN-DBS on the PPN cholinergic system.

Part 2 introduces nanoscale materials for neuromodulation. In this part of the thesis, I investigated the effect of DBS on the monoaminergic system in rodents using MENPs. The goal was to examine MENPs as a potential candidate for a less invasive therapeutic tool for DBS. **Chapter 6** is an editorial review discussing the potential of nanomaterials for neuromodulation. **Chapter 7** assesses the effect of

DBS using MENPs in the SNc, mesolimbic dopaminergic, brainstem serotoninergic, and PPN cholinergic systems in naive mice and compares them to conventional DBS.

These chapters can give more insight into understanding the mechanism of action of DBS on neurotransmitters and ultimately improve and optimize these techniques. In addition, our preliminary insights into MENPs technology are just a first step to understand how this approach affects locomotion and the monoaminergic neurotransmitter systems; further research is warranted to develop this technique to become a less invasive and wireless alternative to the conventional wired DBS.

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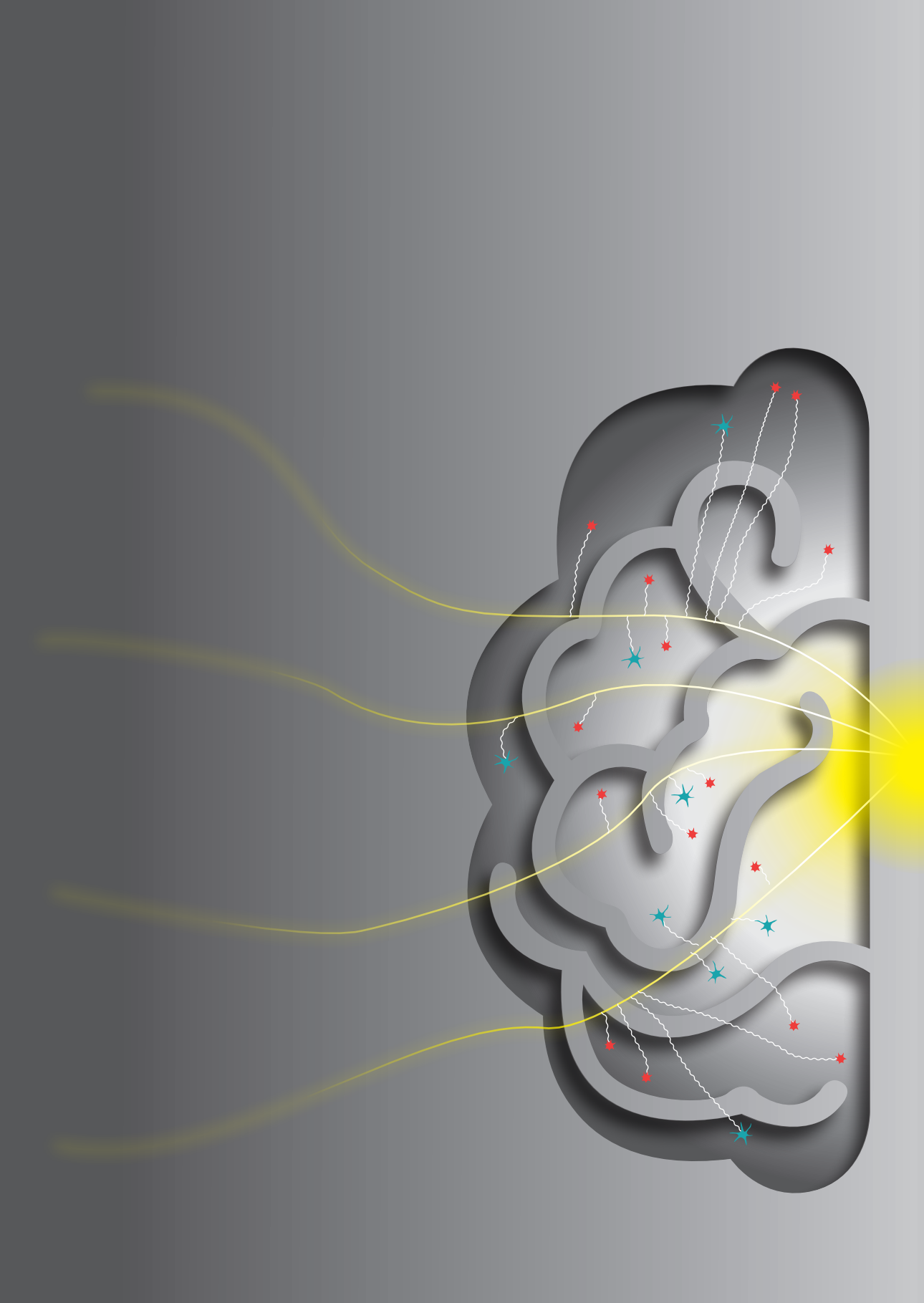
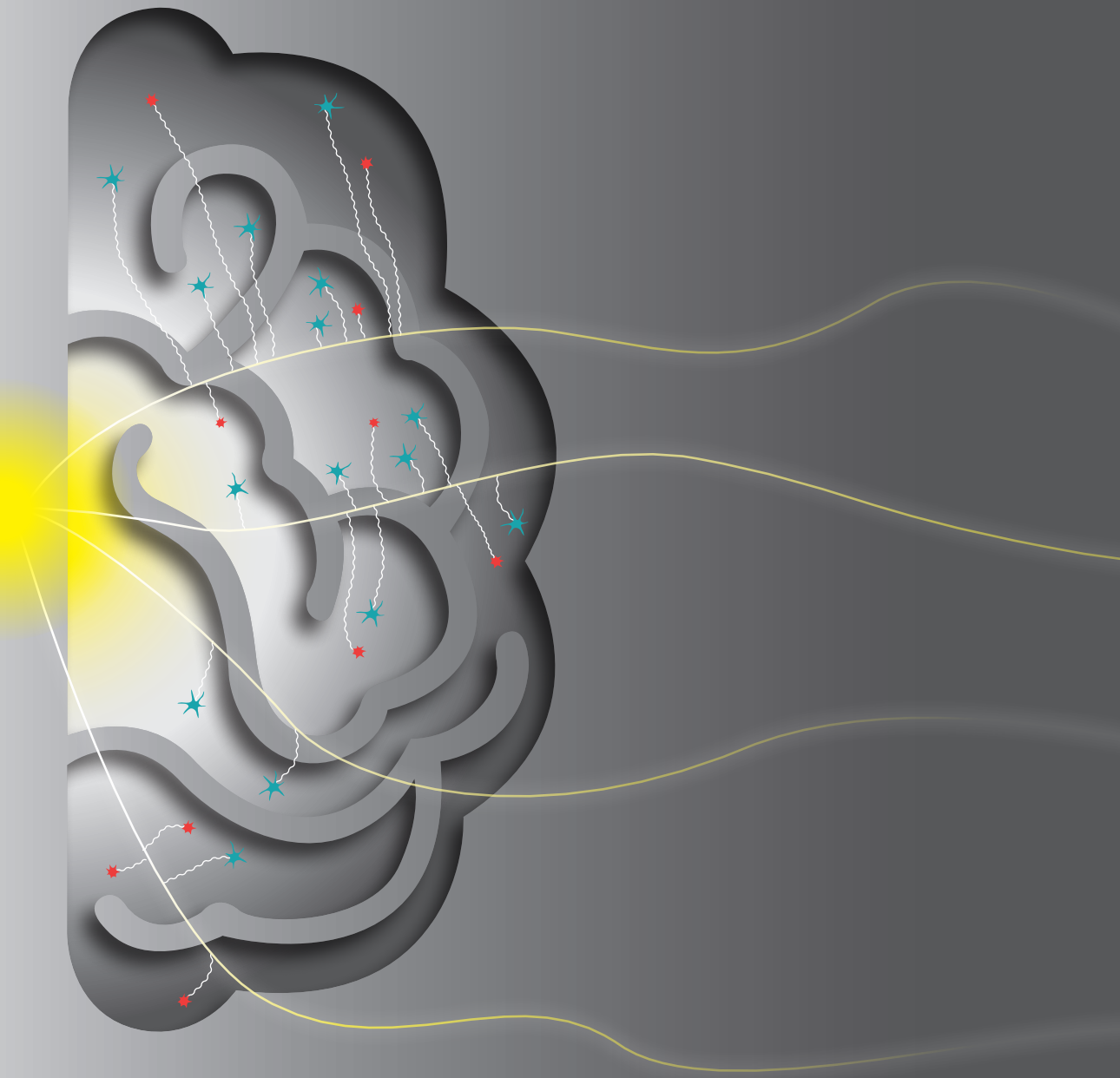
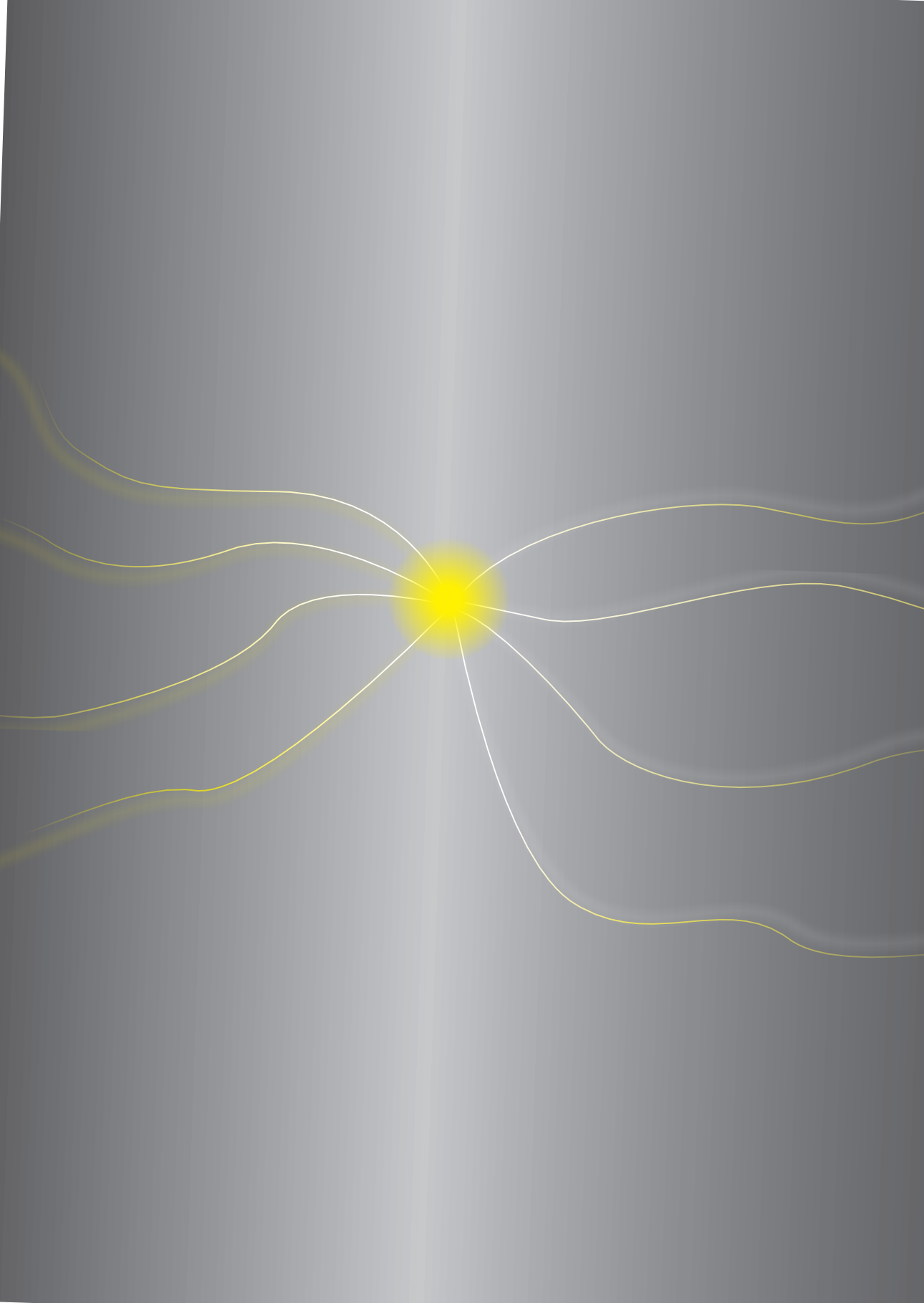


PART 1

Conventional deep brain stimulation





CHAPTER 2

The role of neurotransmitter systems in mediating deep brain stimulation effects in Parkinson's disease

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ABSTRACT

Deep brain stimulation (DBS) is among the most successful paradigms in both translational and reversed translational neuroscience. DBS has developed into a standard treatment for movement disorders such as Parkinson's disease (PD) in recent decades, however, specific mechanisms behind DBS's efficacy and side effects remain unrevealed. Several hypotheses have been proposed, including neuronal firing rate and pattern theories that emphasize the impact of DBS on local circuitry but detail distant electrophysiological readouts to a lesser extent. Furthermore, ample preclinical and clinical evidence indicates that DBS influences neurotransmitter dynamics in PD, particularly the effects of subthalamic nucleus (STN) DBS on striatal dopaminergic and glutamatergic systems; pallidum DBS on striatal dopaminergic and GABAergic systems; pedunculopontine nucleus DBS on cholinergic systems; and STN-DBS on locus coeruleus (LC) noradrenergic system. DBS has additionally been associated with mood-related side effects within brainstem serotoninergic systems in response to STN-DBS. Still, addressing the mechanisms of DBS on neurotransmitters' dynamics is commonly overlooked due to its practical difficulties in monitoring real-time changes in remote areas. Given that electrical stimulation alters neurotransmitter release in local and remote regions, it eventually exhibits changes in specific neuronal functions. Consequently, such changes lead to further modulation, synthesis, and release of neurotransmitters. This narrative review discusses the main neurotransmitter dynamics in PD and their role in mediating DBS effects from preclinical and clinical data.

Keywords: Deep brain stimulation, DBS, neurotransmitters, Parkinson's disease, PD.

1. INTRODUCTION

Deep brain stimulation (DBS) involves a stereotaxic electrode implantation into a specific brain region [1] and has been successful in managing symptoms in several movement disorders including Parkinson's disease (PD) [2]. However, the exact mechanisms behind DBS effects and side effects in PD are not fully understood. Several theories work to explain underlying mechanisms of DBS, the most common being the rate and pattern theories. These theories propose DBS modulates an overactive basal ganglia target in PD patients by normalizing the firing rate at the electrophysiological single-cell level [3, 4], interferes with pathological patterns such as subcortical beta oscillations (13-30 Hz), promotes cortical gamma activity (40-200 Hz), and modulates local field potential readouts [5-7]. However, those theories only involve effects of DBS on local, and to a lesser extent, remote readouts. For instance, electrophysiological recordings of neuronal activity show that ventroanterior (VA) and ventrolateral (VL) thalamus-DBS (10Hz) suppress the beta oscillation and increase the gamma power in the motor cortex in both in vitro and vivo animal models and improves motor function [8]. In addition, subthalamic nucleus (STN) DBS leads to a short-latency excitatory effect that tonically increases the firing rate in the globus pallidus internal/external (GPi/GPe) followed by post-stimulation suppression of the firing rate [9]. Another study showed STN-DBS only suppressed the neuronal activity in the GP and substantial nigra pars recticulata (SNr) in anesthetized rats [10, 11]. Despite this discrepancy, another explanation could be from variations between DBS local effects vs. orthodromic activations from the STN to the GPe and GPi as well as the motor cortex. Furthermore, preclinical studies show that low-frequency stimulation (LFS) of the pedunculopontine nucleus (PPN) suppresses the firing rate in the STN and SNr in PD rat models [12, 13].

Neurotransmitters are largely neglected when discussing mechanisms behind DBS possibly due to technical challenges of recording real-time changes in remote areas distant from the stimulation zone. Both clinical and preclinical PD data show DBS modulates several neurotransmitter networks such as dopaminergic, glutamatergic, GABAergic, serotonergic, and cholinergic systems [14-18]**.** For example, the effect of STN-DBS on motor function could be associated with various effects on striatal dopaminergic and glutamatergic neurotransmission [14, 15], while the effect of GPi-DBS could be associated with changes in the striatal dopaminergic and pallidum GABAergic systems [16, 19]. Likewise, the PPN may be investigated as a new candidate DBS target to assess its effects on the cholinergic system to reduce gait disturbances in PD [18]. Additionally, STN-DBS has been associated with mood-related side effects possibly linked to brainstem serotoninergic systems [17]. STN-DBS therapeutic effects showed to be diminished by severe degeneration of the locus coeruleus (LC) noradrenergic system [20, 21]. Furthermore, DBS has shown to modulate glia cells to induced glutamate release and modulate neurotransmission in nearby neurons in PD [22-24].

DBS has also shown to exhibit long-term effects possibly due to neuroplasticity involving transmitter levels and neuronal communication [3]. Understanding patterned changes at the transmitter level will contribute to the better understanding the underlying mechanisms of DBS and lead to optimized targeting, improved symptom management, lesser side effects, and an improved quality of treatment.

In this article, we discuss neurotransmitter dynamics in PD and briefly revise current theories on the mechanisms behind DBS within the context of neurotransmitter dynamics. We then address the extent of alterations in neurotransmitter systems before and after DBS in PD, the circuits involved, and the impacts such changes can have on PD symptoms.

2. NEUROTRANSMITTER SYSTEMS' DYNAMICS IN PARKINSON'S DISEASES

PD has been pathologically categorized as a disorder of the basal ganglia, a network consisting of three main regions, 1) the striatum (caudate and lentiform nuclei); the lentiform nucleus made up of putamen and dorsal pallidum; dorsal pallidum consists of GPi and GPe, 2) the nigra complex (SNc and SNr), and 3) the subthalamic nucleus (STN) [25]. The neocortex, thalamus and brainstem regions such as PPN are also heavily involved in PD as they are connected to the basal ganglia at multiple levels [25]. The Albin (1989) - Delong (1990) model describes how the basal ganglia regulates movement execution or inhibition by two main pathways [26, 27]; 1) The direct pathway where the striatum projects GABAergic inhibitory activity directly to the GPi/SNr that blocks inhibitory output to the thalamus; the thalamus projects excitatory glutamatergic activity to the cortex allowing for movement execution, and 2) the indirect pathway where the striatum projects GABAergic inhibitory activity to the GPe and STN activating the GABAergic inhibition of the GPi/SNr to the thalamus; thalamic glutamatergic activity is inhibited and ultimately suppresses cortical activity. Moreover, the striatum also receives dopaminergic projections as an output signal from the SNc that regulates both direct and indirect routes (Albin-Delong classic model). In addition, Nambu et al. (2002) described the 'hyper-direct' pathway, a fast pathway forming a loop from the cortex that has a glutamatergic excitatory connection to the GPi via the STN and stimulates the GPi that then inhibits the thalamus and neocortex [28].

The main pathological hallmark of PD is the neurodegeneration of SNc dopaminergic neurons [29]. Dopaminergic neurons from the SNc that innervate the striatum are from the nigrostriatal pathway that regulates both the direct and indirect pathways. The nigrostriatal pathway connects to the thalamus and cortex via striatal interneurons and spinal projection neurons that have dopamine D1 or D2 receptors. Dopamine medications for PD reduce symptoms via their action on D1 receptors but have inhibitory effects on D2 receptors that could partially underlie side effects. This is one reason why medication such as Levodopa combined with D2 receptor antagonist increases the efficacy of the medication [30].

Degeneration of dopaminergic SNc neurons causes dysfunction of the direct and indirect pathways in the basal ganglia leading to less inhibition of the GPe and STN, increased activity of GPi, and decreased overall excitation input from the thalamus to the cortex. Furthermore, the STN also receives afferent glutamatergic innervations from the cortex and the parafascicular nucleus of the thalamus [31, 32]. Direct glutamatergic neuronal connections from the motor cortex (MC) to the STN investigated in 6-hyrdoxydopamine (6-OHDA) hemi-lesioned rats using anterograde tracing [33] showed MC-STN connectivity was impaired and glutamatergic terminals were reduced [33]. In addition, the STN receives

GABAergic input from the GPe, dopaminergic input from the SNc, and cholinergic/glutamatergic input from the PPN [34-36]. As mentioned above, this dysfunctional connection of the MC-STN could lead to overactive STN glutamatergic neurons that may produce excitotoxins projecting to the SNc and GPi [37-39]. Glutamatergic excitotoxicity was found to be related in several validated animal models of PD, such as 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [39]**.** Furthermore, this excitetoxicity can be inhibited by a glutamate antagonist MK801 but not with the GABA antagonist bicuculline [40]. _

3. ROLE OF NEUROTRANSMITTERS IN MEDIATING DBS EFFECTS

Historically, surgical treatment using vascular or chemical lesions of the GP or thalamus were used to reduce PD symptoms before the discovery of Levodopa in 1967 [41, 42]. However, these treatments failed to suppress disease progression and lead to several adverse effects such as dyskinesia, motor fluctuations, and drug-resistance [43]. The development of stereotactic surgery, neuroimaging, and intraoperative electrophysiology enabled more accurate brain region targeting with a higher therapeutic yield and less overall harm [1, 44]. STN-DBS done in 1993 by Benabid then became the standard treatment especially for refractory PD patients [44].

Despite progress in DBS as a treatment for movement disorders, its exact mechanisms of action have not been fully unravelled. Several theories have been proposed, the most common being the neuronal firing "rate-model" and the patterned "synchronized oscillations" hypotheses [3, 45-51]. The rate model hypothesis postulates DBS blocks overactive basal ganglia neuronal firing rates in the STN and GPi caused by PD pathology. The blocking of the overactive basal ganglia was predicted by lesion effects of earlier neuromodulations techniques [41]**.** Interestingly, a post-mortem study on spinocerebellar ataxia disease found a consistent degeneration of SNc, although patients did not exhibit parkinsonian symptoms [52]. Further histological analysis of this post-mortem brain tissue showed a significant lesion in brain regions such as the thalamic ventral anterior and ventral lateral nuclei, pallidum, PPN, and STN. Consequently, these findings suggest that lesion effects of these targets lead to a therapeutic output on PD symptoms. The pattern "synchronized oscillations" hypothesis has also been proposed as a mechanism of DBS, where pathological beta-band oscillatory activity (abnormal synchronized bursts of activity) in loops between the cortex, basal ganglia, thalamus, PPN, and cerebellum contribute to the genesis of PD motor symptoms [53]. DBS is thought to disrupt and suppress pathological beta-band oscillations and promote gamma power, thereby reducing bradykinesia and rigidity symptoms [7].

In addition to local effects, DBS has shown to have downstream effects on remote areas. For instance, STN-DBS in PD has led to increased striatal dopamine release in PD rats [14, 54-56]. In addition, another study found a correlation between an increase in striatal monoamine (MA) levels and STN beta activity suppression via STN-DBS in a rat model of PD (hemi-PD 6-OHDA) [54]. GPi-DBS has also been shown to stimulate striatal dopamine release in rodents [57, 58]. Moreover, preclinical research shows

STN-DBS reduces dorsal raphe nucleus (DRN) serotonergic neuron firing rates in both PD and healthy control rat models [17] while PPN-DBS reduces acetylcholine (ACh) loss in the ventral thalamus [18].

Research suggests DBS has multimodal effects that are not simply due to an inhibition of local axons, but from effects that may pass through fibre, efferent, and afferent axons through orthodromic and antidromic pathways [59, 60]. Moreover, DBS is thought to exhibit long-term effects, including tyrosine hydroxylase (TH) cell survival in the striatum as a possible form of neurogenesis and neuroplasticity [61]. Additionally, alleviating pathological oscillations by continuous DBS showed short-term and long-term synaptic plasticity in the SNr [62, 63]. These long-term effects may be activated via neurochemical changes displayed through neurotransmitters' levels and neuronal communications. The following section will extensively discuss dynamic changes in main neurotransmitter systems involved in PD that mediate DBS effects (*Table1, Figure.1*).

3.1 Dopaminergic system

PD is principally defined as pathological degeneration of dopaminergic SNc neurons; newly diagnosed PD patients can show a 50% loss of SNc dopaminergic neurons [43]. Moreover, the standard medical treatment for PD (drugs like Levodopa) mainly work on dopaminergic receptors, however, long-term use of medical treatments can exhibit unwanted side effects. Therefore, it has been proposed one mechanism of DBS for PD is its action on surviving striatal DA neurons [61]. Several early studies were based on this assumption, with some even attempting to directly stimulate the SNc [64-66]. Overtime, STN-DBS showed to improve the motor symptoms of PD more effectively and reduce the need for Levodopa, eventually leading to fewer adverse drug effects [67].

Nigrostriatal dopaminergic neurons were assessed in several preclinical animal studies and showed that STN-DBS modulate SNc dopaminergic neurons. Sahai et al. 2020 showed that STN-DBS decreases the spiking activity in less than half (43%) of the SNc dopaminergic neurons in naïve rats group while increasing the spiking activity in the other 43% of the cells. However, the PD rat group showed a significant reduction in the spiking activity of 88% of dopaminergic cells [68]. Moreover, an *in vivo* microdialysis study on unilateral 6-OHDA rats showed short-term effects of stimulation and lesioning in the STN increased extracellular levels of dopamine in the striatum [69]. In another microdialysis study in a freely moving rat model of PD (6-OHDA), STN-DBS increased striatal DA metabolites in awake, freely moving animals [55, 56]. When *in vivo* real-time electrical and chemical detection of dopamine concentrations and neural firings in the caudate-putamen (CPU) of PD rats PD were assessed using fast-scan cyclic voltammetry (FSCV), dopamine concentrations increased and striatal neurons firing decreased following GPi-DBS [19]. Additionally, *in vivo* experiments in pigs using a Medtronic 3389 device to perform FSCV combined with a carbon-fiber microelectrode (CFM) in the striatum to track dopamine release evoked by electrical stimulation showed STN-DBS elicited a stimulus-time-locked increase in striatal dopamine release that was both stimulus intensity- and frequency-dependent [70]. Another experiment in monkeys showed STN-DBS induced phasic DA release in the striatum [71].

Table 1. Neurotransmitters dynamics in response to DBS treatment in PD.

NT	DBS target	Study type	Main findings	Reference
Dopamine	STN	In-vivo rat model	Increased striatal dopamine release and improved motor symptoms	**He et al. 2014**
Dopamine	STN	In-vivo rat model	Increased striatal dopamine release	**Yamamoto et al.2014**
Dopamine	STN	In vivo rat model	Increased striatal dopamine metabolites in awake, freely moving animal	**Meissner et al.2002.**
Dopamine	STN	In vivo rat model	Increased expression of DR1 and decrease DR2 and DR3 in the striatum.	**Carcenac et al.2015. Melon et al.2015.**
Dopamine	STN	In vivo rat model	Increased extracellular dopamine release in the striatum.	**Walker al.2009**
Dopamine	STN	In vivo rat model	Increased and decreased the spiking activity of SNc neurons	**Sahai, et al. 2020**
Dopamine	STN	In vivo rat model	Increased striatal dopamine release	**Du, Chen et al. 2018**
Dopamine	STN	In vivo rat model	Long-term effects on survival of striatum dopaminergic cells and cell proliferation in HIPP and olfactory bulb.	**Khaindrava et al. 2011**
Dopamine	STN	In vivo monkey model	Induced phasic DA release in striatum according to in vivo fast-scan cyclic voltammetry	**Nakajima et al. 2017**
Dopamine	STN	In vivo rhesus macaque model	Increased striatal dopamine release. The level of dopamine release was depended on precision of stimulation site.	**Min et al.2016**
Dopamine	STN	In vivo Pig model	Increased striatal dopamine release depended on stimulation intensities and frequencies.	**Shon et al. 2010**
Dopamine	STN	Human clinical trail	PET imaging showed a decrease of VMAT2 transporter in the striatal reflecting a potential increase of dopamine levels	**Smith et al.2019**
Dopamine	STN	Human clinical trail	Increased CSF catecholamine levels and tittered down Levodopa with an improved motor function.	**Yamamoto et al.2015**
Dopamine	STN	Human clinical trail	No changes in DAT availability and an increase in DR2 binding were detected in the striatal.	**Hesse et al.2008**
Dopamine	STN	Human clinical trail	RacloBP that reflects DR2/DR3 density and/or synaptic dopamine levels in the striatal were reduce after STN-DBS.	**Thobois et al.2011**
Dopamine	STN	Human clinical trail	Induced the stabilization of synaptic dopamine concentrations in the striatal.	**Nimura et al.2005**
Dopamine	STN	Human clinical trail	PET imaging showed no evidence of increased striatal dopamine concentration under effective STN-DBS in humans	**Hilker et al.2003**
Dopamine	STN	Human clinical trail	PET imaging showed no evidence of increased striatal dopamine concentration	**Strafella et al. 2003**

Table 1. Continued

Dopamine	GPi	In vivo rat model	No change was found in the striatal dopaminergic metabolism in naïve and 6-OHDA lesioned rats using microdialysis	**Meissner et al.2000**
Dopamine	GPi	In vivo rat model	The striatal dopamine concertation increases and neural firing decrease after GPi-DBS using microdialysis	**Xiao et al.2019**
Dopamine	GPi	Human clinical trail	Increase pallidal dopamine but no correlation with improvement in rigidity	**Martinez et al.2013**
Glutamate	STN	In vivo rat model	STN glutamate level rapidly elevated during stimulation, sustained during stimulation, and descended slowly towards the baseline after stimulation.	**Lee et al.2007**
Glutamate	STN	In vivo rat model	Glutamate levels increased in SNr with both intact and hemiparkinsonian rats	**Boulet et al.2006**
Glutamate	STN	In vivo rat model	Chronic (5 week) STN-DBS of 6-OHDA freely moving rats restored normal levels of glutamate metabolite in the striatum using *in vivo* 11.7 tesla MRS.	**Chassain et al.2016**
Glutamate	STN	In vivo rat model	Increased extracellular glutamate levels in SNr and GPi in both PD and control	**Windels et al.2000**
Glutamate	STN	In vivo rat model	Downregulated of CaMKIIa and Homer1 genes that are involved in glutamate neurotransmission	**Henning et al.2007**
Glutamate	STN	In vivo rat model	Increased extracellular glutamate levels in SNr	**Windels et al.2003**
Glutamate	STN	Human clinical trail	Pontine glutamate levels were lower in PD patients	**Tuura et al.2018.**
GABA	STN	In vivo rat model	Increased GABA levels in the SNr	**Windels et al.2000**
GABA	STN	In vivo rat model	Increased GABA levels in the SNr	**Savasta et al.2002**
GABA	STN	In vivo rat model	Increased GABA levels in the GP	**Salin et al.2002**
GABA	STN	Human clinical trail	Basal ganglia GABA levels were higher in PD patient	**Tuura et al.2018**
GABA	GPi	Human clinical trail	CSF GABA levels increased during stimulation	**Ogura_et al.2004**
GABA	GPi	Human clinical trail	Both STN-DBS and Levodopa reduced GABA levels in the central anterior thalamic nucleus	**A Stefani.2011**
Serotonin	STN	In vivo rat model	Decreased firing rate of DRN 5-HT neurons and induced depressive-like behaviour in both PD and control rat model.	**Temel et al.2007**
Serotonin	STN	In vivo rat model	Decreased both extracellular level and firing of 5-HT neuron.	**Tan et al.2012**
Serotonin	STN	In vivo rat model	STN-DBS inhibited 5-HT release in the PFC, HIPP, and striatum	**Navailles et al.2010.**

Serotonin	STN	In vivo rat model	Firing rates of DRN neurons inhibited by HFS-STN-DBS did not quickly revert to their pre-stimulus firing rates.	**Hartung et al.2011**
Serotonin	STN	In vivo rat model	Severe serotoninergic dysfunction leads to decreased STN-DBS therapeutic efficiency	**Faggiani et al. 2015**
Serotonin	STN	In vivo mouse model	STN-DBS inhibited 5-HT DRN neuronal activity and induced loss of 5-HT cell phenotype	**Alosaimi et al. 2022**
Acetylcholine	PPN	In vivo rat model	DREADD introduced in PPN cholinergic neuron activated residual nigrostriatal dopaminergic neurons and reduced motor symptoms.	**Sharma et al.2020**
Acetylcholine	PPN	In vivo rat model	PPN-LFS mildly reversed the acetylcholine loss in the ventrolateral thalamic nucleus.	**Wen et al.2015**
Acetylcholine	STN/GPi	In vivo monkey model	STN-DBS produced a larger suppression of TAN spiking rate than GPi-DBS	**Nakajima 2017**
Noradrenaline	STN	In vivo rat model	Severe LC noradrenergic dysfunction led decrease STN-DBS therapeutic efficiency	**Faggiani et al. 2015**
Noradrenaline	STN	In vivo rat model	LC noradrenergic degeneration led to weight loss and STN-DBS regained the weight.	**Guimarães et al 2013**
Noradrenaline	STN	Human clinical trail	Clonidine manipulation of LC noradrenergic system inhibit the therapeutic effect of STN-DB	**Albares et al.2015**
Noradrenaline	STN	Human clinical trail	Metoprolol manipulation of LC noradrenergic reduce the STN spiking activity in PD patient and improve rigidity.	**Coenen et al. 2008**

Note. 5-HT; 5-hydroxytryptamine (serotonin), 6-OHDA; 6-hydroxydopamine, Ach; Acetylcholine, CSF; cerebrospinal fluid, DBS; Deep brain stimulation, DA; dopamine, DAT; dopamine transporter, DR1, DR2 &DR3; dopamine 1,2 &3 receptors, DREAD; *designer receptors exclusively activated by designer drugs*, DRN; Dorsal raphe nucleus, GABA; γ-Aminobutyric acid, GPi; globus pallidus internal, HIPP; hippocampus, LC; locus coeruleus, LFS; low-frequency stimulation, MRS; magnetic resonance spectroscopy, NT; neurotransmitter, NA, Noradrenaline, PET; Positron emission tomography, PFC; prefrontal cortex, PD; Parkinson's Disease, PPN; pedunculopontine nucleus, RacloBP ; [11C] raclopride binding potential, SNc; substantial nigra pars compacta, SNr; substantial nigra pars reticulata, STN; subthalamic nucleus, TAN; tonically active neurons; VMAT2; vesicular monoamine transporter 2.

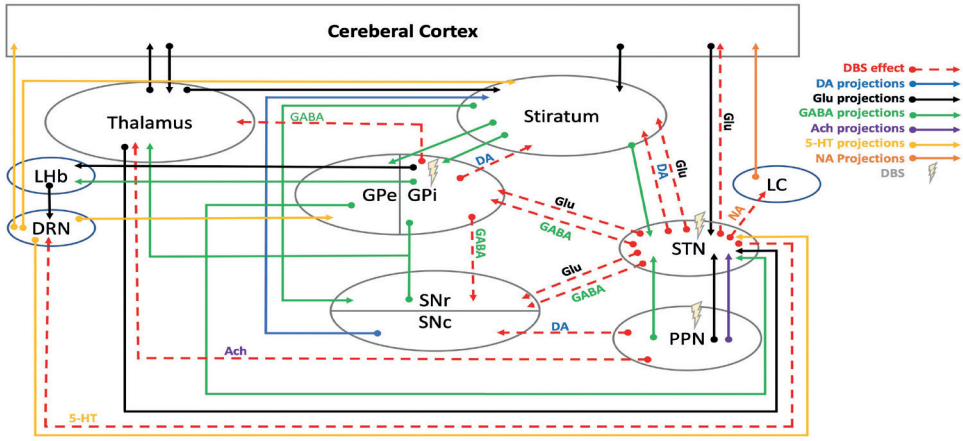

Figure 1. Schematic illustration of DBS neurotransmitter mediation in cortico-thalamic-basal ganglia-brainstem circuits. 5-HT; 5-hydroxytryptamine (serotonin), Ach; acetylcholine, DA; dopamine, DBS; deep brain stimulation, DRN; Dorsal raphe nucleus, GABA; γ-Aminobutyric acid, Glu; glutamate, GPe & GPi; globus pallidus external & internal, LC; locus coeruleus, LHb; lateral habenula, NA; Noradrenaline, PPN; pedunculopontine nucleus, SNc & SNr; substantia nigra pars compacta & pars reticulata, STN, subthalamic nucleus. DBS effects data are cited in *Table 1*

Further animal experimental studies have investigated the STN-DBS neurotherapeutic effects in improving motor symptoms caused by dopamine in non-human primates and in PD rodent models [14, 54]. One study of MPTP rhesus monkeys treated with STN-DBS and microdialysis implanted bilaterally in the putamen and caudate nuclei showed improvement in motor symptoms and increased extracellular DA along with its metabolites in both brain regions [72]. Other researchers used multi-contact DBS electrode, fMRI and FSCV to identify optimum dopamine-recording sites in rhesus macaques and found dopamine release reduces or increases depending on the slight redirection of DBS electrode tip location in the STN while the highest evoked response was shown when the DBS electrode tip contact were in the dorsal part of STN [73]. Lastly, striatal dopaminergic receptors expression was assessed in intact and total nigrostriatal dopaminergic denervated rats after a 4-hour unilateral STN-HFS using particular radioligands ([^{3}H] SCH 23390, [^{125}I] iodosulpride, and [^{125}I] OH- PIPAT) and showed increased D1 receptor (D1R) binding in all areas of the striatum but decreased binding of both D2 and D3, leading some to suggest that D1R effects could explain the therapeutic effect of DBS while side effects could be more due to D2R and D3R [74, 75].

Some evidence suggests that STN-DBS for PD has a long-term neuroprotective effect on the survival of SNc cells in animal models [76-78]. Khaindrava *et al.* investigated DBS of the STN and found no significant increase in cell proliferation, yet cell survival in dopaminergic neurons of the striatum was increased. In addition, lesser amounts of cell proliferation was observed in the hippocampus and olfactory bulb [61]. Another study investigated the neuroprotective effects of STN-DBS on a unilateral 6-OHDA model in rats; post-stimulation effects revealed that nigral TH positive neurons and the protein

phosphatase-2A (PP2A) were blocked inducing autophagy and dissociating the Bcl-2/Beclin1 complex showing a possible neuroprotective mechanism for PD [79]. Another STN-DBS study in 6-OHDA rat model of PD showed BDNF-mediated neuroprotection in the SNc by acute and long-term blocking of TrkB enzyme that decreased phosphorylation of Akt and ribosomal protein S6 [80]. Another study investigated the protective effect of DBS in the STN in unilateral 6-OHDA rats and found apoptosis significantly decreased in the DBS group.

Some clinical findings towards the effects of DBS on striatal dopaminergic neurons have shown to be contradictory. A PET study in PD patients assessed vesicular monoamine transporter 2 (VMAT2) and cerebral glucose metabolism before DBS surgery and 4-6 months after STN DBS and showed improvement of motor and neuropsychiatric symptoms while VMAT2 had decreased in the caudate and putamen along with decreased cerebral glucose metabolism in striatum-[81]. Another PET study showed synaptic dopamine concentrations increase in the putamen and caudate nucleus after STN-DBS alongside medication; Levodopa significantly reduced [11C] raclopride binding potential (RacloBP) in the putamen, while postoperatively the reduced RacloBP-binding reversed [82] and the drug-induced increase in synaptic dopamine concentrations was also higher after stimulation.

A clinical study used SPECT to assess the dopamine D2 receptor and dopamine transporter (DAT) in PD patients pre-surgery and at 12 months post-surgery; the unified PD rating scale (UPDRS) scores post-surgery remarkably improved in those patients and titrated down their medication, but no changes in DAT availability or an increase in D2 receptor binding were detected [83]. Another clinical study investigated the occurrence of apathy and depression following 12-month STN-DBS in PD and control groups and found that PD exhibited more apathy and depression symptoms. Additionally, sections of each group received a PET-scan ([11C]-raclopride) that showed increased binding of D2/D3 receptor density. In sum, these findings reflect that decreased synaptic dopamine levels in the mesolimbic area, particularly the ventral tegmental area (VTA) are more substantial in PD patients, suggesting that apathy and depression occur post-surgery as a postponed dopamine withdrawal syndrome [84].

Clinical PET scans in 6 PD patients receiving STN-DBS showed no difference in RACLO binding (ligand for dopamine D2/D3 receptor) between DBS on and off conditions along with no evidence of increased striatal dopamine concentration under effective STN-DBS [85, 86]. In another clinical trial, cerebrospinal fluid and plasma catecholamine levels in STN-DBS treated PD patients were measured after oral antiparkinsonian drug administration before surgery and an hour after medication while being on DBS; higher preoperative catecholamine levels were linked to better STN-DBS outcomes [87].

Stereotactic microdialysis is feasible in PD patients during STN-DBS surgery [88, 89]. Within 30 minutes of DBS electrode placement in the STN and a microdialysis probe in the STN or SN researchers can get a steady-state baseline level of glutamate, dopamine, and GABA. [89]. A microdialysis study in the GPi during GPi-DBS surgery investigated dopamine levels before and after DBS and found that 4 out of 5 patients had significantly increased pallidal DA after stimulation [90]. However, there was no association

between the improvement in rigidity and pallidal DA increase, suggesting other mechanisms might be involved in this clinical effects of GPi-DBS [90].

In conclusion, the dopaminergic system is essential in PD as is clearly mediated with pharmaceutical and DBS therapies. DBS has shown to treat motor symptoms of PD and decrease the need for DA medication, suggesting DBS has a synergetic effect on DA. Preclinical animal data suggests that STN DBS influences both local and remote dopaminergic systems, while clinical microdialysis data in human subjects point more towards a local effect. Remote effects are less consistent in imaging studies, as inconsistencies stem from limitations innate to PET and SPECT neuroimaging techniques.

3.2 Glutamatergic system

Glutamatergic neurons are distributed throughout the central nervous system [91]. Several theories suggest overactive glutamate in the basal ganglia induces excitotoxin production in SNc neurons contributing to the pathogenesis of PD [37, 92-96]. An experimental stereotactic injection of glutamate receptors antagonist (NMDA blocker) in the striatum of rodents has shown to alleviate parkinsonian symptoms [97, 98]. It then appears that DBS could influence the glutamatergic system in a net positive way as neurons within the striatum act as intermediate excitatory neurons between basal ganglia-thalamocortical circuits [91].

Several pieces of evidence suggest that DBS effects on glutamate is solely a local effect that only lasts during stimulation. In an experimental study of anesthetized rats, a dual enzyme-based electrochemical sensor measured extracellular glutamate concentrations in the STN and found that glutamate concentrations increased rapidly during DBS and were sustained during stimulation. After cessation of stimulation, elevated glutamate levels slowly fell towards baseline [99]. Another study assessed the dyskinesia side-effects of STN-DBS in unilateral 6-OHDA rats using microdialysis in the SNr; STN-DBS increased glutamate and induced dyskinesia in both intact and hemiparkinsonian rats [15]. When the stimulation frequency was lowered, glutamate levels were unaffected suggesting dyskinesia blocks glutamate receptor antagonists and facilitates agonists. In another study, STN-DBS was performed in rats to determine if neurochemical changes in the GP and SNr were frequency-dependent and found that glutamate concentrations were significantly increased at a high frequency (60 and 130 Hz) in the GP and SNr but did not show significant change at low-frequency stimulation (10 Hz) [100, 101]. It should be noted that the measured glutamate levels in the GP refers only to the GPe, and is not a basal ganglia's output structure unlike the SNr. The entopeduncular (EP) nucleus is homologous to the primate GPi in rats [102].

Chassain et al. studied glutamate metabolic, synaptic, and behavioural changes in hemiparkinsonian rats after five weeks of chronic STN-DBS using proton magnetic resonance spectroscopy (11.7T) and found chronic STN-DBS corrects the glutamate metabolites levels associated with neurotransmission in the striatum and SNr, restores corticostriatal synaptic plasticity, and restores motor skills progressively in the staircase test [103]. Consequently, these findings suggest chronic STN-DBS not only has a local effect

but also a remote effect in the basal ganglia. Furthermore, STN-DBS showed a reduction in sensitivity toward glutamate neurotransmission via downregulating calcium/calmodulin-dependent kinase IIa (CaMKIIa) and Homer1 genes in the STN as these genes are associated with glutamate neurotransmission [104]. These findings suggest STN-DBS could have a neurotherapeutic effect by alleviating overactive glutamatergic neurons in the STN. To validate these findings in humans, clinical studies were performed to assess glutamate levels in PD patients and controls using magnetic resonance spectroscopy. Pontine glutamate levels were shown to be lower in PD patients while glutamate levels also emerged as a significant predictor of outcome, further implementing glutamatergic neurotransmission within the mechanisms of DBS [105].

In sum, the glutamatergic system has shown to be involved in PD pathology. STN-DBS modulates glutamatergic neurons and glutamate neurotransmission and exhibits neurotherapeutic effects by improving motor and adverse effects. Acute effects of STN-DBS on the glutamatergic system were thought to be merely local and stimulation dependent, however, chronic effects of STN-DBS show increases in glutamate in the striatum and SNr and further demonstrate to improve motor symptoms of PD in preclinical studies.

3.3 GABAergic system

GABAergic neurons are distributed widely through the entire central nervous system [106] and act mainly as interneurons, having an essential role in regulating cortical and subcortical circuits including the cortico-thalamic-basal ganglia circuits [107]. GABAergic neurons function harmoniously with glutamatergic and dopaminergic neurons in the cortical-thalamic-basal ganglia circuit to control movement. As previously described, glutamatergic neurons have an excitatory effect while GABAergic neurons have inhibitory projections in the basal ganglia. Dopaminergic neurons also have both excitatory and/or inhibitory (depending on momentum) after-effects on glutamatergic and GABAergic modulation balance [107].

STN-DBS has shown to increase the neuronal firing rate in the SNr of PD rats [108]. Moreover, preclinical studies show an increased selective extracellular GABA release in the SNr after STN-DBS in rats [101, 109]. This increase of GABA release in the SNr was shown within a stimulation frequency-dependent range of 60 to 350 Hz, while glutamate release only increased until 130Hz [100]. STN-DBS could then have both local and cumulative effects on GABA release in the SNr that pass from the STN to the SNr, independent of the STN-DBS effect on glutamate release in the GP [59]. Additionally, in situ hybridization of glutamate decarboxylase 67 kDa isoform (GAD67) study also showed an increase in GABA in the GP after STN-DBS in unilateral 6-OHDA rats [110].

Clinical studies demonstrate GPi-DBS to improve bradykinesia and LID (Levodopa-induced dyskinesis) in PD patients through its effects on pallidum GABAergic neurons [111, 112]. Moreover, depending on the specific stimulation site within the GPi it was found that DBS of the ventral GPi reduced LID while stimulation of the dorsal part showed to improve bradykinesia [113, 114].

Using microdialysis in the GPi and ventral anterior (VA) thalamus during the first delivery of STN-DBS or Levodopa, researchers found both treatments reduced GABA levels in the VA thalamus, while STN-DBS also increased cyclic guanosine monophosphate (cGMP) levels in the GPi [115, 116]. A clinical imaging study assessing GABA levels of PD patients and controls using magnetic resonance spectroscopy showed basal ganglia GABA levels to be higher in PD patients [105]. In another clinical study on neurotransmitter levels in CSF of PD patients with GPi-DBS, CSF was collected a day after surgery 1 hour before and 1 hour after GPi stimulation. GABA levels increased during stimulation but no differences were seen in levels of dopamine, noradrenaline, or homovanillic acid [16].

In conclusion, it is clear that GABAergic interneurons play a critical role in movement regulation and within the dynamics of STN- and GPI-DBS. However, GPi-DBS effects depend on whether the ventral or dorsal part of the GPi is stimulated [111-114]. Furthermore, local impacts of STN-DBS show an increase in GABA in the GP while remote effects connected to the SNr are low to high frequency stimulation-dependent and differ from glutamate responses [101, 109, 110].

3.4 Serotoninergic system

The serotonergic system is well known to be involved in mood and anxiety [117], while patients who undergo STN-DBS have shown side effects related to serotoninergic and dopaminergic systems such as depression, suicide ideation, and impulsivity [84, 118-120]. Several lines of evidence show that STN DBS inhibits the serotoninergic system, and while the STN has no direct connection to the DRN, its relay station via the lateral habenula (LHb) suggests an indirect serotonin-modulating mechanism (Insert Reference Tan et al J Psychiatry Res).

Studies have shown that bilateral STN-DBS reduces the firing rate of DRN serotonergic neurons and induces depressive-like behaviour in both PD and control rat models [17]. Furthermore, the firing rate of DRN neurons that were inhibited by STN-DBS did not quickly recover to their pre-stimulus firing rates; many of these neurons remained to show reduced activity throughout the 5-minute post-stimulus recording period. This suggests STN-DBS elicits mechanisms that may cause sustained suppression of the serotonergic system [121]. Moreover, STN-DBS inhibited 5-HT release in forebrain regions such as the prefrontal cortex (PFC), hippocampus (HIPP), and striatum [122, 123]. Recent research shows that long-term (10 weeks) STN-DBS inhibits the serotoninergic neuronal activity during stimulation measured by calcium transients photometry and leads to a loss of serotoninergic cell phenotypes inducing depressive-like behaviour in the MPTP mouse model of PD [124]. Animal studies also show that presence of severe serotoninergic dysfunction reduces STN-DBS therapeutic efficiency [20]. In addition, the 5-HT system has been implicated in dyskinesia [125, 126] [127-129]. However, it can be speculated that a sustained suppression of the 5-HT system via loss of 5-HT cell phenotype could contribute to the lower incidence of dyskinesia following STN-DBS. In the other words, reduced basal ganglia 5-HT function supports the therapeutic effects of DBS. Future research is needed to explore the exact trajectory connections between the STN and the DRN that engender DBS targeting to display fewer adverse effects.

3.5 Cholinergic system

Neurons of the cholinergic system are predominantly located in the nucleus basalis of Meynert, the PPN, and striatum [130-132]. Recent data suggests the degeneration of cholinergic neurons could be involved in the pathogenesis of early-stage PD and linked to its axial and nonmotor symptoms, including cognitive decline and mood disorder [133-137]. Moreover, striatal cholinergic interneurons regulate basal ganglia circuits and could modulate effects of dopaminergic and glutamatergic systems [138].

Animal studies have investigated tonically active neurons (TANs) and putative cholinergic interneurons in the striatum during DBS; STN-DBS produced a more extensive suppression of TAN spiking rates compared to GPi-DBS in healthy monkeys, additionally, a local DA antagonist infusion in the striatum only reduced the spike rate after STN-DBS, but not in the GPi-DBS group [71]. This suggests a more apparent increase of DA after STN-DBS antagonised by the local DA infusion and counterbalanced by the putative cholinergic interneurons.

PPN-DBS has been proposed to treat axial and gait symptoms of PD. PPN-LFS (25Hz) in unilateral 6-OHA rats improved gait symptoms including base of support and maximum contact area in the catwalk test assessing locomotion and gait function [18]. Furthermore, PPN-LFS also mildly reversed acetylcholine loss in the ventrolateral thalamic nucleus in rodents [18]. Sharma et al. 2020 investigated the stimulation effects of designer receptors exclusively activated by designer drugs in the PPN using a PET scan to assess nigrostriatal dopaminergic neurons in a chemogenetic PD model and found that residual nigrostriatal dopaminergic neurons reduced motor symptoms [139]. PPN-HFS has also shown to reduce postural instability of PD-rats (6-OHDA), however, rats stimulated in the PPN showed complex behavior effects [140]. In sum, the cholinergic system regulates motor functions in the basal ganglia and is associated with axial and gait symptoms in PD. Preclinical animal studies of PPN-DBS exhibits an improvement in axial and gait symptoms however the data is mixed. It remains to be determined whether the PPN is a suitable new stimulation target for patients with severe axial and gait dysfunction.

3.6 Noradrenergic system

Noradrenaline (NA) is predominantly produced by the locus coeruleus (LC) located in the pons and projects to cortical, subcortical, and spinal structures [141]. Noradrenergic LC neurons have shown to be involved in a wide range of sensory-motor, behavioral, and cognitive functions [142-149]. Evidence suggests the loss of NA neurons occurs several years prior to presentation of PD symptoms and more extensively than in SNc dopaminergic neurons [150-153]. Additionally, PD exhibits a neuropathological vulnerability to neuromelanin loss that is shown to affect both DA in the SNc and NA in the LC [154-158].

In recent years, the effects of DBS on LC noradrenergic system have gained considerable attention. A preclinical animal study showed that severe noradrenergic dysfunction reduces STN-DBS therapeutic efficiency within a PD rat model [20]. Furthermore, Guimarães et al. 2013 investigated the involvement of the LC noradrenergic system on weight loss in a PD rat model and showed STN-DBS abolished

weight loss in bilateral LC and striatum 6-OHDA lesioned rat without any observed changes in their food or other metabolic parameters. Additionally, the degeneration of the LC was not accompanied by significant changes in motor behavior but led to an extra decrease in striatal monoamine levels reflected by the decrease in the DA/L-3,4-dihydroxyphenylalanine (L-DOPA) ratio [21].

A clinical study suggests a role for the LC noradrenergic system in STN-DBS. When stimulation is combined with administration of clonidine, a selective alpha2 adrenergic agonist, STN-DBS related benefits on akinesia are diminished [159]. On the other hand, one clinical study showed that metoprolol, a beta1-adrenergic antagonist, suppressed STN bursting activities marking a brief decrease in rigidity pre-STN-DBS surgery [160]. Retrospective clinical data showed STN-DBS led to weight regain in PD after DBS surgery [161, 162] and this was suggested to be related to a STN-DBS effect on the LC noradrenergic system [163].

In conclusion, LC noradrenergic dysfunction occurs years before clinical diagnosis and shows a similar pathological root to PD comparable to SNc dopaminergic systems. Furthermore, severe dysfunction of the noradrenergic system diminishes positive effects of STN-DBS, as STN-DBS has shown to improve weight loss in PD possibly through its actions on the LC noradrenergic system. Pharmacological manipulations of the noradrenergic system further impact PD symptoms even alongside STN-DBS and this underlines the need for research combining pharmacological and DBS treatments.

4. THE ROLE OF GLIA CELLS IN NEUROTRANSMITTER HOMEOSTASIS

Glia cells, including astrocytes and microglia, are involved in inflammatory processes and contribute to neurodegeneration of the SNc in PD, as previously reviewed by McGeer et al [23, 24]. In addition, astrocytes have a role in neural communication and exhibit effects on neuronal activities [164, 165]. For instance, astrocytes can store and release glutamate stimulating pre-and post-synaptic receptors of surrounding neurons [166]. Consequently, inflammatory processes activate astrocytes in the SNc to release glutamate which project to the STN and can lead to glutamatergic neuron overactivity. This glutamatergic overactivity has also been shown to promote microglia and pro-inflammatory cytokines release that can contribute further to SNc neuronal damage [167]. Additionally, inflammatory processes can enhance α-synuclein release linked to glutamatergic excitotoxicity in the SNc [168, 169].

DBS has shown to affect glia cells even starting from an initial reaction to the implantation of a DBS electrode [170]. Although the glia cells cannot generate action potentials, their cellular properties allow sensitivity to voltage changes possibly including external electrical stimulation [171, 172]. This electrical stimulation mainly activates astrocytes via intracellular calcium released locally on the stimulation site and to surrounding cells [173-176]. Moreover, calcium propagation to astrocytes could induce glutamate neurotransmission [177, 178]. DBS has been further indicated to modulate different subtypes of glia cells, including astrocytes, microglia, and macrophages that can change glial phenotypes and their functions

contributing to therapeutic effects [22]. A recent experimental animal study showed STN-DBS inhibits neuroinflammatory processes in PD by modulating glia cells including astrocytes in the GP, shown in both in vivo rat models and in vitro cell cultures [179]. Another experimental study showed STN-DBS inhibits microglia and normalizes neuroinflammatory cytokine levels in the SN of a rat PD model [180]. As a consequence, glia cells involved in both PD and STN-DBS modulate neuroinflammatory processes, while a greater understanding of the underlying mechanisms could improve therapeutic outcomes involving the survival of SNc dopaminergic and STN glutamatergic neurons [181].

5. CONCLUSION

It has been shown that multiple neurotransmitters are involved in the neuropathology and pathophysiology of PD, including the SNc dopaminergic, LC noradrenergic, pallidal GABAergic, PPN cholinergic, DRN serotoninergic, and STN glutamatergic systems. Growing evidence supports glia cells, especially astrocytes, to be involved in PD as well, while DBS modulate these cells by mainly effecting glutamatergic neurons [167]. Although DBS improves motor symptoms in PD and continued evidence shows underlying neurochemical alterations involved in PD can be mediated by DBS, the exact mechanisms of action underlying DBS effects on neurotransmitters are still not fully understood.

DBS has demonstrated alleviation of motor symptoms of PD while decreasing the need for pharmaceutical dopamine treatment, indicating DBS impacts dopaminergic system. In preclinical research, STN-DBS has both local and distant effects on dopaminergic systems [14, 54, 55, 68, 69, 71, 73-75, 79]. In contrast, clinical results demonstrate local impacts from STN-DBS using electrophysiological and microdialysis, but such data shows less consistency compared to distant effect from neuroimaging studies [82-87]. Moreover, STN- & GPi-DBS affects glutamatergic neurons and glutamate neurotransmitter release leading to side effects, mainly dyskinesia [15]. The acute effects of DBS on the glutamatergic system were once assumed to be local and stimulus-dependent, but preclinical investigations of prolonged DBS have shown to increase glutamate in distant areas including the striatum and the SNr [100, 101]. DBS effects on the GABAergic system also differ based on specific DBS target; STN-DBS has local influence on GABA in the GPi and a distant effect on the SNr where it is more stimulation-dependent [57, 108]. GPi-DBS also has local effects on GABA release in the GPi and remote effects in the SNr, however, ventral GPi-DBS shows alleviation in LID whereas dorsal GPi-DBS reduces bradykinesia symptoms [113, 114]. STN-DBS has demonstrated superiority in treating bradykinesia and reducing Levodopa requirements [182].

The frequent occurrence of depression among PD patients suggests a role for the DRN serotoninergic system. This is stressed by STN-DBS induced inhibition in serotoninergic neurons in PD. From preclinical data, PPN-DBS has shown to improve axial symptoms in PD, although further research is needed before clinical translation can move forward [18, 139]. Lastly, noradrenergic systems have recently driven attention there way as the degeneration of the LC noradrenergic system has shown

to lead to DBS therapy resistance [20]. Combined pharmacological-DBS treatments should continue be a focus in future research to investigate if other neurotransmitters including the cannabinoid and opioidergic systems may also prove to be involved in mechanistic symptom mediation [183, 184].

One reason neurochemical changes from DBS are not extensively covered within the literature could be from technical limitations innate to detecting transmitter release and transmitter-related charges in remote neuronal areas. Optogenetic studies investigating specific effects of neuromodulation on neurotransmitter release are warranted as they would further help assess DBS cumulative and chronic effects on local and remote neural elements. Further understanding dynamic changes in neurotransmitters will work to improve DBS effectiveness, provide more precise targeting, reduce adverse effects, and provide more appropriate pharmacological intervention options to improve overall quality of PD treatment.

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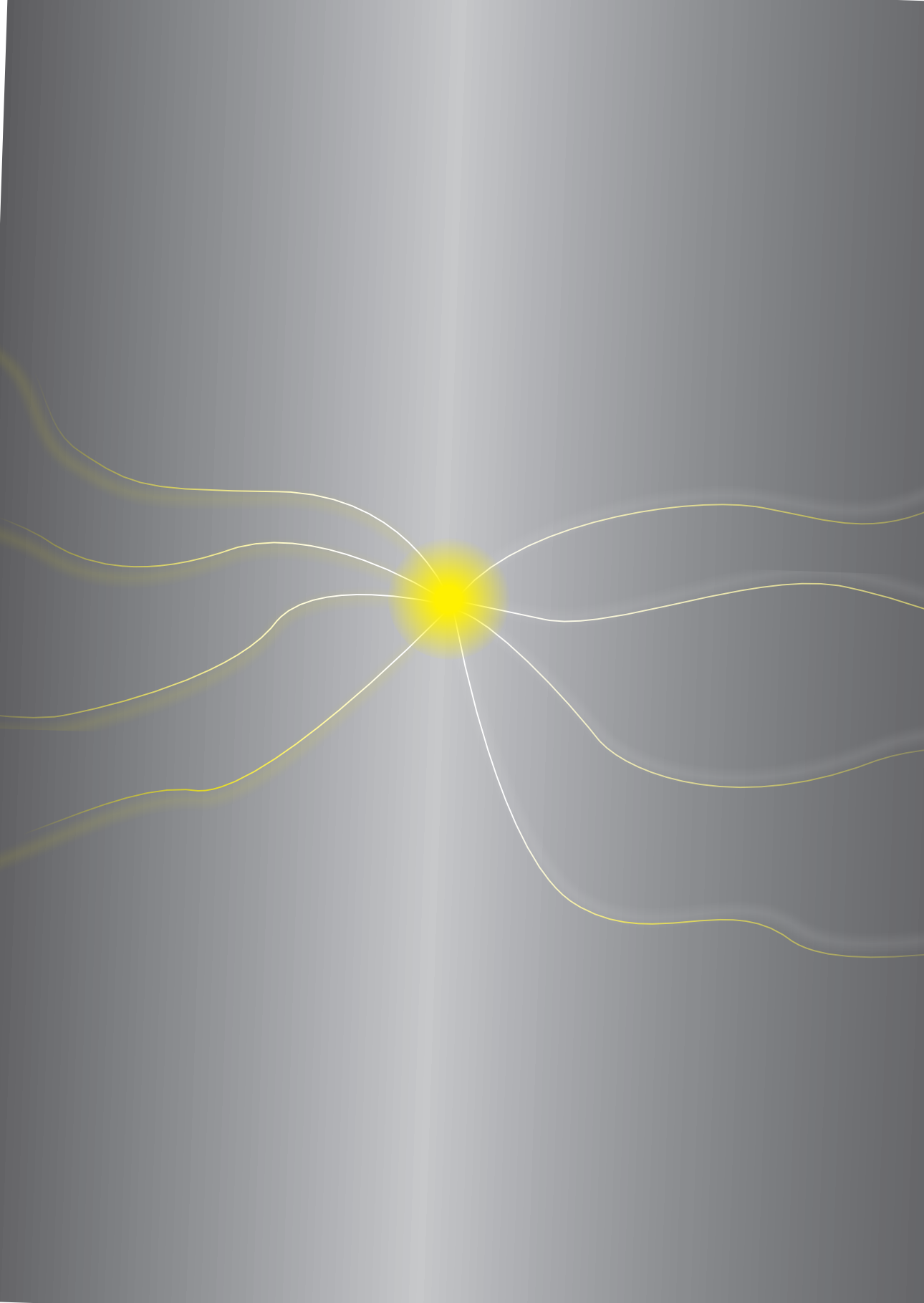

CHAPTER 3

High-frequency stimulation of the subthalamic nucleus induces a sustained inhibition of serotonergic system via loss of cell phenotype

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ABSTRACT

Deep brain stimulation (DBS) of the subthalamic nucleus (STN) has become a standard treatment for Parkinson's disease (PD). However, in a considerable number of patients debilitating psychiatric side-effects occur. Recent research has revealed that external stimuli can alter the neurotransmitters' homeostasis in neurons, which is known as "neurotransmitter respecification". Herein, we addressed if neurotransmitter respecification could be a mechanism by which DBS suppresses the serotonergic function in the dorsal raphe nucleus (DRN) leading to mood changes. We infused transgenic 5-HT-Cre (ePET-Cre) mice with AAV viruses to achieve targeted expression of eYFP and the genetically encoded calcium indicator GCaMP6s in the DRN prior to methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment. Mice received bilateral DBS electrodes in the STN and an optic fiber in the DRN for calcium photometry. MPTP-treated mice demonstrated behavioral and histological PD phenotype, whereas all STN-DBS animals exhibited an increased immobility time in the forced swim test, reduced calcium activity, and loss of tryptophan hydroxylase-2 expression in the DRN. Given the prominent role of calcium transients in mediating neurotransmitter respecification, these results suggest a loss of serotonergic phenotype in the DRN following STN-DBS. These findings indicate that loss of serotonergic cell phenotype may underlie the unwanted depressive symptoms following STN-DBS.

Key words: deep brain stimulation; Parkinson's disease; neurotransmitter respecification; dorsal raphe nucleus

1. INTRODUCTION

Deep brain stimulation (DBS) has emerged as a successful neurosurgical treatment to treat selected neurological and psychiatric disorders [1-4]. DBS of the subthalamic nucleus (STN) has particularly shown to effectively improve medically intractable motor symptoms of Parkinson's disease (PD) [5-8]. Despite long-term improvement in motor function, several PD patients exhibit mood disorders such as depression, suicide ideation and impulsivity after surgery [9, 10].

Our earlier studies have shown that acute bilateral STN-DBS inhibits neurotransmission of the midbrain serotonin (5-hydroxytryptamine; 5-HT) system in the dorsal raphe nucleus (DRN), which is the main source of 5-HT in the central nervous system and its dysfunction has been associated with the onset of mood disorders [11]. Acute STN-DBS in experimental animal studies demonstrated reduced firing rate of DRN 5-HT neurons, decreased 5-HT release in the forebrain and induction of depressive-like behavior in PD rats [12, 13]. However, in clinical settings STN-DBS is applied chronically. Long-term modulation of neuronal networks may induce permanent and neuroplastic changes [14]. More recently, it has been demonstrated that neurotransmitter identity in the mature brain can be influenced by environmental stimuli [15]. Neurotransmitter switching, induction or elimination associated with altered behavioral output are termed neurotransmitter respecification [16-18]. We hypothesized that neurotransmitter respecification plays a role in STN-DBS and occurs in the DRN 5-HT system. To investigate this we used the transgenic mouse line expressing Cre under the enhancer of the transcription factor Pet1 (ePET-Cre), which allows selective targeting of DRN 5-HT neurons [19]. These transgenic mice with PD associated symptoms after methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration were treated with daily STN-DBS for a relatively long period of time compared to existing studies. Behavioral, photometric and immunohistochemical assessments were used to evaluate aspects of neurotransmitter respecification in the DRN 5-HT system.

2. RESULTS

Stimulating electrodes were positioned bilaterally and symmetrically (inter-electrode variation <0.1 mm) in the STN in all mice except two, for which electrodes were located in the zona incerta. Those mice were excluded from the analysis. An example of electrode trajectory in a coronal brain section, and location of all electrode tips in the STN map are shown in the supplementary material (Fig. S1A-B). Fiber photometry probes were placed in the dorsomedial segment of the DRN in all mice except three, which were excluded from signal processing. No signs of significant histological damage due to implantation or electrical stimulation were observed.

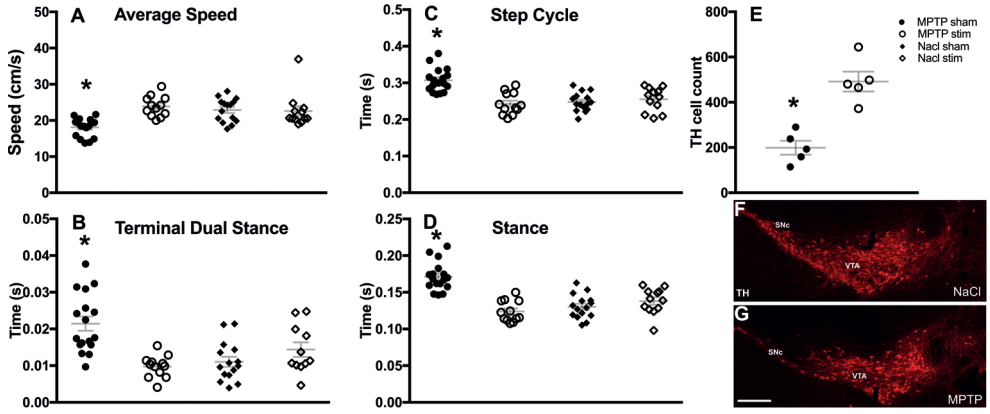

Figure 1. Effect of MPTP treatment and intermittent STN-DBS on Catwalk dynamic and static gait parameters. A-D) Graphs show a significant reduction in speed, and increases of step cycle, terminal dual stance and stance in MPTP-sham mice. STN-DBS restored those parameters to control levels, which is indicated by non-significant differences between MPTP-stim and NaCl-sham groups. E) The graph shows a significant reduction in TH positive cells in the SNc of MPTP-treated mice compared to the NaCl-treated animals. F-G) Representative low-power photomicrograph of coronal brain sections containing the SNc and VTA, stained for TH, show a noticeable TH cell loss in MPTP vs NaCl-treated mice. Data are presented mean +/- SEM; significant difference ($P<0.05$) is indicated by a "*", scale bar = 250µm. Tyrosine hydroxylase, TH; substantia nigra pars-compacta, SNc; ventral tegmental area, VTA; subthalamic nucleus, STN; methyl-4phenyl-1,2,3,6-tetrahydropyridine, MPTP; deep brain stimulation, DBS.

MPTP-treated mice showed a PD-like motor phenotype compared to the NaCl-treated group. MPTP treatment induced significant static and dynamic gait impairments with reduced average speed [MPTP-sham: 18.09 ± 0.62; MPTP-stim: 23.91 ± 0.68; NaCl-sham: 22.90 ± 0.80, and NaCl-stim: 22.60 ± 1.17; Two-way ANOVA; group effect: $F(3,52) = 9.04$, $p < 0.001$; disease*group effect: $F(1,52) = 3.64$, $p < 0.001$; followed by Bonferroni pairwise comparison; MPTP-sham vs NaCl-sham: $p < 0.001$; Fig.1A], increased terminal dual stance [MPTP-sham: 0.021 ± 0.002; MPTP-stim: 0.009 ± 0.001 NaCl-sham: 0.011 ± 0.001, and NaCl-stim: 0.015 ± 0.002; Two-way ANOVA; group effect: $F(3,52) = 10.36$, $p < 0.001$; disease*group effect: $F(1,52) = 2.90$, $p = 0.160$; followed by Bonferroni pairwise comparison; MPTP-sham vs NaCl-sham: $p < 0.001$; Fig.1B], step cycle [MPTP-sham: 0.31 ± 0.008, MPTP-stim: 0.24 ± 0.007; NaCl-sham: 0.25 ± 0.006, and NaCl-stim: 0.26 ± 0.008; Two-way ANOVA; group effect: $F(3,52) = 15.28$, $p < 0.001$; disease*group effect: $F(1,52) = 8.30$, $p < 0.01$; followed by Bonferroni pairwise comparison; MPTP-sham vs NaCl-sham: $p < 0.001$; Fig.1C], and stance [MPTP-sham: 0.17 ± 0.005, MPTP-stim: 0.12 ± 0.004; NaCl-sham: 0.13 ± 0.004, and NaCl-stim: 0.14 ± 0.004; Two-way ANOVA; group effect: $F(3,52) = 23.08$, $p < 0.001$, disease*group effect: $F(1,52) = 8.17$, $p < 0.001$; followed by Bonferroni pairwise comparison; MPTP-sham vs NaCl-sham: $p < 0.01$; Fig. 1D].

Moreover, STN-DBS restored these gait parameters in MPTP-treated mice with a significant increase in average speed and decrease in terminal dual stance, step cycle and stance [Two-way ANOVA; group effects: $F(3,52) = 9.04$, $p < 0.001$; $F(3,52) = 10.36$, $p < 0.001$; $F(3,52) = 15.28$, $p < 0.001$; and $F(3,52) = 23.08$,

$p < 0.001$, respectively; stim*group effects: $F(1,52) = 9.07$, $p = 0.064$; $F(1,52) = 4.75$, $p < 0.05$; $F(1,52) = 12.02$, $p < 0.01$, and $F(1,52) = 17.95$, $p < 0.01$, respectively]. Bonferroni post-hoc pairwise comparison of the means showed significant differences between MPTP-sham vs MPTP-stim in all tests (p's < 0.001; Fig.1A-D). Furthermore, stimulation did not alter gait parameters in NaCl-treated mice (NaCl-sham vs NaCl-stim) in none of the tests (Bonferroni post-hoc pairwise comparison: p's > 0.05). Post-mortem TH-immunohistochemistry revealed a significant loss (average 60%) of SNc dopaminergic neurons after MPTP administration in comparison to NaCl treatment (MPTP-sham: 198.8 ± 30.54 vs NaCl-sham: 491.8 ± 43.82; independent samples T-test $p < 0.005$; Fig. 1E-G).

Fiber photometry assessing calcium transients of DRN neurons showed a significant reduction of GCaMP6s fluorescence, indicating neuronal inhibition upon STN-DBS (Fig. 2A-D). Permutation test showed decreased calcium signaling by STN-DBS in both MPTP and NaCl-treated mice ($p < 0.05$). After stimulation was halted GcaMP6s fluorescence signal returned to baseline within ninety seconds.

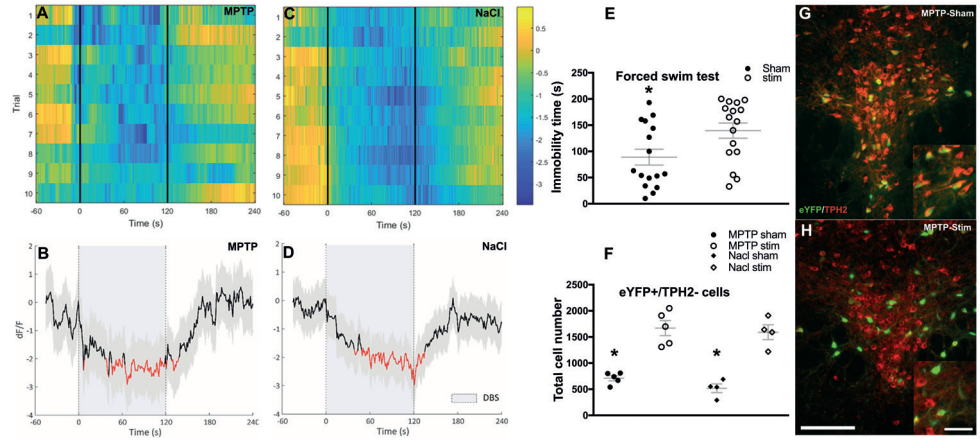

Figure 2. Effect of STN-DBS on serotonergic system. The effect of STN-DBS on activity of 5-HT neurons in the DRN measured with genetically coded calcium sensor GCaMP6s (fiber photometry). A and C) examples of heat-maps of the change in fluorescence (dF/F) before, during (indicated by gray area), and after DBS in MPTP and NaCl-treated mice, respectively. Each row plots one DBS session (total of 10 trials). Color scale at the right indicates dF/F (yellow = high and dark blue = low dF/F). B and D) the bottom plots show the cumulative changes in fluorescence averaged over the ten trials in MPTP- (n=14) and NaCl-treated mice (n=17). The thick black line indicates mean, shaded areas indicate SEM, and red segments indicate statistically significant decrease from baseline ($p<0.05$; permutation test). E) STN-DBS induced depressive like-behavior in forced swim test, shown by an increased immobility time in stimulated animals. F) The graph shows that intermittent STN-DBS significantly reduced TPH2 expression in transfected (eYFP expressing) cells in both MPTP- and NaCl-treated mice. G-H) representative photomicrographs of coronal brain sections containing the DRN display eYFP expressing cells (green) that were double labelled with antibody raised against TPH2 (red; scale bar=150µm). Insets in G-H show higher magnification of eYFP cells that with and without TPH-2 labeling (Scale bar=50µm). Data are presented mean +/- SEM; significant difference ($P<0.05$) is indicated by a "*". Subthalamic nucleus, STN, dorsal raphe nucleus, DRN, enhanced yellow fluorescent protein, eYFP; Tryptophan hydroxylase-2, TPH2; methyl-4phenyl-1,2,3,6-tetrahydropyridine, MPTP; deep brain stimulation, DBS.

FST test was discontinued due to the risk of drowning. As a result, comparing four groups with an ANOVA test was not possible due to the low sample size. Instead, the data of stimulated animals (NaCl-stim and MPTP-stim) were pooled compared to the sham (NaCl-sham and MPTP-sham) animals (stim: 139.56 ± 14.39 vs sham: 88.93 ± 15.13; independent samples T-test, $p < 0.05$; Fig. 2E). STN-DBS induced behavioral despair in the FST, which was apparent by increased immobility time in comparison to non-stimulated mice. This depressive-like behavior after STN-DBS was observed in both MPTP and NaCl-treated mice.

After we established that STN-DBS induced depressive-like behavior and decreased calcium signaling in the DRN, we subsequently evaluated the phenotype of genetically targeted DRN 5-HT neurons. Stereological cell counts of double-labelled enhanced yellow fluorescent protein (eYFP)/ tryptophan hydroxylase-2 (TPH2) expressing neurons in the DRN showed a significant increase of eYFP positive /TPH2 negative neurons in STN-DBS treated mice in comparison to sham stimulated animals [MPTP and NaCl-stim: 1670 ± 144 and 1590 ± 141, vs MPTP and NaCl-sham: 712 ± 50 and 518 ± 83, respectively; Two-way ANOVA; group effect: $F(3,14) = 27.60$, $p < 0.001$; disease*group effect: $F(1,14) = 1.48$, $p = 0.24$; and stim*group effect: $F(1,14) = 81.08$, $p < 0.001$; Fig.2 F-H]. This inhibitory effect of intermittent STN-DBS on TPH2 expression was found to be independent of the integrity of the nigrostriatal dopamine pathway as this observation was present in both MPTP and NaCl-treated mice when tested by Bonferroni post-hoc pairwise comparison of the means [MPTP-sham vs MPTP-stim: $p < 0.001$; NaCl-sham vs NaCl-stim: $p < 0.001$; MPTP-sham vs NaCl-sham: $p = 1.00$ and MPTP-stim vs NaCl-stim: $p = 1.00$; Fig. 2F]. Moreover, neither STN-DBS nor MPTP administration altered neuronal c-Fos expression in the DRN, and no significant changes between groups were found [Two-way ANOVA; group effect: $F(3,18) = 0.31$, $p = 0.81$; Fig. S3]. Finally, quantification of TPH2 and eYFP expressing cells in the DRN did not reveal any significant difference between groups [Two-way ANOVA; group effects: ($F(3,14) = 0.40$, $p = 0.76$; and $F(3,14) = 1.73$, $p = 0.21$, respectively; Fig. S4).

3. DISCUSSION

In this study we investigated the neuroplastic effects of DBS on neurotransmitter phenotype. Recently, neurotransmitter respecification in the adult brain was described in which external cues induced neurotransmitter phenotype switching, neurotransmitter induction or elimination with concurrent behavioral alterations [16-18]. We hypothesized that this phenomenon might play a role in DBS. This may be particularly relevant for STN-DBS as a widely accepted neurosurgical treatment in medically refractory PD with stimulation-dependent motor and non-motor behavioral changes [5-8]. Patients may experience depressive symptoms after surgery, which by itself is a risk factor for post-operative suicide [9, 10]. Understanding the neuronal mechanisms of these behavioral changes is relevant for the more than 208,000 patients that are already treated by DBS worldwide [20].

We used the ePET-Cre mouse line, which enables specific assessment of 5-HT neurons in the DRN synthesizing TPH [19]. MPTP administration in these mice resulted in a significant loss (approximately 60%; Fig. 1E) of SNc dopamine neurons and displayed gait impairments that were alleviated by STN-DBS, overall mimicking dopaminergic degeneration and beneficial motor effects of stimulation in PD patients (Fig. 1A-D).

In addition, STN-DBS elicited behavioral despair in MPTP mice, which is considered as reflecting depressive-like behavior (Fig. 2E). This behavioral change by STN-DBS was independent of the integrity of the nigrostriatal pathway and motor function as NaCl- treated mice showed similar behavioral output (Fig. S2). This observation was also reported by our previous studies [12, 21]. Pretreatment with the selective-serotonin reuptake inhibitor citalopram before STN-DBS was effective in preventing behavioral despair [12]. This pinpointed towards a 5-HT-dependent mechanism and triggered experiments investigating downstream effects of STN-DBS to the brainstem 5-HT system, with the DRN as the major source of 5-HT innervation to the forebrain [11].

Using fiber photometric measurements of calcium signaling, we demonstrated in this study that intermittent STN-DBS decreased calcium signaling and caused neuronal inhibition within the DRN (Fig. 2A-D). This is in line with acute STN-DBS electrophysiological experiments where stimulation decreased 5-HT neuronal firing rate by 40-50 in extra-cellular single cell recordings [12, 22]. Subsequent in vivo microdialysis experiments also found decreased 5-HT release in terminal forebrain regions as expected [13, 23]. Previous studies have focused on the underlying neuronal circuit. Since STN projecting neurons to the DRN are lacking it has been postulated that inhibition of 5-HT neurotransmission is mediated by a multi-synaptic neuronal network. The lateral habenula may contribute to this network as a well-defined major inhibitory input structure to the DRN and has been attributed a critical role in 5-HT feedback mechanisms [21, 22]. Although STN receives 5-HT inputs from the DRN, there is no evidence regarding direct effect of STN-DBS on 5-HT cells via these inputs. Electrophysiological studies have demonstrated that STN-DBS did not induce antidromic or short-latency (< 10 ms) orthodromic responses in peristimulus time histograms recorded from the DRN [12]. We also found that STN-DBS increased neuronal activity with c-Fos expression in the lateral wings of the DRN, which receive major input from various forebrain regions, including the lateral habenula [21]. However, other mechanisms such as 5-HT receptor mediated inhibition or changes in DRN microcircuitry cannot be completely ruled out and may contribute to our observations. Acute STN-DBS has been shown to alter neuronal firing rates of habenular neurons projecting to the DRN [22]. It remains undetermined how STN-DBS influences 5-HT neurotransmission and homeostasis. It has been shown, however, that some cells regain the ability to fire intrinsic spikes of action potential in the presence of continuous stimulation [24], whereas other neurons remain inhibited after cessation of stimulation [22]. These altered activities most likely influence stringent 5-HT feedback mechanisms and may trigger neuroplasticity within the network.

Our earlier study indicated that DBS of the anterior nucleus of the thalamus increased the number of dopaminergic neurons in the ventral tegmental area [25]. This might have been indicative for DBS induced neurotransmitter respecification. In the current study eYFP positive neurons in the DRN should typically express TPH2 in the vast majority (> 90%) [19]. Interestingly, we found STN-DBS to reduce the number of double-labelled eYFP/TPH2 positive neurons quantified by stereological methods (Fig. 2F-H). Although ePET-Cre genetically targets DRN 5-HT neurons specifically, it should be kept in mind that it represents a part of the total 5-HT population [19]. Moreover, 5-HT cells only around the infusion site were transfected in this study. Stereological quantification of eYFP and TPH2 expressing cells in the DRN revealed no significant difference between groups (Fig. S4). In addition, c-Fos expression in the DRN was not altered by STN-DBS, suggesting that overall neuronal activity after intermittent stimulation remained stable (Fig. S3).

Activity dependent intracellular calcium transients play a key role in neurotransmitter respecification by regulating the phosphorylation of transcription factors that are critical in defining the neurotransmitter phenotype of cells [17, 26, 27]. However, how calcium transients alter neurotransmitter respecification, seems to differ across transmitter systems and species. For instance, elevated activity of dopaminergic neurons in the paraventricular nucleus of the hypothalamus in the adult rats was shown to be required for the loss of dopamine expression after long-day photoperiod exposure [18]. Whereas, decreases in calcium spiking by exposure of Xenopus laevis to dark lead to loss of dopamine expression in the hypothalamus [28]. Seemingly, altered calcium transients could lead to opposite effects in serotonergic neurons. Suppression of activity in the Xenopus laevis hindbrain generated an increase in the number of neurons expressing TPH in the raphe nucleus. Whereas, enhancement of activity led to the opposite result [27]. In our study, a decrease in the number of TPH2 expressing neurons was associated with reduced Ca2 + transients. This contrasts with respecification of dopaminergic cells in rats [18], in which an increase in Ca2 + activity correlated with the loss of dopaminergic cell phenotype. It should be noted that the extent to which the 5-HT cells were transfected with the GCaMP6s virus was not quantified in this study. Therefore, it is plausible that Ca2 + transients were not measured in all serotonergic cells.

Initial theories suggested that DBS at stimulation settings commonly used in clinical practice decreases spontaneous firing of neuronal populations and drives axonal projections near the electrode also known as "firing rate model" which was based on real-time and local effects of DBS [14]. Nowadays, ample evidence show that changes in neuronal activity per se are unsustainable states, and neurons regain their intrinsic activity overtime [24] and electrical stimulation results in prolonged plasticity-associated effects even when stimulation is turned off [29]. Similarly, transient changes in Ca2 + activity could lead to transmitter respecification, which can have network and biochemical effects that transcend the time of stimulation. Altogether these behavioral, photometric and immunohistochemical data pinpoint to a key role for stimulus-derived loss of 5-HT cell phenotype. We argue that this loss of 5-HT phenotype plays a key role in unwanted depressive symptoms following STN-DBS. The fade of 5-HT phenotype could also be the mechanism whereby STN-DBS reduces treatment-resistant tardive dyskinesia. The

5-HT system has been implicated in the symptoms of dyskinesia. Extensive 5-HT innervation of the basal ganglia modulates dopamine neurotransmission [30, 31]. The lower incidence of dyskinesia is associated with 5-HT2 receptor antagonism [32, 33]. Moreover, symptoms of dyskinesia can be exacerbated by concomitant treatment with selective serotonin reuptake inhibitors [34-36]. Based on our observation that STN-DBS suppresses 5-HT cell phenotype, one may conclude that reduction in basal ganglia 5-HT function is a key component of the DBS therapeutic mechanism in dyskinesia.

In conclusion, understanding neuroplastic effects is critical to our understanding of network modulation by DBS and symptom reduction or side effects. This study reveals evidence that STN-DBS induces changes in calcium signaling in the midbrain raphe nuclei 5-HT system and results in neurotransmitter respecification, which may play a role in psychiatric side effects in PD. The loss of 5-HT cell phenotype could also be the mechanism whereby STN-DBS reduces treatment-resistant tardive dyskinesia.

4. METHODS

4.1 Animals

Experiments were performed on 56 male transgenic ePET-Cre mice (JAX stock; #012,712). Animals were socially housed under constant temperature, humidity and reversed dark/light cycle (12 h each) with free access to food and water. All animal procedures were performed in accordance with "Animal Research: Reporting of *in vivo* Experiments (ARRIVE)" guidelines. Animal procedures were reviewed and approved by the Institutional Animal Care Committee of Maastricht University in accordance with the Central Authority for Scientific Procedures on Animals (CCD; protocol # AVD107002016543).

4.2 Induction of Parkinson's disease model and stereotactic surgery

Mice were randomly assigned into one of the following four groups: NaCl-sham, NaCl- STN-DBS, MPTP-sham or MPTP-STN-DBS. Mice were injected with MPTP (30 mg/Kg i.p) or normal NaCl (0.9% i.p.) for five consecutive days, two weeks prior to stereotactic surgery. Stereotactic surgery [37] was performed under isoflurane inhalation anesthesia (Abbott Laboratories; induction 4%, maintenance 1.5-2%) after analgesic pretreatment (buprenorphine, 0.1 mg/Kg s.c). The mouse head was positioned and fixated in a stereotaxic frame (Stoelting). A body temperature of 37 °C was maintained with a thermo-regulator pad. After local anesthesia (lidocaine 1% s.c.) the skull was exposed and burr holes were made for implantation of bilateral STN electrodes (coordinates from bregma based on mouse brain atlas: AP -2.00 mm, ML ±1.50 mm, DV -4.55 mm [38]) and a fiber photometry probe (400 µm; 0.48NA Patchcord) was implanted in the DRN (coordinates from bregma based on mouse brain atlas: AP -4.5, ML -0.25, DV-2.9 at a 32º angle from the left).

4.3 Viral transfection

During the same surgery and before implantation took place, two viral vectors were injected into the DRN. A Cre-dependent adeno-associated virus encoding for eYFP (AAV5.EF1a.DIO.eYFP.WPRE.hGH; Penn Vector Core, USA) was injected (1.0 µl, at a rate of 0.1 µl/min) into the DRN. In addition, an AAV vector ensuring targeted genetic encoding of the fluorescent Ca2+ indicator GCaMP6s (AAV5.Syn.Flex. GCaMP6s.WPRE.SV40; Addgene, USA) was also injected into the same coordinates (500 nL; Nanoject I; Drummond Scientific).

4.4 Deep brain stimulation

After 2 weeks of recovery, STN-DBS was performed for 10 weeks with 20 min stimulation sessions (5 times a week) with monophasic high frequency stimulation at 130 Hz, a pulse width of 60 µs and a current intensity of 80 µA. Sham stimulated animals were connected but stimulation was omitted. The DBS construct consisted of two bipolar gold-coated concentric electrodes, with interelectrode distance of 3.0 mm and 5.5 mm length each. The outer stainless steel and inner platinum-iridium parts function as the positive and negative poles, respectively. The outer diameter of the concentric needle is 300 µm (including the insulation), the electrode surface is 0.021 mm2, and the distance between anode and cathode is 50 µm [37]. The surface of the electrode is 0.021 mm^2, so the chosen parameters resulted in a charge density of 22.9 $\mu C/cm^2$, which is well below the limit of 30 $\mu C/cm^2$ based on the Shannon model of neuronal damage [39].

4.5 Fiber photometry

Ca2 + transients of DRN neurons were measured in MPTP and saline-treated mice using an established fiber photometry technique[37]. This method enabled measuring the bulk Ca2 + -dependent fluorescence of GCaMP6 during STN-DBS. A two-wavelength GCaMP fiber photometry system (Doric Lenses Inc., Quebec, Canada) was utilized for calcium signal recording. GCaMP and Ca2+-independent fluorescent signals were alternatingly excited by a 470 nm LED and a 405 nm LED (isosbestic reference signal), respectively. GCaMP6s fluorescence emissions were captured with a Newport 2151 Femtowatt Photoreceiver Module and the signals relayed into a Field Programmable Gate Array (FPGA)-based data acquisition unit which integrates with the Doric Neuroscience Studio software. During the photometry experiment, mice could move freely in their home cage. STN-DBS was applied intermittently (2 min on – 3 min off) for ten trials (5 min per trial) during which photometry measurements were performed in the DBS on/off phases. We extracted, processed and analyzed the calcium transients with a custom MATLAB (Mathworks) script. The first 2.5 minutes of the data during the habituation period were discarded to remove the initial fast bleaching of the fluorescent signal. Next, the original sampling rate of a 100 Hz was downsampled to 1 Hz and low-pass filtered. A two-term exponential model was fitted and subtracted from the decimated data to account for slow bleaching artifacts. Then, a single baseline fluorescence value (F0) was calculated by averaging the fluorescent signals during the 60-sec time period pre-DBS. Subsequently, the normalized change in fluorescence (dF/F) was calculated as F – F0/F0. Data are presented as an average plot with SEM. A permutation test was used to analyze the statistical significance of the DBS-related fluorescent change [40]. To compare the values of dF/F at each

time point with the DBS-related fluorescent change, 10,000 permutations were used. An α-level of ≤0.05 was considered significant.

4.6 Behavioral assessment

4.6.1 Gait analysis

MPTP and STN-DBS related motor effects were assessed by a computerized gait analysis setup (CatWalkXT; Noldus). Mice ran through an enclosed corridor with a hard glass plated floor. Footprints were recorded by a high-speed camera from which gait-related movement parameters were analyzed, including average speed, step cycle, terminal dual stance and stance. Five consecutive uninterrupted straight runs of each mouse were used for statistical analysis [41].

4.6.2 Forced Swim Test

The forced swim test (FST) was used to evaluate despair behavior based on a published protocol [42]. Mice were placed in an inescapable plastic cylindrical container (height 40 cm x diameter 19 cm) filled with a 23-25 °C water (30 cm deep). The duration of immobility was recorded during a trial of 6 minutes. Immobility was defined as the time of not moving or with slight movements to keep the nose above the water surface.

4.7 Tissue processing and immunohistochemistry

At the end of the experiments, mice were deeply anaesthetized with pentobarbital and transcardially perfused with tyrode buffer, followed by ice-cold 4% paraformaldehyde fixative in 0.1 M phosphate buffer. The brains were extracted, fixed in 4% paraformaldehyde overnight and submerged in 20% sucrose for 24 h at 5 °C. The brains were sectioned in coronal slices (thickness: 22 µm) on a cryostat and stored at -80 °C. A standard hematoxylin-eosin staining was performed to assess the electrode tip location (Fig. S1A). Animals with misplaced electrodes were excluded from behavioral and histological analysis.

4.7.1 Tyrosine hydroxylase immunohistochemistry

MPTP-induced dopamine depletion was evaluated by tyrosine hydroxylase (TH) immunohistochemistry. Sections containing the SNc were incubated overnight with primary antibody raised against TH (rabbit polyclonal anti-TH antibody; Santa Cruz Biotechnology Inc; 1:1000). On the next day, sections were incubated with a secondary antibody (donkey anti-rabbit alexa 647, Jackson Immunoresearch Laboratories; 1:400) for one hour. Thereafter, the sections were mounted and coverslipped (Immu-Mount, USA). Photographs of two anatomical bregma levels (coordinated based on mouse brain atlas AP - 2.92 and - 3.16 [38]) were taken with an Olympus DP70 digital camera connected to an Olympus BX50 microscope. A semi-quantitative TH cell count was performed using ImageJ software (National Institutes of Health, USA).

4.7.2 c-Fos immunohistochemistry

To assess overall neuronal activity of the DRN immunohistochemical expression of c-Fos was evaluated. The DRN sections were incubated overnight with a primary anti-c-Fos antibody (rabbit polyclonal anti-c-Fos; Abcam; 1:1000). This was followed by incubation for one hour with a secondary antibody (donkey anti-rabbit alexa 594, Jackson immunoresearch Laboratory; 1:200). Eight slices were selected from bregma - 4.16 to - 4.96 and photographed with an Olympus BX51 fluorescence microscope (Olympus, Germany) connected to an Olympus Camera DP72 (Olympus, Germany). All clear c-Fos expressing neurons were counted (FiJi v2.0.0, National Institutes of Health; Maryland).

4.7.3 Tryptophan hydroxylase-2 immunohistochemistry

To assess whether STN-DBS influenced 5-HT synthesis of eYFP expressing 5-HT DRN neurons, tissue was processed for TPH2 immunohistochemistry, which is the rate-limiting enzyme in 5-HT synthesis. DRN sections were incubated overnight with a primary anti-TPH2 antibody (goat polyclonal anti-TPH2; Abcam; 1:2000). This was followed by incubation with a secondary antibody (donkey anti-goat alexa 647, Jackson Immunoresearch Laboratories; 1:200) for two hours. Stereological analysis of double-labelled eYFP/TPH2 neurons was performed (Stereo Investigator, Microbrightfield Bioscience, Williston, VT, USA) in seven DRN sections per mouse using an immunofluorescence spinning disk confocal microscope (DSU, Olympus BX51, Japan) connected to a digital ultra-high sensitivity CCD camera (C9100-02, Hamamatsu Photonics, Japan). Stereological cell counting was performed using the optical fractionator probe and total double-labelled cell number was estimated using a validated stereological method [43, 44].

4.8 Data analysis

Statistical analysis was performed using SPSS 26.0 software (SPSS Inc., Chicago, USA). Behavioral and immunohistochemical data were analyzed using the two-way ANOVA. Bonferroni post-hoc pairwise comparison was conducted, if (and only if) the global ANOVA test result was significant. To compare the two-groups' data, we used an independent T-test. Data are presented as mean values and standard error of means (± SEM). All data were normally distributed, and statistical significance was defined by a p-value < 0.05. Photometry data was processed and analyzed with custom Matlab (MathWorks) scripts. A permutation test was performed to statistically evaluate calcium transients [40].

5. SUPPLEMENTARY FIGURES

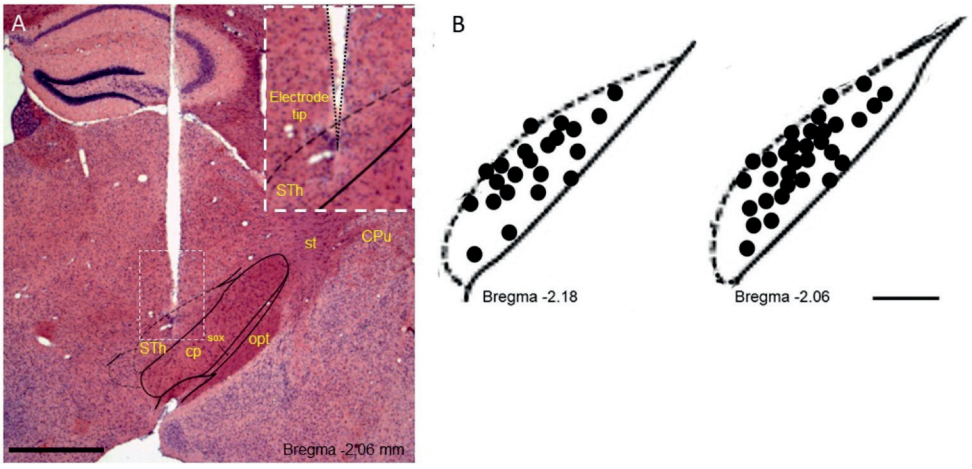

Figure S1. A) Representative low-power photomicrograph of coronal brain section stained with Hematoxylin and Eosin (H&E) shows the deep brain stimulation electrode tip at the subthalamic nucleus (STN, scale bar=500µm). B) Illustrative coronal images showing 54 electrode tip locations in or close to the STN, as verified by post hoc histology (Scale bar=200µm). STh, subthalamic nucleus; cp, cerebral peduncle; CPu, caudate putamen; sox, supraoptic decussation; opt, optic nerve; st, stria terminalis.

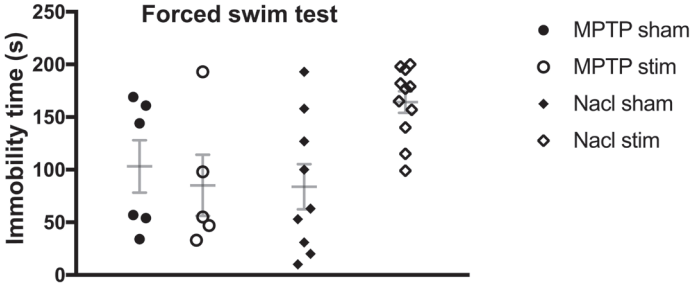

Figure S2. Effect of STN-DBS on depressive-like behavior.

Graph shows the quantification of immobility time of mice in the forced swim test. Statistical analysis revealed no significant difference between groups (F (3,27)=4.56, p=0.11,two-way ANOVA). Data are presented as mean +/- SEM.

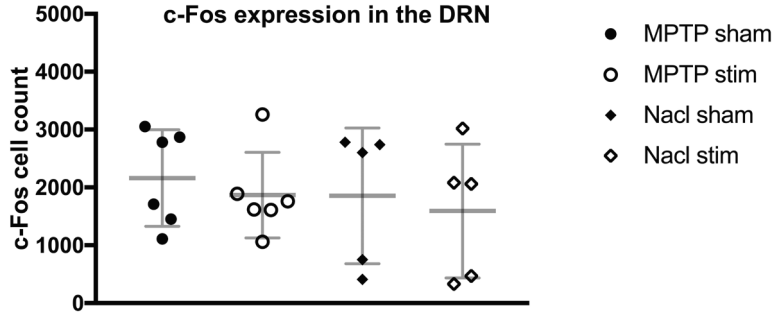

Figure S3. Effect of STN-DBS on the overall neuronal activity in the DRN.

Graph shows the quantification of c-Fos positive cells in the dorsal raphe nucleus (DRN) of mice. Statistical analysis revealed no significant difference between groups (F(3,18)=0.31, p=0.81,two-way ANOVA; F (1,18) =0.001 p=0.97). Comparison between stimulated and non-stimulated mice did not reveal a significant difference (independent samples T-test p=0.48).

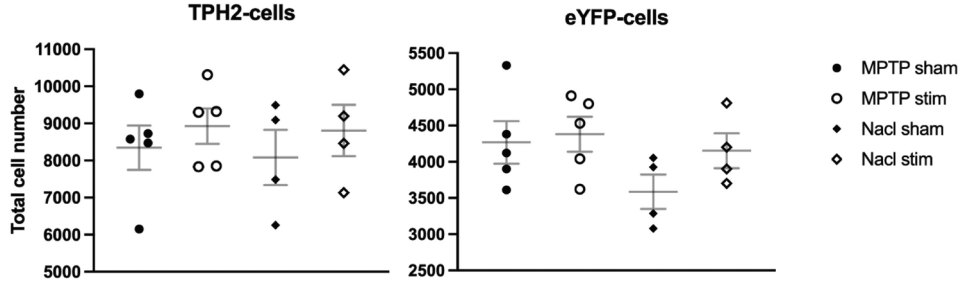

Figure S4. Cell count of TPH2 and eYFP containing cells in the DRN.

Graphs A and B represent the stereological quantification of TPH2 and eYFP containing cells in the DRN of mice, respectively. Statistical analysis did not show any significant difference in number of TPH2 [MPTP-sham: 8345±597 vs MPTP-stim: 8925±477; NaCl-sham: 8083±747; and NaCl-stim: 8810±694, F (3,14) =0.40 p=0.76], and eYFP containing cells [MPTP-sham: 4268±242 vs MPTP-stim: 4380±294; NaCl-sham: 3586±242; and NaCl-stim: 4153±238, F(3,14)= 1.73, p=0.21, Two-way ANOVA, respectively] between groups. Data are presented as mean +/- SEM.

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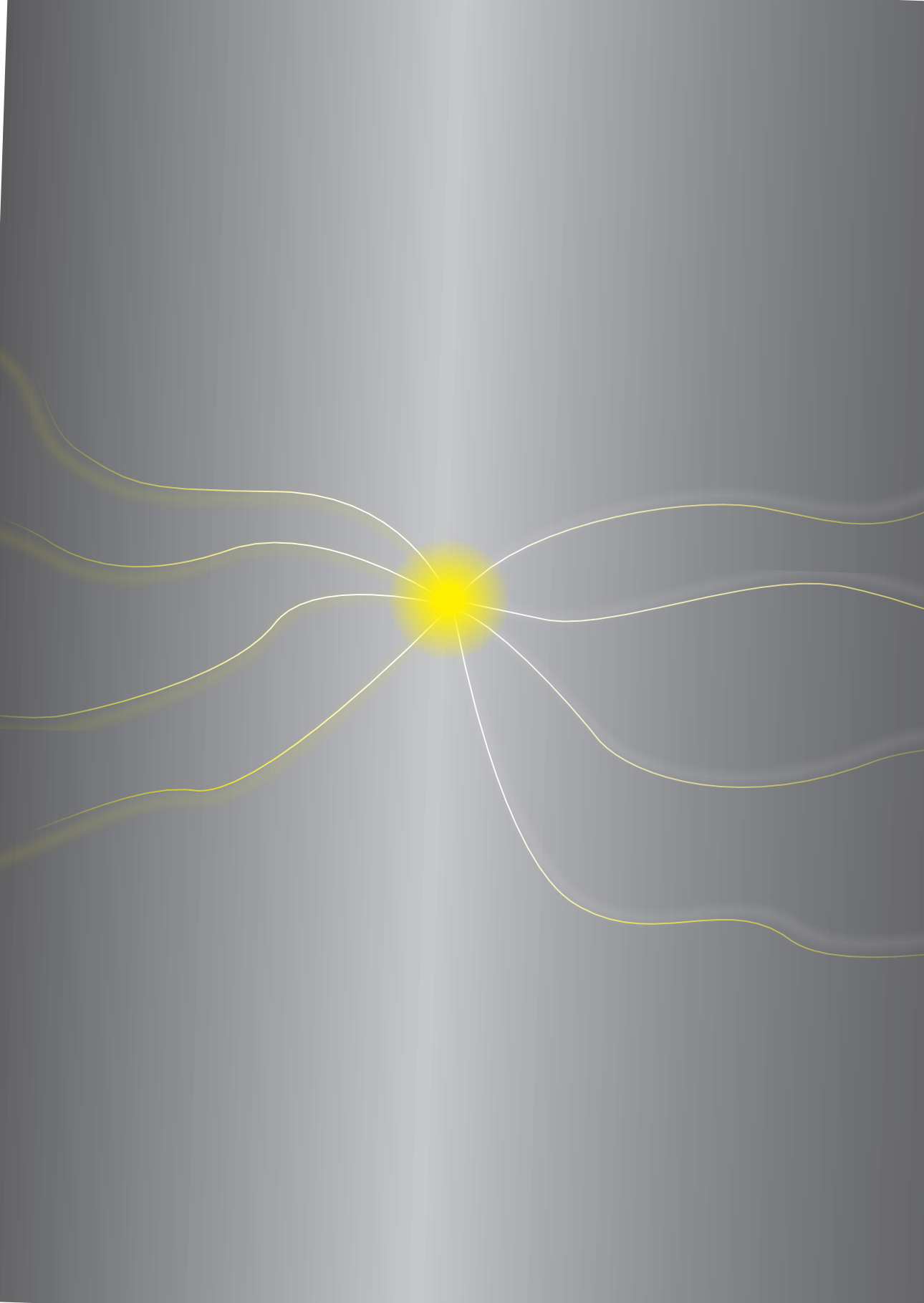

CHAPTER 4

Modulation of the globus pallidus externa using DREADD reveals no effects on the neuronal activity of the dorsal raphe nucleus in mice treated with deep brain stimulation of the subthalamic nucleus

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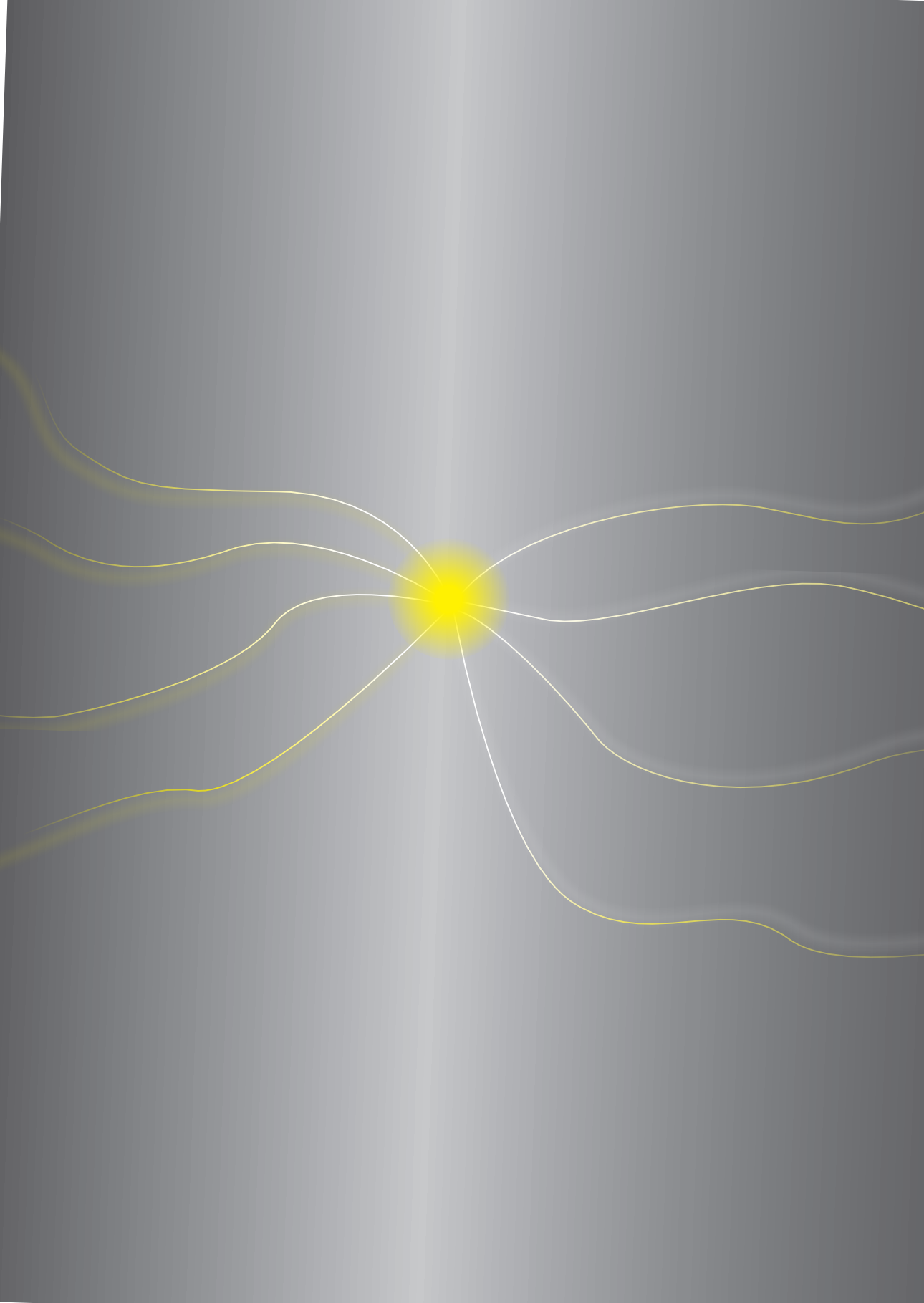

CHAPTER 5

Gait improvement by high-frequency stimulation of the subthalamic nucleus in Parkinsonian mice is not associated with changes of the cholinergic system in the pedunculopontine nucleus

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ABSTRACT

Deep brain stimulation (DBS) of the subthalamic nucleus (STN) is standard care for severe motor symptoms of Parkinson's disease (PD). However, a challenge of DBS remains improving gait. Gait has been associated with the cholinergic system in the pedunculopontine nucleus (PPN). In this study, we investigated the effects of long-term intermittent bilateral STN-DBS on PPN cholinergic neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Parkinsonian mouse model. Motor behavior, previously assessed by the automated Catwalk gait analysis, demonstrated a parkinsonian-like motor phenotype with static and dynamic gait impairments, which were reversed by STN-DBS. In this study, a subset of brains was further immunohistochemically processed for choline acetyltransferase (ChAT) and the neuronal activation marker c-Fos. MPTP treatment resulted in a significant reduction of PPN ChAT expressing neurons compared to saline treatment. STN-DBS did not alter the number of ChAT expressing neurons, nor the number of double-labelled PPN neurons for ChAT and c-Fos. Although STN-DBS improved gait in our model this was not associated with an altered expression or activation of PPN acetylcholine neurons. Motor and gait effects of STN-DBS are therefore less likely to be mediated by the STN-PPN connection and PPN cholinergic system.

Keywords: Deep brain stimulation; Parkinson's disease; acetylcholine; pedunculopontine nucleus, gait

1. INTRODUCTION

Deep brain stimulation (DBS) of the subthalamic nucleus (STN) is considered standard neurosurgical care for medically refractory motor symptoms of Parkinson's disease (PD) [1]. A fast-growing number of PD patients are treated with STN-DBS [2]. Long-term follow-up studies have shown lasting motor benefits [3], [4]. However, PD-related axial dysfunction remains a challenge for STN-DBS, with patients increasingly experiencing gait disturbances and postural instability many years after surgery [3], [4]. There is evidence that STN-DBS influences PD-related aspects of gait with an improvement of body position, standing position and automated gait, but also with persisting problems of gait initiation and posture [5].

The control of gait and balance has been associated with the acetylcholine rich pedunculopontine nucleus (PPN) [6], [7]. In PD, falling and freezing of gait were shown to be acetylcholine dependent in pharmacological and PET studies [8], [9]. Post-mortem studies have found a significant loss of PPN cholinergic neurons among PD patients who suffered from postural instability [10]–[13]. Moreover, PPN lesions in animal models induced gait dysfunction and loss of postural control [14]–[16].

Little is known about the influence of STN-DBS on PPN cholinergic neurons. Anatomical tracing studies in animals have described a sparse STN efferent projection to terminate in the PPN [17]. Dopaminergic depletion of the nigrostriatal pathway by 6-hydroxydopamine injection has resulted in hyperactivity of PPN cholinergic neurons, which was reversed by STN lesions [18]. As far as we know, there is no study which investigated the neuronal changes of PPN cholinergic neurons associated with STN-DBS. Therefore, in this study, methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated parkinsonian mice that underwent long-term intermittent STN-DBS which were analyzed in a previous study concerning motor behavior [19] were further investigated concerning the effects on the cholinergic neurons in the PPN and fibers in the STN by immunohistochemistry.

2. METHODS

Detailed experimental setup and results of nigrostriatal degeneration by induction of the Parkinsonian MPTP model, electrode implantation for deep brain stimulation and behavioral testing of animals are described in Alosaimi et al. [19]. Experiments including MPTP-treatment, stereotactic implantation of electrodes, deep brain stimulation and behavioral testing were not repeated for this work. A subset of animal tissue used in the work of Alosaimi et al. was used for further analysis in this study [19].

2.1 Animals

In this previous study [19], 56 male transgenic ePET-Cre mice (JAX stock #012712; Jackson Laboratory; USA) were housed at 20-24°C with a reversed 12h light/dark cycle, with food and water ad libitum and

social housing in accordance with guidelines of the Federation Laboratory Animal Science Associations (FELASA). Procedures were approved by the Animal Ethical Committee of Maastricht University.

2.2 Induction of Parkinsonian model

Mice were randomly allocated to one of the following groups: saline/sham, saline/stim, MPTP/sham or MPTP/stim. MPTP was administered with a concentration of 30 mg/Kg or saline (0.9% NaCl) intraperitoneally at 24h intervals over five consecutive days. Stereotactic surgery was performed two weeks after the last MPTP injection [19].

2.3 Stereotactic electrode implantation and deep brain stimulation

Stereotactic surgery was performed after analgesic injection of buprenorphine (0.1 mg/Kg s.c) and inhalation anesthesia (isoflurane, 4% induction and 1.5-3% maintenance, Abbot Laboratories). Mice were placed on a thermo-regulator pad to maintain the body temperature at 37°C. The head of the animal was mounted in a rodent stereotactic frame (Stoelting, Ireland). Lidocaine 1% was injected subcutaneously at the incision site. After incision and skull exposure, burr holes were made for bilateral STN-DBS electrode implantation (AP - 2.00 mm, ML ± 1.50 mm, DV - 4.55 mm) [20]. The electrode construction was fixated with composite. Stimulation was applied two weeks after surgery. Mice received long-term intermittent STN-DBS for 20 min per day for a period of ten weeks (three times a week). The electrodes were connected to an external stimulator and monophasic stimulation with a frequency of 130 Hz, pulse width of 60 µs and current intensity of 80µA was applied. Sham animals were connected to the stimulator, but stimulation was omitted. The last stimulation session was conducted two hours prior to sacrificing and perfusion [19].

2.4 Motor behavior

Motor behavior was assessed by the CatwalkXT (Noldus 7.1, Wageningen, the Netherlands). This enabled a computerized analysis of gait parameters. Mice were trained to run over an enclosed straight corridor with a hard glass plated floor. Footprints were detected with a high-speed colored camera and CatWalk XT software analyzed locomotion and gait parameters. A successful trial consisted of at least five uninterrupted runs with no more than 30% of speed run variation. PD relevant gait parameters that were analyzed included average speed, stance, terminal dual stance, and step cycle [19].

2.5 Tissue processing and immunohistochemistry

After the behavioral experiments, mice were once more stimulated, and 2 h afterward deeply anaesthetized with pentobarbital and transcardially perfused with tyrode buffer. This was followed by ice-cold 4 % paraformaldehyde fixative in 0.1 M phosphate buffer. Brains were fixed in 4 % paraformaldehyde overnight and then submerged in 20 % sucrose for 24 h prior to cryoprotective freezing. Coronal brain sections (20 µm) were cut on a cryostat. A standard hematoxylin and eosin staining was used to confirm the electrode positioning in the STN. Two animals with a misplaced electrode in the zona incerta were excluded from both behavioral and histological data analysis.

To identify cholinergic neurons in the PPN and fibers in the STN, a subset of brain sections (n=22 animals) containing these regions was processed for choline acetyltransferase (ChAT), the rate-limiting enzyme for acetylcholine synthesis. PPN-containing sections were incubated with a primary antibody raised against ChAT (Goat polyclonal anti-ChAT, 1:200; Sigma-Aldrich, AB144P) overnight. On the second day, a fluorescent secondary antibody (donkey anti-goat Alexa 488, 1:200; Jackson immunoresearch Laboratory) was applied.

To evaluate the neuronal activity of PPN ChAT neurons by STN-DBS, a subset of sections (n = 22 animals) was immunohistochemically processed for ChAT as well as the protooncogene c-Fos, which is a marker for neuronal activity. Sections with the PPN were incubated overnight with a primary antibody against ChAT (Goat polyclonal anti-ChAT, 1:200; Sigma-Aldrich, AB144P) and simultaneously with a primary antibody raised against c-Fos (Rabbit polyclonal anti-c-Fos; 1:1000; Abcam, ab190289) overnight. On the second day, incubation with fluorescent secondary antibodies (donkey anti-goat Alexa 488, 1:200 and donkey anti-rabbit Alexa 594; 1:200; Jackson Immunoresearch Laboratory) followed for one hour.

2.6 PPN cell counting and STN ChAT expression measurement

Semi-quantitative cell counting was performed on eight consecutive slices per animal reaching from bregma – 4.16 to – 4.96 to estimate the total number of cells in the PPN. Images were taken with an Olympus BX51 fluorescence microscope (Olympus, Hamburg, Germany) connected to an Olympus Camera DP72 (Olympus, Hamburg, Germany) at 10x magnification using the software CellF (Olympus, Hamburg, Germany). PPN was manually contoured by the freehand selection tool of the software FIJI (FiJi v2.0.0, National Institutes of Health (NIH), Bethesda, Maryland). Within the selected area, all cells with distinct shape and clear fluorescence were included in the analysis using the multipoint tool of FIJI (FiJi v2.0.0, National Institutes of Health, (NIH), Bethesda, Maryland), leaving out caps and broken cells. The investigator was blinded for the treatment group. As the STN is innervated by the PPN and contains cholinergic fibers, we analyzed the overall ChAT expression in the STN (bregma -1.94 and -2.06). To analyze the ChAT intensity in STN subregions, we measured the mean grey value within a predefined rectangle positioned within the dorsolateral and medial subdivisions of the STN (Fig 1H; FiJi v2.0.0, National Institutes of Health, (NIH), Bethesda, Maryland).

2.7 Data analysis

Statistical analysis was performed with Prism (Version 9.1.0, GraphPad Software LLC). Immunohistochemical data was analyzed using two-way ANOVA and post-hoc analysis. Data are presented with mean values and standard error of means (+/-SEM). A p-value of < 0.05 was regarded as statistically significant.

3. RESULTS

3.1 MPTP model and gait effects of STN-DBS

Details of behavioral effects of STN-DBS in these mice have been described previously [19]. According to Alosaimi et al., MPTP induced gait impairments with increased stance, terminal dual stance, step cycle and average speed [19]. These static and dynamic gait deficits were reversed by STN-DBS in MPTP mice, as stance, terminal dual stance and step cycle decreased, and average speed increased [19]. Stimulation did not alter gait parameters in saline-treated animals [19].

3.2 Influence of MPTP and STN-DBS on ChAT expressing neurons in PPN

MPTP induced a significant reduction in the number of ChAT expressing neurons in the PPN compared to saline-treated animals (-28.1%, $F(1, 18) = 6.60$, $p = 0.01$; 2-Way ANOVA; Fig 1A-C). STN-DBS did not influence the number of ChAT expressing neurons compared to sham DBS in both saline and MPTP-treated mice ($F(1, 18) = 0.67$, $p > 0.05$; 2-Way ANOVA; n = 5-6 animals per group, Fig 1C).

To investigate whether STN-DBS resulted in activation of the remaining PPN ChAT neurons, an immunohistochemical double-labelling for ChAT and c-Fos was performed (Fig 1D-F). There was no change in the number of double-labelled neurons in the PPN after STN-DBS compared to sham DBS in saline or MPTP-treated subjects ($F(1, 18) = 0.05$, $p > 0.05$; 2-Way ANOVA; n = 5-6 per group; Fig 1G).

3.3 Influence of MPTP and STN-DBS on ChAT expression in STN

The STN is innervated by PPN cholinergic neurons. Measuring the overall STN expression of ChAT positive fibers within the STN did not show differences between groups ($F(1, 16) = 3.71$, $p > 0.05$ for MPTP treatment, $F(1,16) = 0.0016$, $p > 0.05$ for stimulation; 2-Way ANOVA; n = 5-6 per group). It is well defined that the STN has distinct anatomical and functional subregions. Therefore, we analyzed the ChAT density of the dorsolateral and medial STN, which represent the STN motor and limbic subregions respectively (Fig 1H). We found a significant decrease in ChAT expression in the dorsolateral STN after MPTP treatment compared to saline-injected controls ($F(1, 16) = 10.30$, $p = 0.005$; 2-Way ANOVA; n = 5-6 per group; Fig 1I). Stimulation did not influence ChAT density in the dorsolateral STN compared to sham DBS. In the medial STN, there was no change in ChAT expression by MPTP or STN-DBS compared to controls ($F(1, 16) = 2.90$, $p > 0.05$ for MPTP, $F(1, 16) = 0.30$, $p > 0.05$ for stimulation; 2-Way ANOVA; n=5-6 per group).

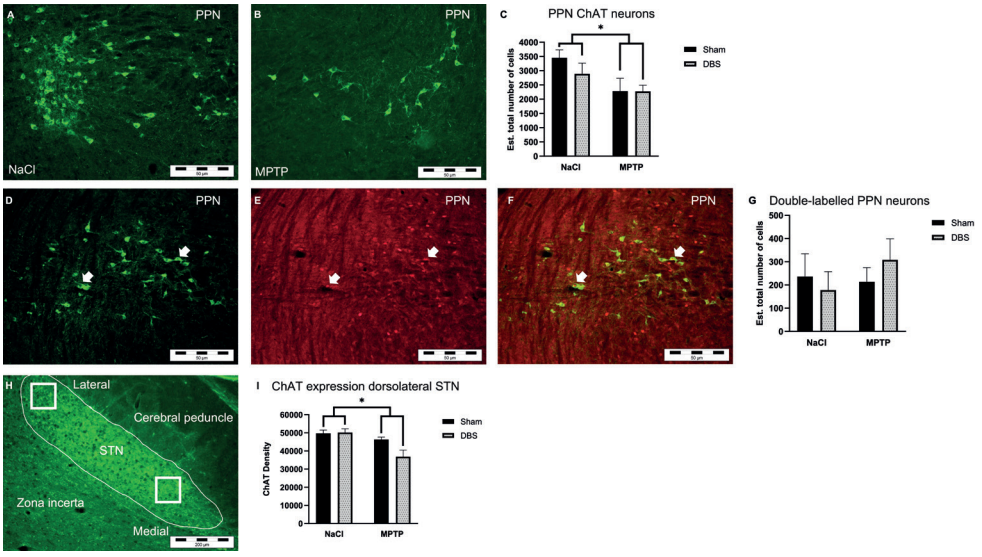

Figure 1. Degeneration of ChAT positive neurons in the PPN in the MPTP mouse model. Representative photomicrographs showing ChAT-positive neurons in saline (A) and MPTP treated animals (B), both underwent sham stimulation. Cumulative data showing a significant decrease in the number of PPN ChAT-positive neurons in MPTP (black) compared to saline (grey). Stimulation did not affect the number of PPN ChAT-positive neurons. (C; *$p < 0.05$; 2-Way ANOVA; n =5-6 animals per group). Representative photomicrographs of ChAT (D), c-Fos (E) and fused image (F) to assess double-labelling of PPN neurons (arrows). There was no change in the number of double-labelled PPN neurons by MPTP toxin and/or STN-DBS (G; $p > 0.05$; 2-Way ANOVA; n =5-6 animals per group). Position of rectangles within the medial and dorsolateral STN where ChAT expression was measured (H). Cumulative data showing significant decreased ChAT expression in the dorsolateral STN in MPTP compared to saline injected animals (I; *$p < 0.05$; 2-Way ANOVA; n = 5-6 animals per group).

4. DISCUSSION

MPTP injections of the animals used for this study resulted in significant neuronal degeneration of the PPN cholinergic system [19]. Cholinergic neurons in the PPN were described to be significantly reduced by 28.1 % in MPTP treated mice compared to saline controls [19]. Neuropathological studies of PD patients reveal a loss of 40–70 % of cholinergic neurons in the lateral PPN [8], [10]–[13], [21]–[24]. As reported in detail in our previous study, MPTP in our mice caused a significant loss of tyrosine hydroxylase expressing neurons (-60 % approximately) in the SNc [19]. We were using slices from the same animals for our current study. We also found a loss of ChAT expression in terminal regions. We observed a loss of ChAT expression in the dorsolateral motor part of the STN, possibly reflecting a loss of ChAT fibers. This parallel degeneration of the cholinergic and dopaminergic systems in PD underlines its widespread neuropathology across multiple neurotransmitter systems. This also highlights the validity of our MPTP mouse model and enables us to study the effects of STN DBS on neurotransmitters in PD.

The loss of PPN cholinergic neurons is associated with the occurrence of gait and balance deficits. In our MPTP mouse model Alosaimi et al. have observed a phenotype with gait impairments in stance, step cycle and speed which was reversed by STN-DBS [19]. Clinical studies describe various effects of STN-DBS on gait and balance in PD patients. UPDRS-scales are reduced and STN-DBS improves gait velocity, distance, postural control and gait balance [5], [25]–[27].

From an anatomical perspective a downward projection from the STN to the brainstem and the PPN has been described across species. Although this projection is sparse, the influence of the STN on PPN cholinergic neurons is profound. Previously, 6-hydroxydopamine injections into the SNc in rats caused hyperactivity of PPN neurons, and this increased neuronal activity was reversed by ibotenic STN lesions [18]. However, our study found no indication that STN-DBS influenced PPN cholinergic neurons. There was no change in the number of immunohistochemically double-labelled PPN neurons for ChAT and the neuronal marker c-Fos. This suggests that STN-DBS does not activate the remaining PPN cholinergic neurons. The improvement of gait parameters by STN-DBS may therefore depend on other STN-related pathways. Recently, a structural connectivity study demonstrated that gait improvements in STN-DBS treated PD patients were related to stimulation of fiber tracts between the STN and motor cortex [28]. Similarly, optogenetic stimulation of the STN has attributed the beneficial motor effects of STN stimulation to the modulation of upstream connections between the STN and frontal cortices [29].

The STN has anatomical and functional distinct subregions. The dorsolateral STN is centrally located in the basal ganglia-thalamo-cortical motor circuit. A dysfunction of this circuit in PD models the onset of several key PD motor symptoms. The STN itself receives cholinergic innervation from the PPN and we found a significant loss of cholinergic fibers in the dorsolateral STN subregion. Interestingly, this loss was not found in the medial STN, which is part of the basal ganglia limbic circuit**.** The specific loss of cholinergic fibers in the motor subregion of the STN may contribute to a dysfunction of the motor circuit and development of motor symptoms and probably is not involved in neuropsychiatric symptoms of PD. Anterograde tracing studies in the squirrel monkey using the markers [^{3}H]-Leucine and the lectin *Phaseolus vulgaris*-leucoagglutinin (PHA-L) injected into the central PPN showed dense ipsilateral innervation of the dorsal surface of the STN, while more peripheral injections of the PPN showed connections to the ventromedial rather than dorsolateral areas of the STN [30]. Other tracing studies described profuse arborization and even distribution of PPN afferents throughout the whole STN [30]–[33]. Biotinylated dextran amin (BDA) injections into ChAT positive regions of the PPN in rats resulted in dense plexus with thin fibers and small varicosities in the STN. Large boutons were only moderately present [33]. The specific regional loss within the STN that we observed may be the result of degeneration of PPN afferents but a direct effect of MPTP in the STN cannot be ruled out. In our study STN-DBS did not alter the density of cholinergic fibers. The gait improvements by STN-DBS in our model, observed by Alosaimi et al. [19], is therefore also not likely to be related to local cholinergic changes within the STN. However, we did not investigate alterations in local cholinergic receptor function and potential alterations induced by STN-DBS.

5. CONCLUSION

Altogether in this study we observed a degeneration of the PPN cholinergic system as well as cholinergic fibers in the STN. In parallel, in our previous study, Alosaimi et al. found MPTP-induced motor deficits with gait impairments [19]. Although STN-DBS was able to improve gait, this was not associated with a direct activation of PPN cholinergic neurons or alterations of local STN cholinergic fibers. STN-DBS associated gait improvements may be mediated by other neuronal pathways, such as upstream connections between the STN and frontal cortices.

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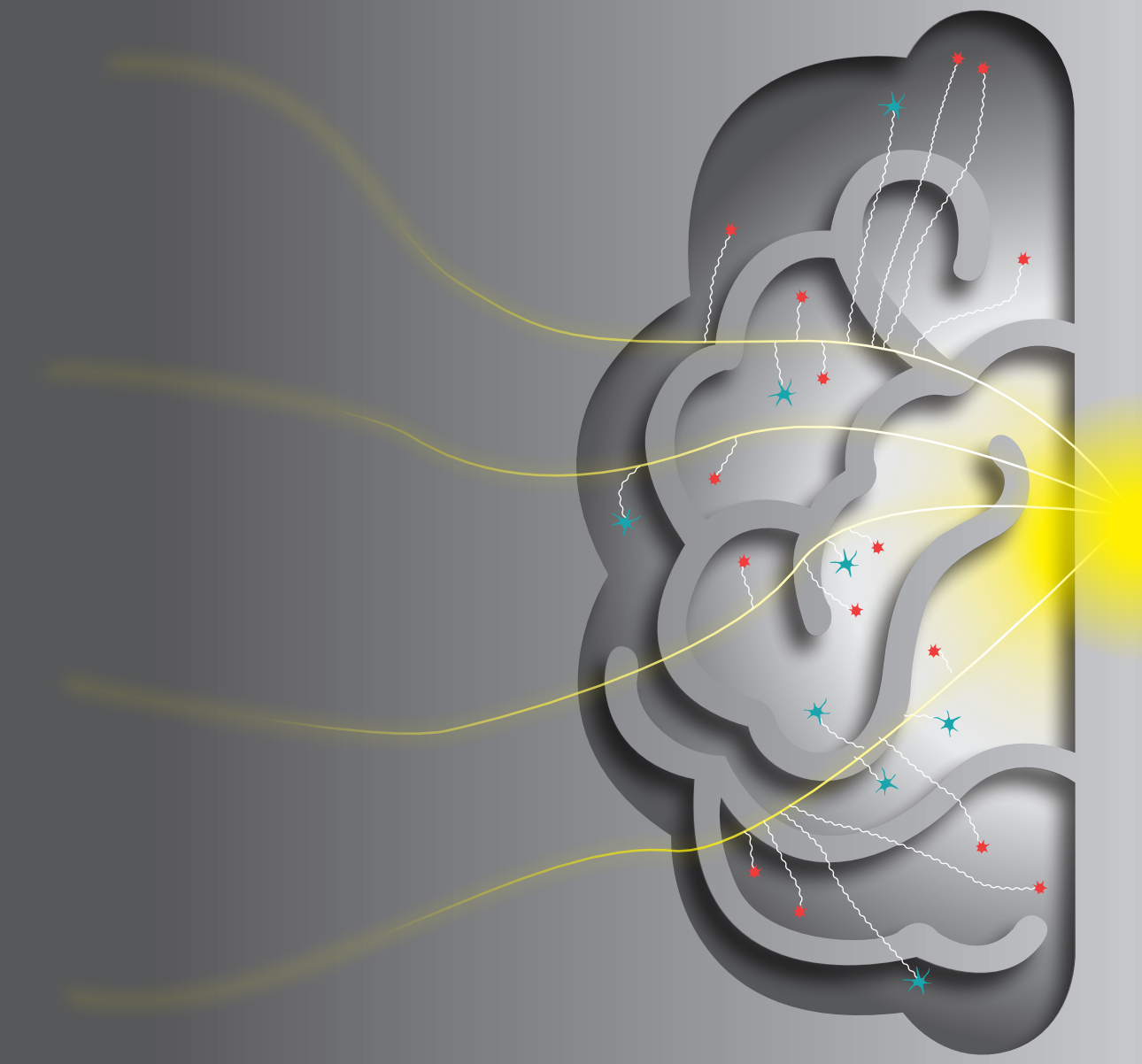
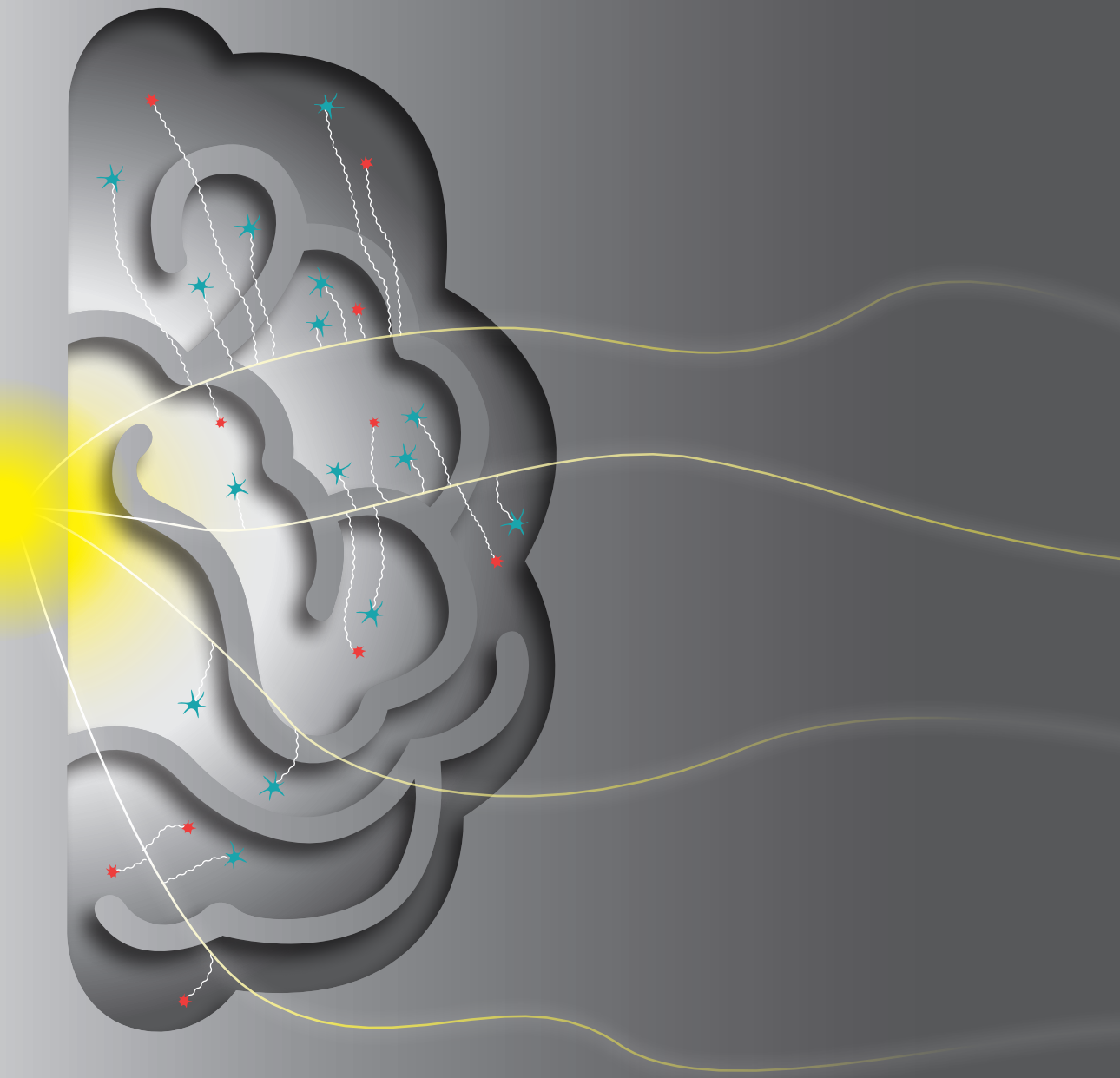
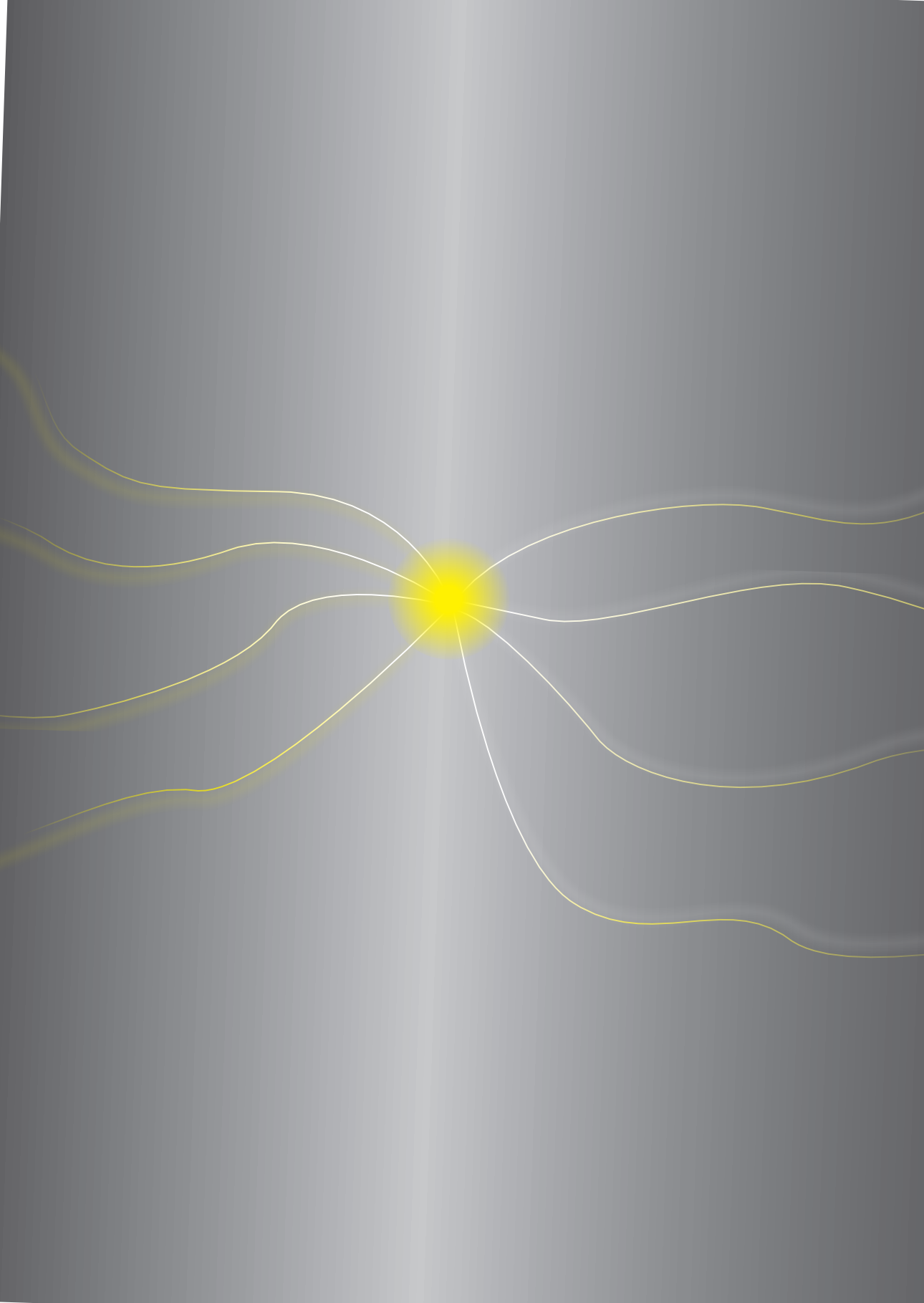


PART 2

Nanoscale materials for neuromodulation





CHAPTER 6

Nanomaterials in neuromodulation: what is the potential?

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SHORT SUMMARY

Deep brain stimulation (DBS) has become a standard neurosurgical treatment for several neurological diseases such as Parkinson's disease. However, it is still an invasive procedure that comes with the cost of surgical complications, discomfort and requires follow-ups interventions. Furthermore, it has an additional financial burden on the healthcare system. Scientific advances in nanotechnology, pave the way for the possibility to develop novel DBS approaches using nano-milli-size materials. These technologies have the potential to be minimally invasive and cost-effective. However, many challenges and obstacles still need to be resolved before reaching this goal. These include the long-term safety and delivery of nonmaterial into the brain parenchyma and at the related location. Yet, research ethical issues necessities to be considered and acknowledged regarding the use of nanomaterials.

1. INTRODUCTION

A wide range of neuromodulation technologies has been explored for their applicability and effectiveness in modulating neural tissue, ranging from tethered devices to nano-scale approaches [1-3]. Neuromodulation often involves controlled electrical alterations of neuronal activity in real-time. Deep brain stimulation (DBS) has emerged as one of the most successful techniques in managing clinical symptoms in a number of neurological disorders such as Parkinson's Disease (PD) [1]. Despite improving motor symptoms, it requires an invasive surgical procedure that carries the potential risk of complications. In fact, 15-34% of the patients undergoing DBS require follow-up interventions for electrode replacement or removal due to hardware malfunctions or infection [4]. Moreover, the average cost of DBS for a patient with PD over 5 years is US$186,244 [5]. As a consequence, many patients are reluctant to undergo this procedure, which has led the technique to be under-utilized for the eligible patient population [6]. Alternatively, several noninvasive neurostimulation techniques have been developed and used, such as transcranial magnetic stimulation [7], transcranial direct current stimulation, vagal nerve stimulation [8], transcranial alternating current stimulation [9], and focused ultrasound for alleviating symptoms of neurological and psychological disorders [10]. However, compared to DBS, these techniques lack targeting precision and adequate penetration when deep subcortical structures are concerned.

To overcome these obstacles and meet the rising demand for better neuromodulation therapies, alternative neurostimulation approaches inspired by material sciences have been proposed. In pre-clinical research, recently developed nano-scale particles and milli-scale devices have been investigated due to their versatile implementation potentials. The main advantage of using nanomaterials and/or milli-scale devices for neuromodulation is that they have the potential to be minimally invasive, cost-effective, and biocompatible compared to conventional tethered devices. Here we highlight the key advances in utilization of these technologies using the body-of-literature available at the time of writing.

2. NANOTECHNOLOGY ENABLES NOVEL MODALITIES FOR NEUROMODULATION

In neuromodulation, nanomaterials act as signal transducers for an external energy source such as a magnetic field. The core elements of these nanomaterials are often metallic, which allow them to transduce magnetic energy into either mechanical, thermal, or electrical energy, eventually generating action potentials [11]. Nevertheless, several other energy sources such as light and ultrasonic waves have also been used to stimulate the administrated nanomaterials. For instance, lanthanide-based up-conversion nanoparticles (UCNPs) enable the conversion of low-energy photons into high-energy photons, and vice versa, when using near-infrared (NIR) radiation. Excited UCNPs can generate multiple emission bandwidths, which can serve as a multi-color light source for optogenetic systems. In these studies, a combined NIR source and multi-chromatic UCNPs with excitation-specific luminescence were shown to enable the selective activation or inhibition of distinct neuronal populations that were expressing specific opsins [12,13]. In addition, ultrasonic waves have also been used to power

internally implanted barium titanate (BaTiO3) piezoelectric stimulators for the restoration of involuntary movements in a rat model of spinal cord injury [14,15]. The piezoelectric stimulators were stimulated at 1 MHz in short 200 µs sinusoidal burst pulses at the lumbosacral spinal cord using an external transceiver to generate evoked potentials in the hind limbs of paralyzed animals [14].

Magnetic fields, in particular, are the most commonly used energy source to power nanomaterials, partially because they are cheap to generate and mostly inert to non-magnetic substances. For instance, magneto-mechanic nanoparticles (MMNPs) can act on mechanosensitive ion channels, effectively modulating or inducing neuronal action potentials. The m-Torquer system, as used by Lee and coworkers, is composed of a magnetic torquer and a rotating circular magnet array (CMA). The m-Torquer system is composed of octahedral magnetic nanoparticles with a diameter of 500 nm. The M2 region of the premotor cortex of mice was bilaterally targeted with adenovirus-containing Myc897-Piezo1 (Ad-Piezo1) followed by the delivery of m-Torquer into the right hemisphere. This was done to specifically target the m-Torquer with the Myc-antibody. Magneto-thermal nanoparticles (MTNPs), on the other hand, act by activating a heat-sensitive element, such as the capsaicin receptor (transient receptor potential cation channel subfamily V member 1, TRPV1). When exposed to an alternating magnetic field, the nanoparticles dissipate energy as heat, which triggers the reversible firing of TRPV1-transfected neurons [3]. Recent studies have shown that neuromodulation via these particles can reverse motor deficits in a neurotoxin-induced mouse model of PD. This was indicated by an increased expression of c-Fos cells (a neural activity marker) in motor pathways [3]. With regards to targeting, the introduction of exogenous molecules along with matching antibodies has shown to enable highly specific binding to the target and a better MTNPs distribution [16,17]. Although these technologies allow for cell-specific neuromodulation, they require genetically modifying cells, which could create regulatory barriers to their clinical translation. In addition, gene editing impairs the translatability of some of those studies as they introduce and target exogenous molecules. Therefore, attempts have been made to circumvent this issue by using materials that transduce the applied energy into electrical charges directly so that there is no need for the transfection of exogenous actuators.

Two-phase magnetoelectric nanoparticles (MENPs) consisting of magnetostrictive and piezoelectric components generate electric charges in the presence of a magnetic field, which have been used to electrically stimulate neurons. In a recent study, Nguyen and coworkers have conducted magnetic stimulation using MENPs in cortical slices ex vivo. After the application of external alternating (AC) and direct current (DC) magnetic fields, the MENPs produced local and network neuromodulation. In addition, neurostimulation was achieved without affecting the cell viability and astroglia activity, indicating the safety of this approach [18]. In line with this, our recent *in vivo* study demonstrated that we could power the MENPs with a magnetic field to remotely generate electric polarization of the MENPs and locally modulate neuronal activity. This was sufficient to alter specific motor pathways and change animal behavior [2]. DBS with MENPs does not require genetic tissue modification to express cell membrane ion channels or any other actuator. However, this is at the cost of being unspecific for cell-types. To improve the selectivity of this approach, different antibody coatings can be used for targeted

delivery. In pre-clinical settings, nanomaterials have shown to be compatible with precise cell targeting by tailored antibodies. However, it should be noted that using antibodies for cell targeting, as seen in MMNPs and MTNPs, may slow down molecular turnover in the membrane with uncertain effects on cell excitability [19].

2.1. Surface coating of the nanomaterials

When using nanomaterials for neurostimulation, different coatings and core components are preferred for each specific purpose. Coatings can be used to stabilize the nanomaterials in aqueous solutions; to direct them towards specific targets, and/or to improve their biocompatibility. For the particle's core, different materials and structures will yield different transducing effects. This variety of nanomaterials and their potential applications is wide and has been recently reviewed by Dominguez-Paredes and colleagues [11]. As such, additional layers are often added to the core to provide added functionalities. Covalent modifications require highly controlled reaction conditions while providing more stable bonds with the transducing nanomaterial. On the other hand, non-covalent modifications require less precise matching and milder reaction conditions. They can also undergo a higher number of chemical interactions per molecule, albeit the stoichiometry and orientation of these interactions are hard to control [11]. Nanomaterials need to be stable in the blood and the brain parenchyma, hence coatings such as silica-shells or propylene glycol monomethyl ether acetate (PMA)-shells are sometimes applied to stabilize the core metallic materials. For example, in magnetic nanomaterials, a non-covalent coating with an amphipathic polymer such as dodecyl-grafted-poly-isobutylene-alt-maleic anhydride/PMA) or PMA-shell has been used to functionalize magnetic nanoparticles in aqueous solutions [20]. Furthermore, surface modifications are also applied to facilitate the crossing of biological barriers and to enable tissue targeting [21]. In addition, the immune system will identify the nanomaterial as foreign and therefore immune recognition needs to be avoided to enhance the nanomaterial's functionality and distribution. For instance, poly-ethyl-glycol (PEG) is one of the immune-recognition-avoiding layers which can be added in nanomaterial designs [11].

2.2. Tissue delivery of the nanomaterials

Once the nanomaterials are stabilized for blood circulation, they need to be introduced into the brain parenchyma. Most studies to date have been conducted by invasively delivering the nanomaterial in question into the targeted area. However, current advances in blood-brain barrier (BBB) disruption could provide the possibility of delivering them via the bloodstream. This can be done by transiently opening the BBB using focused ultrasounds (FUS); osmotic disruption of the BBB, or hijacking ligand-receptor interactions without disrupting the BBB [22,23]. In addition, after intravenously injecting nanomaterials, external magnetic guidance can be applied to directly extravasate the nanomaterials and guide them to the brain [18,24].

2.3. Parenchymal retention and tissue clearance

Lastly, the dynamics of nanomaterial retention in the brain parenchyma is poorly understood to date. Ideally, nanomaterials should remain in the parenchyma for as long as possible, in the minimal concentration needed, whilst causing the least amount of cell damage. Super-paramagnetic iron-oxide nanoparticles (SPIONPs) coated with dextran were injected into the striatum of rats and were gradually cleared out from the brain parenchyma at the injection site in about two weeks (presumably with a contribution of glial cells), with clearing times of up to eight weeks depending on nanoparticle concentration [25]. In addition, striking novel studies have discovered that gadolinium-based magnetic resonant imaging (MRI) contrast agents show long-term brain retention and deposition. Seemingly these particles (3-350 nm in size) had been well-tolerated in the brain parenchyma and tissue clearance was slow or absent [26]. Other experimental reports *in vivo* also indicate that using nanomaterials appears to be safe and to not cause significant tissue damage. For instance, MENPs were well tolerated in the brain parenchyma and did not affect cell viability in our rodent study [2]. This could predict that those nanomaterials can remain safely in the brain for prolonged periods of time, yet future studies will be key to appropriately characterize their retention and clearance over time.

2.4. Milli-scale materials

Advances in material sciences have also led to an increase in using milli-scale devices for neural stimulation and have shown promising results that could benefit the scientific community. Magnetoelectric (ME)-converters are milli-scale devices that are placed subdurally and act similarly to MENPs. These ME transducers convert low magnitude (<1 mT) and low-frequency (~300 kHz) magnetic fields into electric fields that can power custom integrated circuits or stimulate nearby tissue. In addition, the ME-converter was able to electrically stimulate a rat sciatic nerve at a distance of 4 cm from the energy source [27]. Clinical application of milli-scale devices is more promising as these can be used to modulate the peripheral nervous system as well as cortical areas in the central nervous system with none or minimally invasive procedures. Future research will be indispensable to optimize this technology and investigate whether it allows targeting deep brain areas.

3. CONCLUSION

In summary, the use of nanomaterials as nanoelectrodes shows promise as a new solution for wireless neural devices. As this field is still in its infancy, future research will be critical to understanding the potentials and limitations of this technology. Furthermore, research into noninvasive delivery routes, toxicity, and cell/tissue targeting specificity will help bring this technology closer to clinical application. A substantial number of studies have tested nanomaterials for neuromodulation, but the majority of those have used *in vitro* or *in silico* models. The application of these nanomaterials in animal models of neurological and psychiatric disorders will help to clarify the main components driving their therapeutic effect, and the mechanisms that may underlie patient responses. Clarifying these aspects will direct more rational and effective decision making in translating the use of the nanomaterials to the clinic. To

conclude, it is important to recognize that several novel ethical considerations arise when developing brain implants with nano-scale materials. Especially, the excitement regarding the use of innovative nano-scale materials should not be at the cost of compromising patient safety and long-term safety assurances. In this regard, research ethics guidelines will likely require reconsideration to acknowledge these issues [28].

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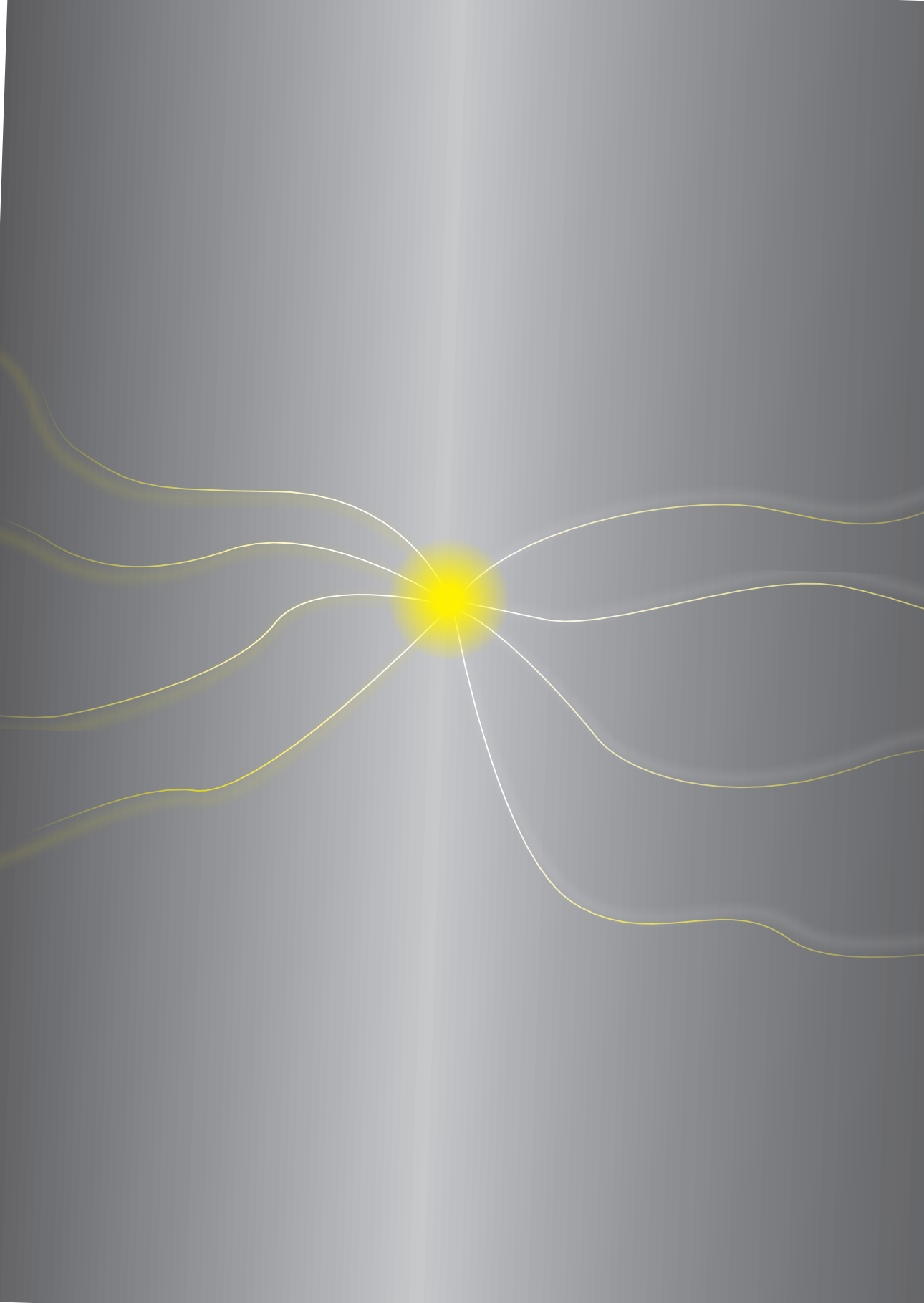

CHAPTER 7

Wireless stimulation of the subthalamic nucleus with nanoparticles modulates key monoaminergic systems similar to contemporary deep brain stimulation

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ABSTRACT

Background

Deep brain stimulation (DBS) is commonly used to alleviate motor symptoms in several movement disorders. However, the procedure is invasive, and the technology has remained largely stagnant since its inception decades ago. Recently, we have shown that wireless nanoelectrodes may offer an alternative approach to conventional DBS. However, this method is still in its infancy, and more research is required to characterize its potential before it can be considered as an alternative to conventional DBS.

Objectives

Herein, we aimed to investigate the effect of stimulation via magnetoelectric nanoelectrodes on primary neurotransmitter systems that have implications for DBS in movement disorders.

Methods

Mice were injected with either magnetoelectric nanoparticles (MENPs) or magnetostrictive nanoparticles (MSNPs, as a control) in the subthalamic nucleus (STN). Mice then underwent magnetic stimulation, and their motor behavior was assessed in the open field test. In addition, magnetic stimulation was applied before sacrifice and post-mortem brains were processed for immunohistochemistry (IHC) to assess the co-expression of c-Fos with either tyrosine hydroxylase (TH), tryptophan hydroxylase-2 (TPH2) or choline acetyltransferase (ChAT).

Results

Stimulated animals covered longer distances in the open field test when compared to controls. Moreover, we found a significant increase in c-Fos expression in the motor cortex (MC) and paraventricular region of the thalamus (PV-thalamus) after magnetoelectric stimulation. Stimulated animals showed fewer TPH2/c-Fos double-labeled cells in the dorsal raphe nucleus (DRN), as well as TH/c-Fos double-labeled cells in the ventral tegmental area (VTA), but not in the substantia nigra pars compacta (SNc). There was no significant difference in the number of ChAT/ c-Fos double-labeled cells in the pedunculopontine nucleus (PPN).

Conclusions

Magnetoelectric DBS in mice enables selective modulation of deep brain areas and animal behavior. The measured behavioral responses are associated with changes in relevant neurotransmitter systems. These changes are somewhat similar to those observed in conventional DBS, suggesting that magnetoelectric DBS might be a suitable alternative.

Keywords: Deep brain stimulation; magnetoelectric nanoparticles; serotonin; dopamine; acetylcholine

1. INTRODUCTION

Deep brain stimulation (DBS) requires invasive stereotactic surgery for the implantation of the electrodes and a tethered pulse generator [1]. Despite its great success in symptom management in movement disorders such as Parkinson's disease (PD) [2-8], DBS has potential surgical complications such as cerebral hemorrhage, and infections [8]. In addition, 15-34% of the patients undergoing DBS procedures require a follow-up surgery for DBS electrode replacement or removal due to hardware malfunctions, displacement, bleeding, or infection [7-9]. For instance, a recent study demonstrate that over 10% of 132 treated patients showed 17 electrode lead migration of more than 3 mm in 16 patients, due to their dystonic phenotype and problems with the lead fixation at the bur-hole [10]. A minimally invasive DBS system could address some of these challenges and accommodate the growing demand for neuromodulation treatments [11, 12]. Among others, several noninvasive neurostimulation techniques have been investigated and used, such as transcranial magnetic stimulation, or transcranial alternating current stimulation for neurological and psychological diseases [13, 14]. However, these techniques lack precise targeting and appropriate penetration depth of subcortical structures, such as the subthalamic nucleus (STN) [15]. Recently, we have shown that we can stimulate deep brain targets of mice with wireless nanoelectrodes *in vivo* [16]. These two-phase magnetoelectric nanoparticles (MENPs) are composed of magnetostrictive and piezoelectric materials, which when strain coupled, generate electric fields in an applied magnetic field. The generated electric field can then elicit specific and local modulation at the injection site [16]. On the other hand, magnetostrictive-only nanoparticles (MSNPs) do not generate electrical fields under a magnetic field and as such, were used as a control [16].

Given these early but promising results, we sought to examine how this novel wireless approach alters the basal ganglia and related circuitry that underlay DBS-related motor and non-motor responses. Clarifying whether this approach induces similar changes in the brain could help establish magnetoelectric DBS as a suitable alternative to conventional DBS. In PD research, subthalamic nucleus (STN)-DBS has been shown to alter the activity of dopaminergic, serotonergic, and cholinergic systems in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), dorsal raphe nucleus (DRN), and pedunculopontine nucleus (PPN) both in healthy and PD conditions [17-20]; reviewed in [21].

The effect of STN-DBS on neuronal activities of dopaminergic SNc neurons has been investigated in several electrophysiological studies in naïve animals [22, 23]. Experimental data has shown that STN-DBS decreases the spiking activity in less than half (43%) of the SNc dopaminergic neurons in naïve rats, while increases the spiking activity in another 43% of the dopaminergic cells [22]. However, the effect of STN-DBS was more consistent in PD animals with decreasing spiking activities in 88% of SNc neurons [22]. Another experiment shows that STN-DBS increases the firing rate of 76% of SNc dopaminergic neurons in naïve animals [23]. To date, there is no clear evidence on the effect of STN-DBS on the activity of the VTA dopaminergic neurons in naïve animals. However, the activity of these neurons has been known to be inhibited in response to movement learning behavior activities [24]. In other words, as animals learn to predict rewards, reward-related activity in dopaminergic neurons is decreased [24, 25].

Although a few studies have linked the activity of dopaminergic neurons to a particular behavior [26, 27], the activity of dopaminergic neurons was somewhat related to the speed of the animal [24].

Previous studies have indicated that STN-DBS inhibits serotonergic neuron activity in the DRN in PD and naive animals [19, 28, 29]. Ample evidence suggests that the disruption of the serotonergic raphe system plays a key role in mood disorders [30]. As aforementioned, changes in the activity of the serotonergic system are critical, as it plays an important role in not only the therapeutic but also the adverse effects of DBS. Additionally, both dopaminergic and cholinergic systems are linked to axial symptoms of neurological diseases such as PD [31, 32]. Although STN-DBS does not seem to improve all of these axial symptoms [33, 34], it is still important to assess whether magnetoelectric DBS could similarly influence the cholinergic system.

Herein, we aimed to address how and to what extent the dopaminergic, serotonergic, and cholinergic systems are altered after magnetoelectric DBS with MENPs in naïve mice. Moreover, we also wanted to assess whether these changes are similar to conventional DBS, and to relate these changes to the behavioral effects observed. Magnetic stimulation was applied to animals injected with either MENPs or control MSNPs in the STN. C-Fos co-expression with tyrosine hydroxylase (TH), tryptophan hydroxylase-2)TPH2(, and choline acetyltransferase (ChAT) was determined with immunohistochemistry (IHC) to assess the activity of dopaminergic, serotonergic, and cholinergic neurons, respectively.

2. MATERIALS AND METHODS

2.1. Animals

Experiments were performed on 16 male naïve mice (C57BL/6 J; the Jackson Laboratory). Animals were housed under constant temperature and humidity with a 12-hour/12-hour dark/light cycle with food access *ad libitum*. All animal experiments were carried out under a protocol approved by the Institutional Animal Care Committee of Maastricht University in accordance with the Central Authority for Scientific Procedures on Animals.

2.2. Stereotactic nanoparticle injection

The mice were injected with an analgesic (buprenorphine, 0.1 mg/Kg s.c), 30 min prior to the stereotactic surgery. After injection, inhalation anesthesia (isoflurane, Abbot Laboratories, Maidenhead) was induced at 4% and maintained at 1.5–2%. After adequate anesthetic induction, the mouse was positioned in a small animal stereotaxic frame (Kopf, Los Angeles, USA). Body temperature was maintained at 37 °C using a thermo-regulator pad. An ocular ointment was applied to avoid eye dryness. Lidocaine 1% was subcutaneously administered at the incision site as local anesthesia after disinfection of the skin. Burr holes were made into the skull to aim for bilateral STN (AP −2.0 mm, ML ± 1.5 mm, DV −4.5 mm) to inject a total of 2 ml (100 mg/ml) with infusion rate (100 nL/min) of either MENPs or MSNPs using a micro-infusion pump (Nanoject II, Drummond Scientific).

2.3. Magnetic stimulation and behavioral testing

After a 1-week recovery from stereotactic surgery, mice underwent three minutes magnetic stimulation by applying a 220 mT DC magnetic field with a 6 mT, 140 Hz AC magnetic field to the MENPs and control MSNPs as seen in [16] prior to each behavioral testing. Behavioral tests were performed in a repeated-measures design where both MSNPs and MENPs mice were stimulated in the first trial and then reassigned to off-stimulation in the second trial with a 3-week interval in between the sessions (Fig. 1). Animals were tested in the Catwalk, Rotarod, and Open Field test (OFT). Catwalk and Rotarod testing and data are described and published in our earlier report [16]. For this study, we conducted a follow-up analysis of OFT data and post-mortem immunohistochemistry investigations on animals who underwent behavioral testing in our previous study [16]. Half of the mice in each group were randomly subjected to magnetoelectric DBS (Stim-ON groups) 90 min prior to the perfusion and sacrificing of the animals. The other half served as a control by being placed in the coil while the coil remained off (Stim-OFF groups). The animals were thus sorted into the following groups: MENPs Stim-ON, MENPs Stim-OFF, MSNPs Stim-ON, and MSNPs Stim-OFF, with four mice per group. The experimenter and data analyst were blinded to animal identity during behavioral testing, post-mortem histology and data analysis.

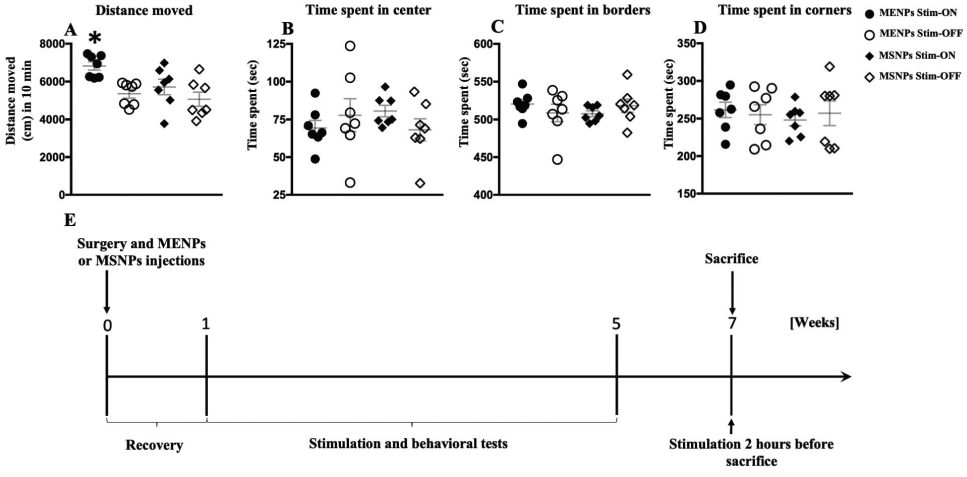

Figure 1. Magnetic stimulation of MENPs-treated mice in the STN induced behavioral activity changes in the open field test (OFT). A) The graph shows a significant increase in distance moved in mice with magnetic stimulation. B-D) There was no significant difference in time spent in neither the center, borders, nor corners arena in the OFT. E) Timeline of the experiment (Phase-III experiment in our former study [16]). Data are presented as means and ± SEM; the significant difference ($p < 0.05$) is indicated by an "*". Abbreviations: Magnetoelectric nanoparticles, MENPs; subthalamic nucleus, STN.

2.4. Open Field Test

The open field test (OFT) consisted of a clear Plexiglas square arena measuring 100 x 100 cm with 40 cm high walls and a dark floor, as described previously [35]. The OFT measures time spent and the distance moved in the arena to provide an indication of the animal's locomotor activity following magnetoelectric DBS. Animals were individually placed in the center of the arena and were allowed to move freely in the arena for 10 min. The behavior of each mouse was recorded on a computer using the Ethovision tracking software (Ethovision, Noldus Information Technology, Wageningen, The Netherlands). This software automatically calculated and analyzed data including the locomotion and distance moved and the time spent in the center, borders, and corners areas. After each trial, the testing area was cleaned with 70% ethanol solution to diminish the odors of other mice.

2.5. Tissue Processing

Mice were deeply anesthetized with pentobarbital and transcardially perfused with Tyrode buffer and 4% paraformaldehyde fixative. Then, brains were extracted and fixed in 4% paraformaldehyde overnight and submerged in 20% sucrose (24 h at 5 °C). The brains were then immediately frozen with CO2 and stored at – 80°C. After fixation, coronal brain sections (20 mm) were cut on a cryostat and stored at – 80 °C.

2.6. C-Fos immunohistochemistry

Tissue sections series were incubated with a primary antibody raised against c-Fos (rabbit polyclonal; 1:1000; Abcam, ab190289), for two nights followed by a donkey anti-rabbit biotin secondary antibody (Jackson Immunoresearch Laboratories, west grove, USA, 1:400) and avidin-biotin peroxidase complex (ABC kit Vestastatin, Burlingame, CA, USA; 1:800). The staining was visualized with 3,3'-diaminobenzidine (DAB).

2.7. Double immunofluorescence of tyrosine hydroxylase, tryptophan hyroxlayse-2 and choline acetyltransferase with c-Fos

Tissue sections containing the VTA, SNc, DRN, and PPN were incubated overnight with either primary antibodies against TPH2 (Goat polyclonal 1:2000, Abcam, ab121013), TH (Sheep polyclonal 1:2000, Sigma-Aldrich, AB1542), or ChAT (Goat polyclonal 1:200; Sigma-Aldrich, AB144P), respectively in combination with primary c-Fos antibody (rabbit polyclonal Abcam; 1:1000). Donkey anti-goat Alexa 488 and anti-rabbit Alexa 594 secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, USA, 1:200) were incubated, as well as donkey anti-sheep biotin; secondary antibody Jackson (Immunoresearch Laboratories, West Grove, USA, 1:200) and streptavidin Alexa 488 Jackson (Immunoresearch Laboratories, West Grove, USA, 1:5000).

2.8. Quantification of immunohistochemically stained sections

For c-Fos staining, photographs of stained tissue sections containing the motor cortex (MC), the paraventricular region of the thalamus (PV-thalamus), and the centromedial region of the thalamus (CM-thalamus) from three rostrocaudal anatomical levels from Bregma (AP: −0.58, −0.94, and −1.22) were taken at 10X magnification. We used Cell P software (Olympus Soft Imaging Solutions, Münster,

Germany) from an Olympus DP70 digital camera with a motorized condenser connected to an Olympus AX70 microscope (Olympus, Zoeterwoude, The Netherlands). In the area of interest, the number of c-Fos cells was counted using ImageJ software [version 1.52; National Institutes of Health (NIH), Bethesda, USA]. A cell was considered positive if the intensity of the cell staining was higher than the surrounding background. In each subject, the average value of three sections was used for statistical analysis.

The double-labeled sections (TH/c-Fos co-expressed in the VTA and the SNc; TPH2/c-Fos in the DRN; and ChAT/c-Fos in the PPN) were analyzed using a fluorescence spinning disk confocal microscope (DSU; Olympus BX51, Hamamatsu City, Japan). 3D virtual tissues were acquired using a digital ultra-high sensitivity CCD camera (C9100-02, Hamamatsu Photonics, Hamamatsu City, Japan). Cell counting was performed in all counting frames using the optical fractionator. Total cell numbers were estimated using a validated stereological method which is previously described [36], and practiced routinely at our laboratory [37].

2.9. Data analysis

Statistical analysis was performed using GraphPad Prism 9.4.0 (GraphPad Software, San Diego, California, USA). Behavior tests were performed in a repeated-measures design where both MSNPs and MENPs mice were stimulated in the first trial and then reassigned to off-stimulation in the second trial. We performed repeated-measures ANOVA and Bonferroni post-hoc analysis to compare between two sets of measurements. Furthermore, immunohistochemical data were analyzed using two-way ANOVA and Bonferroni post-hoc analysis. Data were presented as the mean and standard error of means (± SEM) and statistical significance was defined as P-value < 0.05.

3. RESULTS

3.1. Open field test

In the OFT, MENPs mice showed a significant increase in the distance moved after magnetic stimulation (MENPs Stim-ON: 6822 ± 221 versus MENPs Stim-OFF: 5359 ± 231, MSNPs Stim-ON: 5715 ± 404 and MSNPs Stim-OFF: 5063 ± 376 cm per 10 min, respectively) compared to the nonstimulated trial and the MSNPs mice [$F(1,12) = 11.78$, $p < 0.05$, pairwise comparison p's < 0.05; Fig. 1A]. However, there was no significant difference in the time spent in neither the center, borders, nor corners in the OFT of all groups [$F(1,12) = 0.10$, $p < 0.05$, $F(1,12) = 0.30$, $p < 0.05$, and $F(1,12) = 0.17$, $p < 0.05$, respectively; Fig. 1B-D].

3.2. Immunohistochemistry

In stimulated mice treated with MENPs, c-Fos expression was significantly increased in the MC (MENPs Stim-ON: 944 ± 65 versus MENPs Stim-OFF: 645 ± 34, MSNPs Stim-ON: 740 ± 22 and MSNPs Stim-OFF: 737 ± 42 cell count/mm^2, respectively) and PV-thalamus (MENPs Stim-ON: 678 ± 49 versus MENPs Stim-OFF: 384 ± 45, MSNPs Stim-ON: 396 ± 12 and MSNPs Stim-OFF: 386 ± 6 cell count/mm^2, respectively) compared to nonstimulated as well as MSNP-treated mice [$F(1,12) = 12.04$, $p < 0.01$; Fig. 2A, D-E and $F(1,12) = 20.21$, $p < 0.001$, pairwise comparison p's < 0.05; Fig. 2B, D-E, respectively]. In the CM-thalamus, there was no statistical difference between the groups [$F(1,12) = 0.52$, $p = 0.48$; Fig. 2C-E].

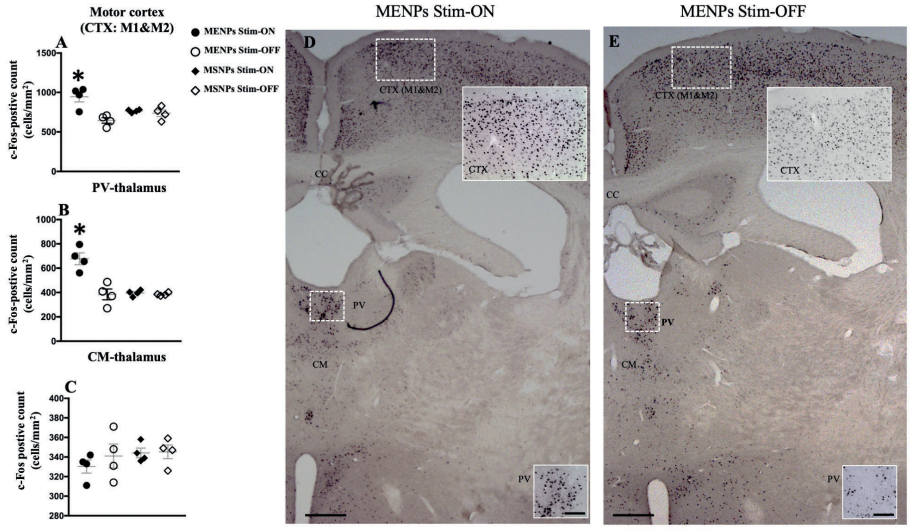

Figure 2. Magnetic stimulation of MENPs-treated mice in the STN resulted in neuronal activity changes. A-C) Graphs show that magnetic stimulation significantly increased c-Fos expression in the MC and PV-thalamus of MENPs-stim mice, but not in the CM-thalamus. D-E) Representative photomicrographs of coronal sections stained for c-Fos showing the MC, PV- and CM-thalamus, for both stimulated and nonstimulated MENPs-treated mice; scale bar= 250 µm (overview) and 50 µm (inset). Data are presented as means and ± SEM; the significant difference ($p < 0.05$) is indicated by an "* ". Stimulated (Coil-ON), Stim-ON; non-stimulated (Coil-OFF), Stim-OFF; magnetoelectric nanoparticles, MENPs; motor cortex, MC; paraventricular region of the thalamus, PV-thalamus; centromedial region of the thalamus, CM-thalamus.

In addition, c-Fos co-expression with dopaminergic, serotonergic, and cholinergic cells was examined using stereological quantification of double-labeled cells. Stimulated mice treated with MENPs and showed a significantly lower amount of double-labeled TH/c-Fos cells in the VTA (MENPs Stim-ON: 47 ± 21 versus MENPs Stim-OFF: 276 ± 70, MSNPs Stim-ON: 309 ± 81 and MSNPs Stim-OFF: 281 ± 28 cells, respectively), compared to nonstimulated as well as MSNP-treated mice [$F(1,12) = 5.82$, $p < 0.05$, pairwise comparison p's < 0.05; Fig. 3A, E-F]. However, no statistical difference was found between groups when analyzing the number of TH/c-Fos cells in the SNc [$F(1,12) = 0.0003$, $p = 0.98$; Fig. 3B, G-H]. In addition, the VTA and SNc TH cell count showed no statistically significant difference between the groups (Fig. 4A-B).

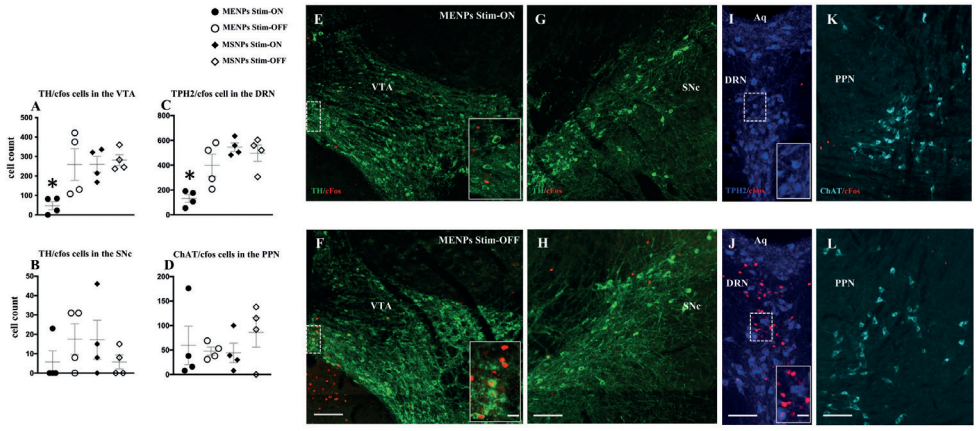

Figure 3. Magnetic stimulation of MENPs-treated mice in the STN modulates the neuronal activity of the VTA dopaminergic and DRN serotonergic neurons, but neither in the SNc dopaminergic nor the PPN cholinergic neurons. A-B) Graphs show magnetoelectric stimulation significantly decreased the TH/c-Fos double-labeled cells in the VTA of MENPs-stim mice, but not in the SNc. It also decreased the TPH2/c-Fos double-labeled cells in the DRN (C) but did not significantly alter the number of c-Fos/ChAT double-labeled cells in the PPN (D). E-H) Representative photomicrographs of coronal brain sections, double-labeled for TH (green)/c-Fos (red) in the VTA and SNc scale bar= 150 µm (overview) and 15 µm (inset); I-K) TPH2 (blue)/c-Fos in the DRN; K-L) ChAT (cyan)/c-Fos in the PPN, for both stimulated and non-stimulated MENPs mice; scale bar= 100 µm (overview) and 15 µm (inset). Data are presented as means and±SEM; the significant difference ($P < 0.05$) is indicated by an "*". Stimulated (Coil-ON), Stim-ON; non-stimulated (Coil-OFF), Stim-OFF; Ventral tegmental area, VTA; substantia nigra pars compacta, SNc; dorsal raphe nucleus, DRN; pedunculopontine nucleus, PPN; cerebral aqueduct, Aq; tyrosine hydroxylase, TH; tryptophan hydroxylase 2, TPH2; choline acetyltransferase, ChAT. I, J, K, and L) pseudocolours were used for both TPH2 (blue pseudocolor) and ChAT (cyan pseudocolor).

In stimulated mice treated with MENPs, double-labeled TPH2/c-Fos cells in the DRN showed to be significantly decreased (MENPs Stim-ON: 132 ± 33 verses MENPs Stim-OFF: 417 ± 65, MSNPs Stim-ON: 545 ± 31, and MSNPs Stim-OFF: 497 ± 77 cells, respectively), compared to nonstimulated as well as MSNPs-treated mice [$F(1,12) = 19.28$, $p < 0.001$, pairwise comparison p's < 0.01; Fig. 3C, I-J]. Quantification of TPH2 cells in the DRN showed no statistically significant difference between groups (Fig. 4C).

Lastly, stimulated mice treated with MENPs revealed no statistical significance in ChAT/c-Fos cells in the PPN between the treatment groups [$F(1,12) = 0.31$, $p = 0.59$; Fig. 3D, K-L]. Quantification of the PPN ChAT-positive cell count showed no statistically significant difference between groups (Fig. 4D). Finally, magnetic stimulation of MENPs-treated mice significantly decreased the c-Fos expression in the VTA [$F(1,12) = 23.18$, $p < 0.001$), but not in the SNc, DRN or PPN [$F(1,12) = 0.09$, $p = 0.77$; $F(1,12) = 0.62$, $p = 0.44$ and $F(1,12) = 0.128$, $p = 0.727$, respectively (Fig. 4 E-H)].

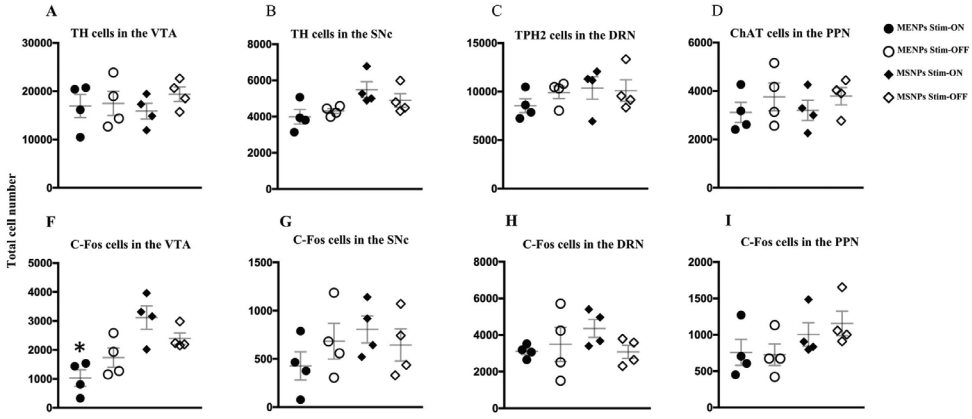

Figure 4. A-D) Graphs show that magnetic stimulation of MENPs-treated mice in the STN did not significantly alter either the TH, TPH2 or ChAT cell counts in the VTA, SNc, DRN or PPN [$F(1,12)=0.04$, $p=0.84$; $F(1,12)=0.14$, $p=0.70$; $F(1,12)=0.35$, $p=0.57$; and $F(1,12)=1.87$, $p=0.20$, respectively]. E-H) Graphs show that magnetic stimulation of MENPs-treated mice significantly decreased the c-Fos expression in the VTA, but not in the SNc, DRN nor PPN. Data are presented as means and ± SEM; the significant difference ($p<0.05$) is indicated by an "* ".

4. DISCUSSION

The main goal of this study was to assess the effects of magnetoelectric DBS of the STN on the primary neurotransmitter systems implicated in the working mechanisms of conventional DBS. This is critical for the characterization and validation of this potentially novel DBS approach. Stimulated animals exhibited an increase in c-Fos expression in the MC and PV-thalamus and distance moved in the OFT. Furthermore, TH and TPH2/c-Fos co-expressing cells were reduced in the VTA and DRN, respectively. However, magnetoelectric stimulation did not show a significant difference in the co-expression of ChAT/c-Fos cells in the PPN. Those histological and behavioral findings are somewhat similar to the known effects of high frequency STN-DBS [21].

In this study, we initially replicated our earlier findings where magnetoelectric stimulation increased c-Fos expression in both the MC and the PV-thalamus (Fig. 2A, B, D-E). This explains the behavioral outcomes of our former study, in which an enhanced dynamic and speed-related gait parameters were observed in the Catwalk test [16]. Additionally, stimulated animals showed a significant increase in the distance-moved in the OFT (Fig. 1A). This hyperlocomotion could be due to the effect of magnetoelectric stimulation on the activity of dopaminergic cells in the VTA [38-40].

In stimulated animals, we observed a significant reduction in TH/c-Fos double-labelled cells in the VTA, but not in the SNc (Fig. 3A, B, E and F). This indicates that magnetoelectric stimulation affects the mesolimbic dopaminergic pathway in naïve animals, while the nigrostriatal pathway is relatively spared. Notably, the TH expressing cell populations were unchanged in both the VTA and the SNc (Fig S1 A-B).

The dopaminergic neurons of the VTA play an important role in the mesolimbic circuitry and its function in the reward, limbic, cognitive as well as psychomotor behavior [41-43]. -

The STN and its glutamatergic neurons can activate the VTA in the mesolimbic circuitry by neurons in the medial tip of the STN that project to the limbic-related VTA cells [40, 44]. Ablation of VTA with radiofrequency has been shown to induce hyperactivity in non-goal specific movements in rats [38], which is in line with lower dopaminergic neuronal activity and hyperlocomotion observed in this study. In addition, antidromic propagation in the VTA projections, and/or orthodromic activation of GABAergic cells in the VTA or passing-by fibers from the subthalamic area to the VTA could inhibit VTA dopaminergic cells [39, 40]. Current literature present ample evidence that challenged VTA dopaminergic system could affect psychomotor behavior [38, 43].

We found a selective increase in c-Fos expression in the limbic thalamus (PV-thalamus) and the MC (Fig. 2A, B, D-E). Increased c-Fos expression has also been observed in the MC of naïve rats following electrical STN-DBS also [45]. The implications of this regional c-Fos activity pattern on locomotion have been extensively discussed in our previous work [16]. We postulated that the enhanced activity in the PV produces states of arousal that result in hyperlocomotion [46], as it relays information projected from the brainstem and subthalamic areas to the nucleus accumbens and the amygdala, as well as the cortical areas associated with these subcortical regions.

STN-DBS has been shown to elicit debilitating mood effects such as depression, suicide ideation, and impulsivity in some PD patients [47, 48]. Our earlier studies have shown that acute bilateral STN-DBS reduced the firing rate of the DRN serotonergic neurons, decreased serotonin release in the forebrain, and induced depressive-like behavior in PD rats. Given the absence of direct projections from the STN to the DRN, those effects were thought to be relayed via areas such as the lateral habenula [19, 49, 50]. Moreover, in a recent study we demonstrated that STN-DBS induces a sustained suppression in serotonergic system, which was accompanied by depressive like behavior both in PD and naïve mice [29, 51]. These could explain the adverse mood effects following STN-DBS in PD patients, given the fact that the DRN is the main source of serotonin in the central nervous system and its dysfunction has long been associated with the onset of mood disorders [52]. In line with these results, magnetoelectric stimulation of the STN inhibited the activity of the serotonergic neurons in the DRN (Fig. 3C, I-J), indicating that nanoelectrode neurostimulation could be comparable to conventional DBS in terms of local and remote network effects.

We also observed that the activity of the cholinergic neurons in the PPN was not altered between groups (Fig. 3D, K and L), despite that there is a known dopaminergic-cholinergic imbalance in axial symptoms of movement disorders, especially in PD [53, 54]. A descending projection from the STN to the brainstem and, in particular the PPN has been described in mammals [55]. However, there is no indication that high frequency stimulation of the STN influences PPN cholinergic neurons. On the other hand, an optogenetic study has demonstrated that gait improvement in STN-stimulated animals was

related to the modulation of upstream connections between the STN and frontal cortices [56]. Likewise, a recent structural connectivity study has attributed the beneficial motor effects of STN stimulation in PD patients to the modulation of fiber tracts between the STN and motor cortex [57], suggesting that STN-DBS does not activate the PPN cholinergic neurons. Similarly, in our study we found no indication that magnetic stimulation in the STN influenced PPN cholinergic neurons. Therefore, the observed motor effects are more likely due to changes in the mesolimbic dopaminergic system rather than the motor circuity, such as the PPN.

Nevertheless, the current study has some limitations. First, the study was performed in naïve and not parkinsonian animals. Still, this is a necessary first step to understanding the mechanism and effects of MENPs stimulation on the transmitter systems before moving forward to more complex models. While significant work is required to realize this technology as a minimally invasive DBS replacement (e.g., designing the powering device, using less invasive delivery routes) [12], it is important at this technological development stage to explore its effects on local and remote neural elements. Furthermore, in its current state, the proposed technology compromises some freedom that is essential to tailoring the delivery of neuromodulatory effects to the targeted brain region derived from the multiple contacts of the existing DBS lead technology. Further research could explore whether multiple MENPs can be placed and differentially activated to sculpt the volume of activated brain tissue to maximize efficacy and minimize the side-effects .

Despite that, here we report that MENPs stimulation has similar molecular effects to conventional DBS. Comparing the effects of conventional DBS and MENPs stimulation on monoaminergic systems was challenging, especially in naïve animals, as conventional DBS is usually tested in parkinsonian models. Future research will be required to understand these changes more extensively, particularly in PD models, and eventually compare the clinical outcomes of both conventional and MENPs technology, which is the ultimate goal of investigating this novel technique.

5. CONCLUSION

We have previously demonstrated that magnetoelectric nanoelectrodes enable selective modulation of specific brain areas and related behavior in mice [16]. Herein we aimed to investigate the mechanisms of action of wireless DBS compared to known aspects of the conventional DBS mechanisms. We showed that the stimulation of the STN with this approach suppresses the mesolimbic dopaminergic and brainstem serotonergic pathways. These observations which are in line with the changes in cell activity as well as animal behavior measured. These changes are comparable to those that have been observed in conventional DBS, suggesting that magnetoelectric DBS alters the neural pathways and corresponding behavioural outcomes in a similar fashion, and thus shows promise as a neuromodulatory therapy.

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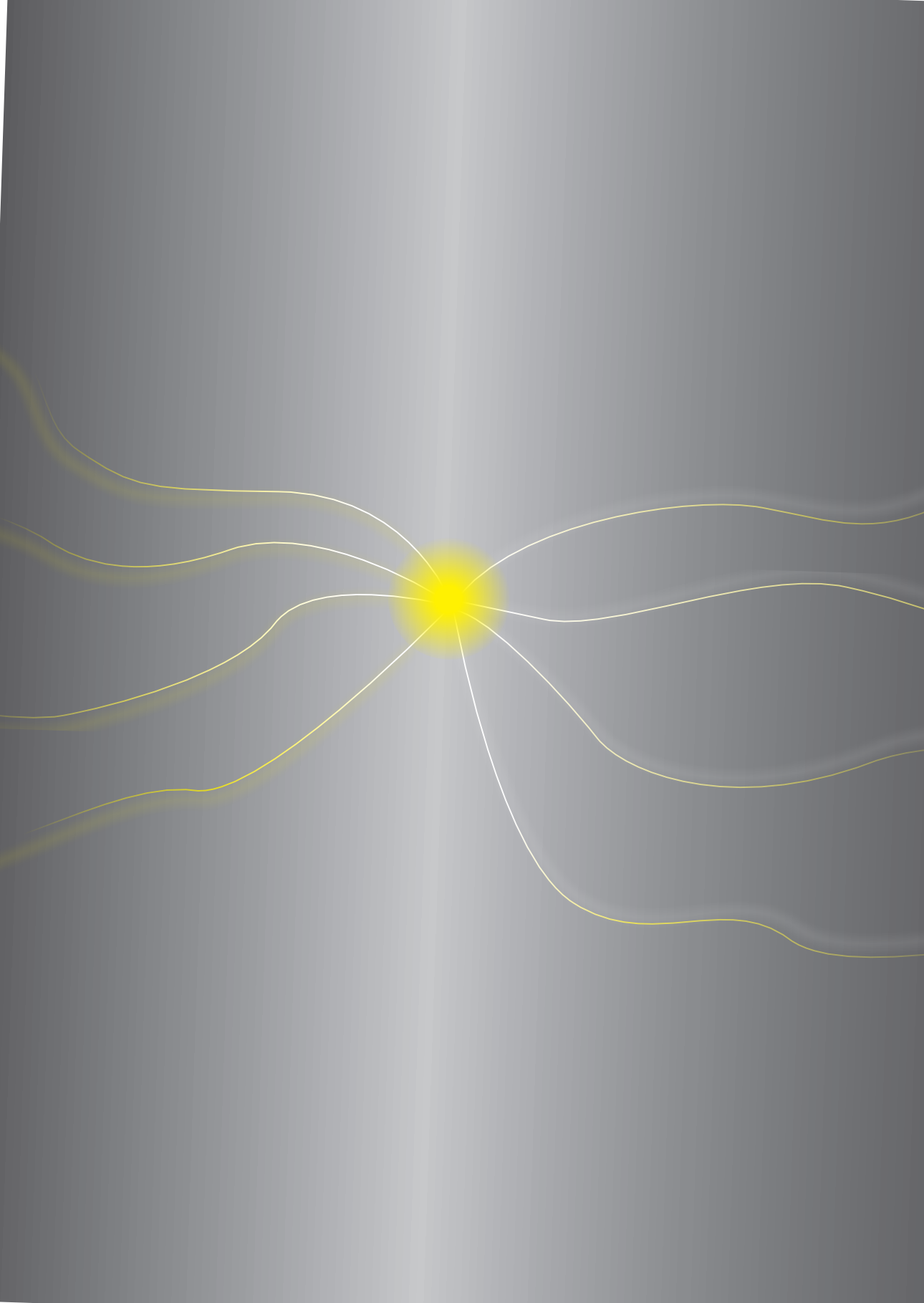

CHAPTER 8

General discussion

1. GENERAL DISCUSSION

In this thesis, I investigated the effects of deep brain stimulation (DBS) on monoaminergic neurotransmitter systems.

Particularly, in the first part of this thesis, I provided a literature review on changes in monoaminergic neurotransmitter systems in Parkinson's disease (PD) and deep brain stimulation (DBS). Then, I investigated the neuroplastic effects of long-term DBS of the subthalamic nucleus (STN) on the neuronal activity and phenotype of the dorsal raphe nucleus (DRN) serotonergic neurons. STN-DBS inhibited the DRN serotonergic neuronal activity and had neuroplastic effects on the phenotype of the serotonergic neurons. Afterward, I investigated whether the inhibitory effect of STN-DBS on serotonin (5-hydroxytryptamine; 5-HT) cells are relayed via the globus pallidus externa (GPe). To address this, I conducted *designer receptors exclusively activated by designer drugs* (DREADDs) modulation of the GPe in mice treated with STN-DBS. GPe modulation did not influence the DRN serotonergic neurons in mice treated with STN-DBS. Furthermore, STN-DBS improved the gait symptoms of methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated mice. It is well known that the cholinergic system is involved in the gait symptoms of PD [1], however, STN-DBS did not affect the PPN cholinergic system. In the second part of this thesis, I provided a literature review on the potential use of nanomaterials as a wireless tool for minimally invasive DBS. Lastly, I conducted a proof-of-concept study on mice stimulating the STN using magnetoelectric nanoparticles (MENPs) and assessed how the principal monoaminergic neurotransmitter systems are involved compared to conventional DBS. Magnetic stimulation of the MENPs exhibits effects on locomotion, local neuronal activities and inhibits the serotonergic neurons in the DRN and dopaminergic neurons in the ventral tegmental area (VTA) but has no effect in the substantia nigra pars compacta (SNc). However, magnetic stimulation of the MENPs did not affect the PPN cholinergic system, which was also in line with the conventional STN-DBS outcomes. Below, I will discuss the major findings of each chapter in more detail.

Monoaminergic systems, including the SNc dopaminergic and DRN serotonergic are implicated in the neuropathology and pathophysiology of PD as intensively discussed in **chapter 2**. In brief, growing evidence shows that DBS can influence the underlying neurochemical changes associated with PD pathology and improves motor symptoms in PD patients. Furthermore, DBS has been shown to reduce the need for pharmaceutical dopamine treatment while improving the motor symptoms of PD in clinical research [2]. However, the precise effects of DBS on neurotransmitters are still not entirely understood. Nevertheless, several theories attempt to explain the basic mechanism of DBS. The most common theories are the rate and pattern theories [3, 4]. According to these theories, DBS regulates overactive basal ganglia neuronal firing rates in PD patients by restoring the normal electrophysiological single-cell firing rates, and also interferes with pathological patterns such as the subcortical beta oscillations (13–30 Hz), promoting cortical gamma activity (40–200 Hz), and modifying local field potentials [5-8]. However, these hypotheses merely emphasize on how DBS affects local readouts and, to a lesser extent, distant areas.

In fact, preclinical studies indicate that STN-DBS has an effect on local and remote striatal dopaminergic systems [9]. In addition, STN-DBS induces inhibition of serotonergic neurons in PD [10], which is particular interesting, because the DRN serotonergic system may have a role in the frequent incidence of depression in PD patients [11]. Preclinical data have also demonstrated that PPN-DBS reduces axial symptoms in PD, but further studies are required before it is considered a new brain region target for clinical practice [12].

Due to the technical difficulties in detecting real-time transmitter release and transmitter-related changes in distant neural locations, neurochemical alterations following DBS may not be widely discussed in the literature. Moreover, several neurotransmitter changes occur in remote areas. For instance, STN-DBS affects projections to several distant areas including the striatal dopaminergic, DRN serotonergic, pallidum GABAergic neurons, and global glutamatergic neurons in several brain areas, including the striatum, substantia nigra pars reticulata (SNr), and GPe [9, 10, 13, 14]. This suggests that the impact of DBS is not only because of local axon inhibition, as shown in electrophysiological studies, but also affect orthodromic and antidromic pathways to distant nuclei. As a result, most of these network neurochemical changes remain uninvestigated. For example, the exact anatomical pathways explaining the effect of STN-DBS on the DRN serotonergic system are still not fully explained.

Recent approaches have been developed, such as optogenetic and transgenic animal lines, and they can provide a possibility to address the aforementioned limitations by targeting a specific cell type to assess the changes in remote areas in real-time instances [15]. Consequently, it is necessary to conduct optogenetic experiments to examine the precise effects of neuromodulation on neurotransmitter release. Those approaches would make it feasible to evaluate the cumulative and chronic impact of DBS on local and distant neural components. Ultimately, it could provide a better understanding of the dynamic changes in monoaminergic neurotransmitters.

STN-DBS is widely used to treat motor symptoms in PD. However, STN-DBS exhibits adverse mood effects such as depression. Previous studies show that STN-DBS inhibits the serotonergic neurons and exhibits despair-like behaviour in MPTP mice [10]. However, the exact mechanisms behind this side effect are still not fully explained. In addition, it is challenging to address the abovementioned issue with conventional techniques. Therefore, **chapter 3**, I used a specific cell-type approach to investigate long-term effects of STN-DBS on the neuronal activity and phenotype of DRN serotonergic neurons in ePet-Cre transgenic mice, which enabled specific assessment of the serotonergic neurons in the DRN. For this, adeno-associated virus (AVV) expressing a genetically encoded $Ca2+$ indicator, GCaMP6, was injected in the DRN to assess the neuronal activity using fibre photometry and AVV-enhanced yellow fluorescent protein (eYFP) was used to assess serotonergic neurons in the same target. Furthermore, mice received either systemic MPTP injections, which caused a considerable 60% loss of SNc dopamine neurons, or saline for the control group. MPTP mice showed gait impairments that STN-DBS improved in the Catwalk test.

I observed that long-term STN-DBS reduced calcium signalling in the DRN, which indicates neuronal inhibition of the DRN serotonergic neurons. This is consistent with electrophysiological studies, where acute STN-DBS reduced the firing rate of serotonergic neurons by 40–50% in extracellular single-cell recordings [10]. Subsequently, in vivo microdialysis studies noticed a low serotonin release in terminal forebrain regions [16, 17]. Moreover, I also found that STN-DBS caused behavioural despair in MPTP mice, which is thought to be a sign of depression. Furthermore, mice treated with saline also showed the same behavioural outcomes. This suggests that the STN-DBS-induced behavioural change was not dependent on the structural integrity of the nigrostriatal pathway or motor function. Earlier studies are also in line with our findings [10]. This indicated a serotonin-dependent mechanism of STN-DBS in the brainstem, with the DRN serving as the primary source of serotonergic innervation to the forebrain [18].

In addition, most (> 90%) of the eYFP-positive neurons in the DRN generally expressed Tryptophan hydroxylase 2 (TPH2) in our study. Interestingly, I found that STN-DBS decreased the amount of double-labelled eYFP/TPH2-positive neurons using stereological techniques. Moreover, STN-DBS did not affect c-Fos expression in the DRN, suggesting that the total neuronal activity during intermittent stimulation remained stable. This might be a sign that DBS caused neurotransmitter respecification while the fate of these switch cells was not investigated.

Several studies have demonstrated neurotransmitter respecification in the adult brain, in which external cues can lead to neurotransmitter phenotypic switching, neurotransmitter induction, or neurotransmitter elimination with concurrent behavioural alterations [19, 20]. I proposed that this neuroplasticity also occurs with DBS. This is especially important for STN-DBS, a commonly used neurosurgical therapy for PD with stimulation-dependent motor and non-motor behavioural alterations [21-25].

Although the STN receives serotonergic inputs from the DRN, there is no proof that STN-DBS directly impacts the serotonergic neurons through this projection. In fact, electrophysiological data histograms during the peristimulus period collected from the DRN have shown that STN-DBS did not cause an antidromic or short-latency (10 ms) orthodromic response [10]. Moreover, the lateral wings of the DRN, which receive a significant amount of input from multiple forebrain areas, including the lateral habenula (LHb), showed to enhance neuronal activity with c-Fos expression of DRN [26]. However, additional pathways might explain our findings, such as inhibition mediated by the serotonergic receptor or DRN network circuitry to other brain structures. Moreover, preclinical studies suggest that the effect of STN-DBS on DRN serotonergic neurons bypass through relay stations via the LHb and prefrontal cortex [26-28]. Furthermore, an optogenetic study found that several neuronal inputs project from the basal ganglia (BG), including the GPe, to DRN serotonergic neurons [29]. In addition, the STN has a direct projection to GPe [30]. In fact, the STN-DBS showed to change the neuronal activities of the GPe [30]. In **chapter 4**, Our data suggest that STN-DBS inhibits the DRN serotonergic neuronal activity. However, the DREADDs modulation of the GPe did not significantly influence this outcome. I did not observe a

significant difference when the GPe was both excited and inhibited and targeted via viral vectors, which were then activated by Clozapine-N-oxide (CNO) medication.

As previously described, STN-DBS improved the gait symptoms in MPTP mice. In addition, it has been shown that the PPN cholinergic system is involved in the control of gait and balance functions [31]. Moreover, preclinical studies show that the degeneration of the PPN cholinergic system led to gait impairment and postural instability in animal PD models [32-34]. However, the effect of STN-DBS on the cholinergic system is still not thoroughly investigated. As a result, in **chapter 5** I investigated the PPN cholinergic system of MPTP-treated mice. Our findings showed that MPTP-treated mice had 28.1% fewer cholinergic neurons in the PPN than saline-control mice. In fact, neuropathological assessment of post-mortem brains from PD patients found the degeneration between 40 and 70 percent of lateral PPN cholinergic neurons [35-37]. In addition, gait impairments in PD patients have been linked to the loss of PPN cholinergic neurons [38-40]. Nevertheless, there was no evidence in my study that STN-DBS affected PPN cholinergic neurons. The number of choline acetyltransferase (ChAT) and the neuronal marker c-Fos immunohistochemically double-labelled PPN neurons remained unchanged. This shows that STN-DBS does not activate the remaining PPN cholinergic neurons.

From an anatomical perspective, a downward projection from the STN to the brainstem and the PPN has been reported in animal studies. Despite the limitation of this projection, the STN has an impact on PPN cholinergic neurons. In a previous study, 6-hydroxydopamine injections led to severe degeneration of SNc and induced the hyperactivity of PPN neurons, and this enhanced neuronal activity was reversed by ibotenic STN lesions [41]. Hence, other STN-related pathways may be required for STN-DBS to enhance gait parameters. Moreover, a structural connectivity analysis showed that the stimulation of fiber tracts connecting the STN, and motor cortex was responsible for the gait improvements in PD patients treated with STN-DBS [42]. Similarly, optogenetic activation of the STN has shown that the modification of upstream connections between the STN and frontal cortices is responsible for the motor benefits of STN stimulation [43].

As stated in previous chapters, conventional STN-DBS is used to treat motor symptoms in PD patients. However, conventional STN-DBS is still an invasive approach that requires surgical intervention. As it can lead to surgical complications such as infections, cerebral hemorrhage, and electrode lead dislocation [44]. Furthermore, DBS surgery also requires follow-up interventions for electrode replacement or removal due to hardware malfunctions or infection [45]. Consequently, in part II of my thesis, in **chapter 6** I introduce a potential new approach for wireless neural devices using nanomaterials as nanoelectrodes to address this issue. Future studies are essential to comprehend the potentials and limitations of this technology because this technology is still in its infancy. Moreover, studies into toxicity, cell/tissue targeting selectivity, and noninvasive delivery methods will advance this technology into clinical use. Several research studies have examined the neuromodulatory potential of nanomaterials, although most have relied on in vitro or in silico simulations [24]. Using these nanomaterials in animal

models of neurological and psychological disorders will aid in clarifying the key elements behind their therapeutic efficacy and potential use for PD patients.

In the current thesis, in **chapter 7** the main objective or my final study was to evaluate how the principal monoaminergic neurotransmitter systems are involved in the mechanisms of magnetoelectric stimulation of the STN compared to the conventional wired STN-DBS. I found that magnetoelectric stimulation of the STN increases the locomotion and c-Fos expression in the motor cortex (MC) and paraventricular (PV) thalamus of naïve mice. Additionally, the number of cells co-expressing tyrosine hydroxylase (TH) and TPH2/c-Fos was decreased in the VTA and DRN, respectively. However, magnetoelectric stimulation of the STN did not reveal any significant difference in the co-expression of ChAT/c-Fos cells in the PPN. These histological and behavioral findings resemble the known conventional STN-DBS effects discussed in **chapter 2**.

This aligns with previous findings where the magnetoelectric stimulation of the STN also elevated c-Fos expression in the MC and PV thalamus [46]. In addition, the behavioral findings from previous research showed improved dynamic and speed-related gait parameters during the Catwalk test [46]. Moreover, an earlier study showed that conventional STN-DBS induces an increase in c-Fos expression has also been seen in the motor cortex of naïve rats [47]. As the PV relays information projected from the brainstem and subthalamic regions to the nucleus accumbens and the amygdala, as well as the cortical areas associated with these subcortical regions, I hypothesized that the increased activity in the PV produces states of arousal that result in hyperlocomotion [48].

Our data suggest that the magnetoelectric stimulation of the STN primarily affects the mesolimbic dopaminergic system while the nigrostriatal network in naïve animals was spared. However, the total TH-expressing cell population was maintained in both the VTA and the SNc. This indicates that the impact of magnetoelectric stimulation of the STN on the activity of dopaminergic cells in the VTA may cause this hyperlocomotion. Interestingly, the medial tip of the STN projects to the VTA neurons, and also the STN and its glutamatergic neurons can activate the VTA of the mesolimbic pathway [49, 50]. Previous research showed that the radiofrequency ablation of the VTA caused hyperactivity in non-goal specific movements in rats [51], which is consistent with the decreased dopaminergic neuronal activity and hyperlocomotion seen in our work. Moreover, dopaminergic cells in the VTA may be inhibited by antidromic and orthodromic projections from the VTA GABAergic cells or bypass fibers from the subthalamic region [49, 52].

Magnetoelectric stimulation of the STN decreased the activity of serotonergic neurons in the DRN, suggesting that the local and distant network effects of nanoelectrode neurostimulation are similar to those of conventional wired STN-DBS [10]. Although there is a known dopaminergic-cholinergic imbalance in axial symptoms of movement disorders, especially in PD [53, 54], I did not observe a change in the activity of the PPN cholinergic neurons. Additionally, this is in line with our findings of conventional wired STN-DBS in **chapter 5**. It is another piece of evidence that the therapeutic effect

of STN-DBS is more related to other pathways, such as direct projection to the cortex [42] and the mesolimbic dopaminergic system.

There were some limitations of this study. First of all, naive animals were used instead of parkinsonian animal models. However, before moving on to more complicated models, it is essential to understand the mechanism of magnetoelectric stimulation of the STN on monoaminergic neurotransmitter systems. Although future studies are still needed to develop this technology into a minimally invasive DBS tool (such as developing the powering device and employing less invasive delivery methods), it is crucial to investigate its effects on local and distant neuronal components at this current stage.

2. FINAL CONCLUSIONS

The monoaminergic neurotransmitter system is essential in PD pathophysiology and mediates a number of effects as well as side effects of therapeutic approaches, including DBS. However, the mechanism of action of DBS on neurochemical changes is still not fully revealed due to the practical obstacles to monitoring live changes for both local and distant effects. I found that long-term STN-DBS inhibited the serotonergic neuronal activity in the DRN and led to a loss of the serotonergic phenotype. These findings will help understand the mechanism behind the adverse mood effect caused by STN-DBS in PD patients. Furthermore, I found that the effect of STN-DBS on the DRN neuronal activities is not influenced by the DREADDs modulation of the GPe. However, more evidence suggests that it relays through the LHb. Despite the role of the cholinergic system on gait and balance functions, STN-DBS did not affect the cholinergic system. This suggests that the therapeutic effect of STN-DBS on gait parameters can be due to cortical and mesolimbic pathways.

I also found that magnetoelectric stimulation of the STN and conventional STN-DBS has comparable locomotion and neurochemical effects. Future research is critical to evaluate MENPs technology in PD animal models.

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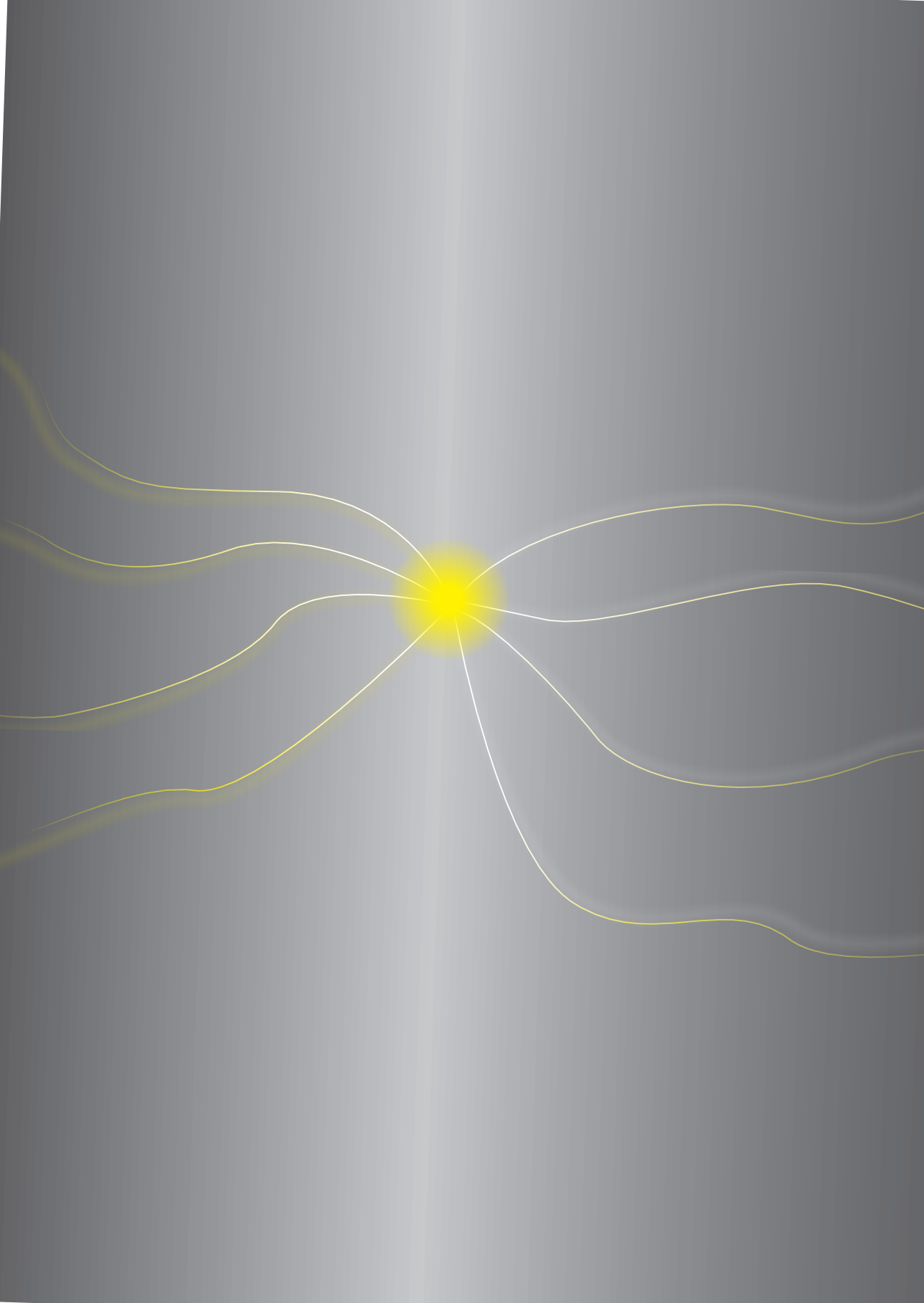

ADDENDUM

Impact paragraph/Volarization

Summary

Summary in Arabic

Biography

List of publications

IMPACT PARAGRAPH/VOLARIZATION

Relevance for the academic community and society

Deep brain stimulation is a well-established surgical procedure used to manage the symptoms of many neurological diseases, in particular Parkinson's disease. The procedure involves implanting a small device into the brain to deliver electrical impulses to specific areas that regulate movement. Moreover, deep brain stimulation has also helped us understand the brain and the mechanisms behind movement disorders. For instance, researchers have utilized it to map the neural connections responsible for movement and learn how alterations in these circuits might cause movement problems.

Among ten million people who are suffering from Parkinson's disease, roughly 208,000 patients have received deep brain stimulation treatment worldwide. Although this surgery is relatively safe, it still requires surgical intervention with costs and complications such as cerebral bleeding and infections. In fact, deep brain stimulation surgeries cost each Parkinson's disease patient roughly US$186,244 over five years of healthcare and follow-up surgeries that are required to optimize deep brain stimulation parameters or battery replacements. Consequently, patients are reluctant to undergo deep brain stimulation surgery, leading to the underutilization of this approach. This is why developing less invasive deep brain stimulation alternative approaches is necessary. Although deep brain stimulation has shown remarkable therapeutic outcomes for Parkinson's patients, it also indicates some side effects, including mood disorders like depression. Consequently, it is crucial to comprehend the neurochemical mechanisms behind deep brain stimulation, which could remarkably improve the current treatment and help optimize the pharmacological treatment combined with deep brain stimulation for Parkinson's patients and healthcare providers.

I reviewed the literature about the significant impacts of conventional deep brain stimulation on the most relevant monoaminergic neurotransmitter systems, namely the dopamine, noradrenaline and serotonin system and their counterpart-related neurotransmitter system, especially the cholinergic system. I have learned that deep brain stimulation has considerable local and remote effects on the neurotransmitter systems in several brain regions responsible for movement and mood regulation. The disbalance of these neurotransmitters contributes significantly to the development of neurological and psychological disorders. Consequently, the current thesis aims to investigate the underlying mechanism behind these neurochemical changes, which could improve this treatment and minimize the side effects of deep brain stimulation therapy. In addition, novel deep brain stimulation techniques using nano-scale materials were reviewed and tested the neurochemical effect in naive animals.

Target groups

The target groups of the research presented in this thesis are broad. I will start with the prioritized target groups, including patients with Parkinson's disease and their caregivers. Deep brain stimulation has been extensively studied and effectively improves motor symptoms in patients with Parkinson's disease. Furthermore, it has also been shown to enhance the quality of life, reduce dopamine medication use,

and decrease the number of hospitalizations for patients with Parkinson's disease. Individuals who underwent this surgery notice a considerable positive impact on their capacity to work, interact with others, and participate in daily activities. This can also reduce the stress on caregivers, who might need to help them with daily tasks and medication administration.

The general public is another target group. Parkinson's disease is the second most common neurogenerative progressive disorder. Ten million are suffering from this disease worldwide. It substantially affects the physical and mental health of patients. Pharmacological treatment has many drawbacks, such as the fluctuation of responses to the medication and medication resistance, especially in the advanced stage of Parkinson's disease. Deep brain stimulation has been successful in treating the motor symptoms of Parkinson's disease and improve the overall life quality for patients, which has an impact on the economy and society because patients can work, consume and invest longer. However, deep brain stimulation sometimes induces some mood side effects. Therefore, further research is required to understand the underlying mechanism of deep brain stimulation.

In line with this, another target group is the critical scientific mass. Previous research found that deep brain stimulation of the subthalamic nucleus reduces the neuronal serotonin activity in the dorsal raphe nucleus. This is particularly interesting because serotonin in the dorsal raphe nucleus is associated with mood disorders. The thesis found that brain stimulation of the subthalamic nucleus reduces neuronal activity in the dorsal raphe nucleus, which was accompanied by the loss of their cell phenotype. In addition, conventional deep brain stimulation requires an invasive procedure and leads to surgical complications such as brain bleeding and infections. Novel techniques using nano-scale materials may offer less invasive approaches with fewer complications.

Lastly, the academic community and companies developing neuromodulation techniques are also a target group as they can use these findings to improve their electrode designs with regard to inducing fewer side effects and more precise benefits and support future research.

Activity products

The primary product/outcome of the current thesis is divided into two parts. First, the outcome of deep brain stimulation on the main monoaminergic and related neurotransmitters. The second part tested a less invasive deep brain stimulation approach using nano-scale materials.

In Chapter 3, I started with investigating the neurochemical effect of deep brain stimulation of the subthalamic nucleus in the dorsal raphe nucleus serotonin neurotransmitter system. I found that it interferes with cellular serotonin balance, which could lead to side effects of brain stimulation surgery, such as depression. Understanding the exact mechanism of how brain stimulation surgery reduces serotonin will help improve the surgical treatment and may offer the optimal pharmacological intervention combined with brain stimulation surgery. In addition, I tested whether the globus pallidus externa is a relay of the subthalamic nucleus to the dorsal raphe nucleus anatomically, which inhibits the

serotonin release, as there is no direct connection between the subthalamic nucleus and dorsal raphe nucleus. It has been recently discovered that there is a connection between the globus pallidus externa and the dorsal raphe nucleus. In addition, there is a well-known connection between the globus pallidus externa and the subthalamic nucleus. Our findings did not verify that the globus pallidus externa is a relay station between the subthalamic and dorsal raphe nuclei. However, earlier studies suggest other relay pathways, such as lateral habenula and medial prefrontal cortex.

Furthermore, deep brain stimulation of the subthalamic nucleus treats the gait and balance symptoms of Parkinson's disease. The cholinergic system has a significant role in regulating gait and balance. I have assessed whether deep brain stimulation of the subthalamic nucleus affects the cholinergic system as a possible mechanism of improving the gait by this system. However, deep brain stimulation of the subthalamic nucleus did not affect the cholinergic system. This suggests that other pathways, such as the cortical and mesolimbic pathways, could explain the cause of improvement in gait and balance.

In the second part of my thesis, I reviewed novel approaches for less invasive deep brain stimulation. Then, in the final chapter of the current thesis, I assessed the effect of wireless deep brain stimulation using nanoparticles on the main monoaminergic (dopamine and serotonin) and cholinergic neurotransmitters and compared them to conventional deep brain stimulation. They have the same molecular effects in naïve animals compared to conventional deep brain stimulation. The findings of this thesis are a first step to understand this technology, and future research is necessary to develop this nanotechnology further.

Innovation

There are several innovative aspects of the current thesis. Deep brain stimulation of the subthalamic nucleus has reduced the effect of serotonin activity and caused the serotonin cell to lose its original phenotype. This indicates that deep brain stimulation may induce neuroplastic changes. However, the fate of the phenotype switch of the cells was not investigated. Future research is warranted to have a better understanding of this phenomenon.

In addition, in the thesis, I tested the globus pallidus externa as a potential relay route from the subthalamic nucleus to the dorsal raphe nucleus, as there is no direct connection between them. My findings suggest that the globus pallidus externa is not a relay station; however, more detailed, and specific cell-type studies are required to verify our results. Also, the cholinergic system is not involved in the treatment benefits of the deep brain stimulation of the subthalamic nucleus.

The thesis also innovatively introduces a novel technique to be developed into a less invasive approach. This technique uses nano-scale materials (nanoparticles) and can be wirelessly stimulated using a specific magnetic field. The findings of the thesis suggest that this nanoparticle induces locomotion and neuronal activities and has a neurochemical and cellular outcome similar to conventional (wired)

deep brain stimulation. However, this is the first step to understanding this technology. Scientists and researchers need to investigate this novel nanotechnology in future research on disease animal models.

Implementation

Overall deep brain stimulation surgery for Parkinson's disease has remarkable positive scientific and social impacts. However, it must be used cautiously, followed thoroughly, and evaluated independently for each patient, considering potential risks and benefits. Future research needs to unravel the exact mechanism further and thoroughly describe how to optimize this operation and minimize the harmful effects of these techniques. Lastly, improving less invasive approaches might help to make it more feasible and cost-effective for patients and healthcare.

SUMMARY

Parkinson's disease (PD) is a neurogenerative disease affecting over ten million people worldwide. PD was first described in 1817 by a British physician named James Parkinson. Its key motor symptoms include tremors, rigidity, bradykinesia, and postural instability. In addition, PD patients suffer from non-motor symptoms such as cognitive impairments and mood disorders. The disease is caused by the death of dopamine cells in the substantia nigra pars compacta. The exact cause of this cell death is not well understood. While there is no cure for PD, dopamine replacement therapy can only treat motor symptoms in the early stages but have side effect and fluctuation in medical treatment, especially in advanced stages.

Deep brain stimulation (DBS) is a surgical procedure involving implanting electrodes in specific brain regions to apply an electrical impulse that can improve motor symptoms in PD patients. In the past, traditional surgical approaches were used to treat these conditions. However, they were abandoned due to their irreversible nature and the availability of more effective drug treatments. Advancements in neurosurgery have led to improvements in stimulation equipment and neuroimaging techniques, making targeting brain structures more precise. Furthermore, intraoperative electrophysiology techniques have also improved the targeting of brain regions, particularly the subthalamic nucleus (STN). High-frequency stimulation (HFS) of the STN, discovered in 1993, is now a standard treatment for PD.

Monoaminergic neurotransmitter systems are essential for regulating various functions of the central nervous system, such as mood, behavior, and movement. The primary monoaminergic neurotransmitters involved are dopamine, noradrenaline and serotonin. Dopaminergic neurons primarily project to the basal ganglia, which are involved in movement regulation, while serotonergic neurons project to several brain areas, including the basal ganglia and prefrontal cortex. In addition, noradrenergic neurons projects to cortical, subcortical, and spinal structures. Dysregulation of these systems can lead to neurological like PD. The cholinergic system may also interact with the monoaminergic systems, particularly in movement regulation. Understanding the involvement of these systems in neurological will help optimize treatment options to improve patient healthcare.

Monoaminergic neurotransmitters, such as dopamine, noradrenaline and serotonin, play an essential role in DBS's therapeutic and side effects. In **Chapter 2**, the literature review the DBS effects on the activity of the striatal and mesolimbic dopaminergic systems, locus coeruleus (LC) noradrenergic, dorsal raphe nucleus (DRN) serotoninergic system, and the pedunculopontine nucleus (PPN) cholinergic system. DBS has been shown to improve motor symptoms and increase dopamine release in the striatum. However, it can also cause non-motor side effects such as mood disorders, which might be related to its impact on the DRN serotonergic and LC noradrenergic systems. Recent research has shown that external stimuli can drive neurotransmitter switching in the mature brain, providing evidence for

DBS-induced neurotransmitter respecification. Understanding the effects of DBS on the monoaminergic systems is essential for optimizing its benefits and minimizing its side effects.

Recent approaches such as optogenetic, chemogenetic, and transgenic animal lines can address the limitations of detecting real-time transmitter release and neurotransmitter-related changes in distant neural locations. In addition, these approaches make it possible to target specific cell types. Therefore, in **Chapter 3**, we have conducted optogenetic experiments to examine the precise effects of neuromodulation on neurotransmitter release and evaluate the cumulative and chronic impact of DBS on local and distant neural components. While using a genetically encoded calcium indicator protein using a viral vector, we could assess the neuronal activity and phenotype of serotonergic neurons in the DRN. Our findings suggest that long-term STN-DBS may have a negative impact on the serotonergic system, specifically by inhibiting the activity of DRN serotonergic neurons, which may contribute to mood-related side effects such as depression. The use of a specific cell-type approach using transgenic mice allowed for the assessment of the effects of STN-DBS on DRN serotonergic neurons with greater specificity. The observed reduction in calcium signaling in the DRN is consistent with previous studies that have demonstrated the inhibitory effect of STN-DBS on serotonergic neurons in the DRN.

Additionally, the behavioral despair observed in mice treated with STN-DBS suggests the negative impact of STN-DBS on the serotonergic system. The findings highlight the importance of investigating the long-term effects of DBS on the brain's neuromodulatory systems, particularly those implicated in mood regulation. Post-mortem immunohistochemistry analysis reveals that the STN-DBS may cause a change in the phenotype of DRN serotonergic neurons. This is supported by previous studies showing that STN-DBS can lead to changes in neurotransmitter release and the expression of specific neurotransmitter-related proteins in various brain regions.

Neuroplasticity refers to the ability of the brain to adapt and reorganize in response to environmental stimuli or experiences. There is increasing evidence that neuroplasticity also occurs with DBS, including STN-DBS. As previously mentioned, STN-DBS can affect local neural circuits and distant brain areas, leading to changes in neurotransmitter release and behavioral outcomes. The stimulation-induced changes in neurotransmitter release may cause neuroplastic changes in the target neurons, resulting in the respecification of neurotransmitter phenotypes. This may contribute to the long-term effects of STN-DBS on both motor and non-motor symptoms in PD.

There is no direct connection between STN and DRN, and STN-DBS could bypass through other relay nuclei such as lateral habenula and prefrontal cortex. Nevertheless, a novel link between globus pallidus externus (GPe) and DRN has been discovered. In addition, the known connection of the GPe to STN. As a result, in **Chapter 4,** we assessed the effect of GPe on DRN using a chemogentic approach in mice treated with STN-DBS. Our findings suggest that the GPe does not directly mediate the effect of STN-DBS on DRN serotonergic neurons. Further studies are needed to investigate the precise pathways involved in STN-DBS. In **Chapter 5**, our findings suggest that STN-DBS has no significant effect on the

PPN cholinergic system. These findings indicate that STN-DBS may improve gait in PD through other pathways, including the STN projection and modulation of cortical connectivity.

DBS requires invasive surgery and can lead to complications. In **Chapter 6**, we have reviewed the use of nanomaterial for neuromodulation. Novel approaches are being explored, such as using magnetoelectric nanoparticles (MENPs) to deliver DBS wirelessly. MENPs generate an electric field in response to a magnetic field, which can stimulate the brain without genetic modification. In **Chapter 7**, our findings suggest that magnetoelectric stimulation of the STN may have similar effects as conventional STN-DBS, particularly concerning enhancing locomotion and an increase in the neuronal activity in the motor cortex and paraventricular thalamus. Notably, magnetoelectric stimulation of the STN decreases the activity of the ventral tegmental area (VTA) dopaminergic and DRN serotoninergic neurons, respectively. However, it's important to note that magnetoelectric stimulation did not affect neuron activity in the PPN cholinergic and SNc dopaminergic neurons. These results suggest that the principal monoaminergic neurotransmitter systems may play a role in the mechanisms of magnetoelectric stimulation of the STN. These findings may have implications for developing noninvasive neuromodulation approaches for treating PD. In addition, the increased activity in the PV following magnetoelectric stimulation of the STN may lead to hyperlocomotion due to the relay of information from the subthalamic and brainstem regions to the nucleus accumbens and the amygdala, which are associated with cortical areas.

These findings suggest that magnetoelectric stimulation of the STN may modulate dopaminergic activity in the VTA, which could lead to the observed hyper locomotion. The potential involvement of GABAergic cells in the VTA in this mechanism warrants further investigation. In addition, it is possible that the lack of observed effects on the nigrostriatal network in naïve animals could be due to compensatory mechanisms within the system. Future studies could investigate the long-term effects of magnetoelectric stimulation of the STN on both the mesolimbic and nigrostriatal pathways, as well as potential downstream effects on behavior and cognition. Overall, these findings provide insight into the neural mechanisms underlying the therapeutic effects of magnetoelectric stimulation of the STN and could inform the development of new neuromodulatory approaches for PD. In addition, magnetoelectric stimulation of the STN did not significantly impact the activity of cholinergic neurons in the PPN. This is consistent with previous research on conventional STN-DBS, which also suggests that the therapeutic effect of STN-DBS may be related to other pathways, such as the direct projection to the cortex and the mesolimbic dopaminergic system, despite a known dopaminergic-cholinergic imbalance that exists in axial movement disorder symptoms, especially in PD. However, it appears that magnetoelectric stimulation of the STN did not affect this particular pathway.

Nevertheless, this technology is still new and needs more research to optimize it, including designing a powering device and less invasive delivery routes to the brain. In addition, several challenges must be addressed before nanomaterials can be used in clinical applications. It needs to be explored whether multiple MENPs can be used simultaneously. Delivering nanomaterials to the brain requires noninvasive methods that do not damage neural tissue. Moreover, studying the optimal stimulation parameters and

patterns of MENPs would be essential to optimize their therapeutic effects while minimizing side effects. Overall, further research is needed to understand the potential of MENPs technology for PD treatment.

In summary, These findings provide valuable insight into the mechanisms of action of STN-DBS and its effects on the monoaminergic neurotransmitter system. The role of the serotonergic system in mood regulation and its relationship with STN-DBS-induced adverse mood effects highlight the importance of monitoring and managing the serotonergic system in PD patients. The lack of effect on the cholinergic system suggests that other pathways may be responsible for the therapeutic effects. STN-DBS surgery for PD has overall had a beneficial impact. It must, however, be taken with caution, monitored closely, and individually assessed for each patient while considering possible harmful effects. Future studies must fully detail how to enhance this process, reduce the negative impacts of these procedures, and uncover the precise neurotransmitters mechanism. In addition, developing less-invasive techniques could make it more practical and affordable for patients and healthcare.

أنظمة النواقل العصبي أحادي الأمين الآثار العلاجية والجانبية للتحفيز العميق للدماغ

مرض باركنسون هو اضطراب عصبي المنشأ يؤثر على أكثر من عشرة ملايين مريض في جميع أنحاء العالم. تشمل أعراضه الحركية الرئيسية الارتعاش، والصلابة، وبطء الحركة، وعدم التوازن الحركي. تم تشخيص لأول مرة في عام 1817 من قبل طبيب بريطاني يدعى جيمس باركنسون. بالإضافة إلى ذلك، يعاني مرضى شلل الرعاش من أعراض غير حركية مثل الإعاقات الإدراكية واضطرابات المزاج. ينتج المرض عن موت خلايا الدوبامين في المادة السوداء بارس كومباكتا. السبب الدقيق لموت هذه الخلية غير مفهوم جيدًا. في حين أنه لا يوجد علاج لمرض باركنسون، فإن العلاج ببدائل الدوبامين يمكن أن يعالج الأعراض الحركية فقط في المراحل المبكرة، ولكن له آثار جانبية وتقلبات لاستجابة للعلاج الدوائي، خاصة في المراحل المتقدمة.

التحفيز العميق للدماغ هو إجراء جراحي يتضمن زرع أقطاب كهربائية في مناطق معينة من الدماغ لتطبيق نبضة كهربائية يمكن أن تحسن الأعراض الحركية لدى مرضى شلل الرعاش. في الماضي، تم استخدام أساليب الجراحية التقليدية لعلاج هذه الحالات. ومع ذلك، فقد تم التخلي عنها بسبب طبيعتها المدمرة لأنسجة الدماغ بالمناطق المستهدفة وتوفر علاجات دوائية. لكن أدت التطورات في جراحة الأعصاب إلى تحسينات في معدات التحفيز وتقنيات التصوير العصبي، مما يجعل استهداف هياكل الدماغ أكثر دقة. علاوة على ذلك، حسنت تقنيات الفيزيولوجيا الكهربائية أثناء العملية أيضًا من استهداف مناطق الدماغ، الذي تم إعادة الضوء على التحفيز العميق للدماغ في عام 1993، ليكون أحد الخيارات العلاجية المعتبرة لمرض باركنسون.

تعد أنظمة النواقل العصبي أحادي الأمين ضرورية لتنظيم الوظائف المختلفة للجهاز العصبي المركزي، مثل المزاج والسلوك والحركة. من أهم النواقل العصبية الأولية أحادية الأمين هي الدوبامين السيروتونين. يمكن أن يؤدي عدم انتظام هذه الأنظمة إلى الإصابة بأمراض عصبية مثل شلل الرعاش. قد يتفاعل النظام الكولين أيضًا مع أنظمة أحادي الأمين، لا سيما في تنظيم الحركة. سيساعد فهم مشاركة هذه الأنظمة في علم الأعصاب على تحسين خيارات العلاج لتحسين الرعاية الصحية للمرضى. تلعب الناقلات العصبية أحادية الأمين، مثل الدوبامين السيروتونين، دورًا أساسيًا في الآثار العلاجية والجانبية الناتجة عن التحفيز العميق للدماغ لمرضي باركنسون. في هذي الاطروحة تم استعرض تأثيرات التحفيز العميق للدماغ على نشاط أنظمة الدوبامين السيروتونين، واتضح لنا أن التحفيز العميق للدماغ يحسن الأعراض الحركية. ومع ذلك، يمكن أن يسبب أيضًا آثارًا جانبية غير حركية مثل اضطرابات المزاج، والتي قد تكون مرتبطة بتأثيرها على نظام ن السير وتونين. يعد فهم تأثيرات التحفيز العميق للدماغ على أنظمة أحادي الأمين أمرًا ضروريًا لتحسين فوائده وتقليل آثاره الجانبية.

يمكن أن تعالج الأساليب الحديثة مثل الخطوط الحيوانية الوراثية والكيميائية والمعدلة وراثيًا قيود اكتشاف إطلاق جهاز الإرسال في الوقت الفعلي والتغيرات المرتبطة بالناقلات العصبية في المواقع العصبية البعيدة. بالإضافة إلى ذلك، تتيح هذه الأساليب استهداف أنواع خلايا معينة. لذلك أجرينا تجارب علم البصريات الوراثي لفحص التأثيرات الدقيقة للتحوير العصبي على إطلاق الناقل العصبي وتقييم التأثير التراكمي والمزمن للتحفيز العميق للدماغ على المكونات العصبية المحلية والبعيدة.

أثناء استخدام بروتين مؤشر الكالسيوم المشفر وراثيًا باستخدام ناقل فيروسي، يمكننا تقييم نشاط الخلايا العصبية والنمط الظاهري للخلايا العصبية. تشير النتائج التي توصلنا إليها إلى أن التحفيز العميق للدماغ على المدى الطويل قد يكون لها تأثير سلبي على نظام هرمون السيروتونين، وتحديداً عن طريق تثبيط نشاط عصبونات هرمون السيروتونين، والتي قد تساهم في الآثار الجانبية المرتبطة بالمزاج مثل الاكتئاب. سمح استخدام نهج محدد من نوع الخلية باستخدام الفئران المعدلة وراثيًا بتقييم آثار التحفيز العميق للدماغ على الخلايا مع دقة أكبر.

بالإضافة إلى ذلك، يشير اليأس السلوكي الذي لوحظ في الفئران المعالجة بـالتحفيز العميق للدماغ إلى التأثير السلبي على نظام هرمون السيروتونين. تسلط هذي النتائج الضوء على أهمية التحقيق في الآثار طويلة المدى للتحفيز العميق للدماغ على أنظمة التنظيم العصبي للدماغ، لا سيما تلك المتورطة في تنظيم الحالة المزاجية. يكشف تحليل الكيمياء المناعية بعد الوفاة أن التحفيز العميق للدماغ قد تسبب تغييرًا في النمط الظاهري للخلايا العصبية السيروتونين. هذا مدعوم من قبل الدراسات السابقة التي تبين أن التحفيز العميق للدماغ يمكن أن يؤدي إلى تغييرات في إطلاق الناقل العصبي والتعبير عن بروتينات محددة مرتبطة بالناقل العصبي في مناطق الدماغ المختلفة.

تشير المرونة العصبية إلى قدرة الدماغ على التكيف وإعادة تنظيم استجابةً المؤثرات الخارجية أو التجارب السلوكية. هناك أدلة متزايدة على أن المرونة العصبية تحدث أيضًا مع التحفيز العميق للدماغ على الدوائر العصبية المحلية ومناطق الدماغ البعيدة، مما يؤدي إلى تغييرات في إطلاق الناقل العصبي والنتائج السلوكية. قد تسبب التغيرات التي يسببها التحفيز في إطلاق الناقل العصبي تغيرات في اللدائن العصبية في الخلايا العصبية المستهدفة، مما يؤدي إلى إعادة التعرف على الأنماط الظاهرية للناقل العصبي. قد يساهم هذا في التأثيرات طويلة المدى التحفيز العميق للدماغ على كل من الأعراض الحركية وغير الحركية في مرض باركنسون.

تم استكشاف أساليب جديدة، مثل استخدام الجسيمات النانوية المغناطيسية الكهربية لتوصيل التحفيز العميق للدماغ لاسلكيًا. تولد الجسيمات النانوية المغناطيسية الكهربية مجالًا كهربائيًا استجابةً لمجال مغناطيسي، والذي يمكن أن يحفز الدماغ دون تعديل جيني. تشير النتائج التي توصلنا إليها إلى أن التحفيز الكهرومغناطيسي قد يكون له تأثيرات مماثلة التحفيز العميق للدماغ بالطرق التقليدي، خاصة فيما يتعلق بتعزيز الحركة وزيادة نشاط الخلايا العصبية. والجدير بالذكر أيضا أن هذه النتائج تشير أن أنظمة الناقل العصبي أحادي الأمين الرئيسية قد تلعب دورًا في آليات التحفيز الكهرومغناطيسي. بشكل عام، توفر هذه النتائج نظرة ثاقبة للآليات العصبية الكامنة وراء الآثار العلاجية للتنبيه الكهرومغناطيسي ويمكن أن تساعد في تطوير مناهج تعديل عصبي جديدة ليتم تطبيقها على مرض باركنسون. ومع ذلك، لا تزال هذه التكنولوجيا جديدة وتحتاج إلى مزيد من البحث لتحسينها، بما في ذلك تصميم جهاز توليد طاقة مغناطيسي معين وطرق توصيل أقل توغلًا إلى الدماغ. بالإضافة إلى ذلك، يجب معالجة العديد من التحديات قبل استخدام المواد النانوية في التطبيقات السريرية. يجب استكشاف ما إذا كان يمكن استخدام الجسيمات النانوية المغناطيسية الكهربية المتعددة في وقت واحد. يتطلب إيصال المواد النانوية إلى الدماغ طرقًا غير باضعة لا تلحق الضرر بالأنسجة العصبية. علاوة على ذلك، فإن دراسة معلمات التحفيز الأمثل وأنماط الجسيمات النانوية المغناطيسية الكهربية ستكون ضرورية لتحسين آثارها العلاجية مع تقليل الآثار الجانبية. بشكل عام، هناك حاجة إلى مزيد من البحث لفهم إمكانات تقنية الجسيمات النانوية المغناطيسية الكهربية في علاج مرض باركنسون. باختصار، توفر

هرمون السيروتونين في تنظيم الحالة المزاجية وعلاقته بتأثيرات الحالة المزاجية الضارة الناجمة عن التحفيز العميق أهمية مراقبة وإدارة نظام هرمون السيروتونين في مرضى باركنسون.

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BIOGRAPHY

Faisal Alosaimi was born on January 12, 1986, in a calm weather mountain city of Taif, Saudi Arabia. In 2006, After studying biomedical science for two years, he decided to quit and join the King Abdullah Scholarship program to study medicine at Maastricht University in 2009, where he graduated in 2014.

After returning to Saudi Arabia, he worked as a physician for almost two years. Throughout his medical career, he had a passion for teaching and research. In 2016, Faisal had the opportunity to become a teaching assistant at the Physiology Department of King Abdulaziz University. This experience further fueled his interest in academia and research.

In 2018, Faisal was awarded a scholarship from his home university to pursue his doctoral degree at the Neurosurgery Department of Maastricht University. This opportunity allowed him to continue his research and teaching pursuits while furthering his education. Faisal's journey has been shaped by his dedication to learning, teaching, and research.

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