PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
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Doctoral Thesis

Exploring the therapeutic potential of non-invasive spinal cord stimulation in a parkinsonian animal model

By

María Florencia Alamos Grau

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Thesis presented to the Pontificia Universidad Católica de Chile to qualify for the degree of PhD in Neuroscience

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Dedicated to

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
Agvm	ventral medial agranular cortex
APA	anticipatory postural adjustments
DBS	deep brain stimulation
DLS	dorsal lateral striatum
FoG	freezing of gait
GPi	globus pallidus pars interna
iSCS	invasive spinal cord stimulation
LB	Lewy body
LFP	local field potential
M1	primary motor cortex
MEP	motor evoked potentials
PD	Parkinson's disease
PIGS	postural instability and gait disorders
PSD	power spectral density
S1	primary somatosensory cortex
SNpc	substantia nigra pars compact
SSCS	superficial spinal cord stimulation
STN	subthalamic nucleus
SMA	supplementary motor area
TH	tyrosine hydroxylase
UPDRS	unified Parkinson’s disease rating scale
VPL	ventral posterolateral thalamic nuclei

1. ABSTRACT

1.1 ENGLISH VERSION

Invasive spinal cord stimulation has shown positive results for axial symptoms of Parkinson's disease, advancing its potential use as a novel treatment option. However, invasive spinal cord stimulation is an expensive and invasive surgical procedure. There are also associated risks such as infection, hemorrhages and lead migration. This is the first study to date to explore the effects of superficial spinal cord stimulation, a non-invasive technique that delivers current by electrodes located on the skin surface above the spinal cord at the thoracolumbar level. We used adult Sprague Dawley male rats with unilateral nigrostriatal dopaminergic lesions induced by the toxin 6-hydroxydopamine to evaluate the effectiveness of superficial spinal cord stimulation by measuring the neural activity in the cortico-basal ganglia circuit and the motor function. For the first outcome, we compared the local field potential (before, during, and after superficial spinal cord stimulation) from 5 different areas of the sensory-motor circuit, using the uninjured hemisphere as a control. For the second outcome, we evaluated motor performance during 11 days of superficial spinal cord stimulation, using different motor tests. We compared the motor performance between the superficial spinal cord stimulation group (parkinsonian rats treated by superficial spinal cord stimulation), sham group (non-treated parkinsonian rats), and peripheral group (parkinsonian rats treated with peripheral stimulation). The superficial spinal cord

stimulation was able to modulate the pathological neural activity and also motor function in some animals. However, due to the model's important limitations, further experiments in a bipedal model are needed to evaluate the motor impact of this therapy. Since this strategy is less expensive, safer, and easier to administer than other neuromodulation techniques, our findings might be relevant to the clinical practice and open a new area of research for Parkinson's disease treatment.

1.2 VERSIÓN EN ESPAÑOL

La estimulación invasiva de la médula espinal ha mostrado resultados positivos para los síntomas axiales de la enfermedad de Parkinson, potenciándola como una nueva opción de tratamiento. Sin embargo, la estimulación invasiva de la médula espinal es un procedimiento quirúrgico invasivo y costoso. También tiene riesgos asociados, como infecciones, hemorragias y migración de los electrodos. Este es el primer estudio hasta la fecha que explora los efectos de la estimulación superficial de la médula espinal, una técnica no invasiva que administra corriente mediante electrodos ubicados en la superficie de la piel sobre la médula espinal a nivel toracolumbar. Utilizamos ratas macho Sprague Dawley adultas con lesiones dopaminérgicas nigroestriatales unilaterales inducidas por la toxina 6-hidroxidopamina para evaluar la efectividad de la estimulación superficial de la médula espinal, midiendo la actividad neuronal en el circuito cortico-basal y la función motora. Para el primer objetivo, comparamos el potencial de campo local (antes, durante y después de la estimulación superficial de la médula espinal) de 5 áreas diferentes del circuito sensorio-motor, utilizando el hemisferio no lesionado como control. Para el segundo objetivo, evaluamos el rendimiento motor durante 11 días de estimulación superficial de la médula espinal, utilizando diferentes pruebas motoras. Comparamos el rendimiento motor entre el grupo de estimulación superficial de la médula espinal (ratas parkinsonianas tratadas con estimulación superficial de la médula espinal), el grupo sham (ratas parkinsonianas no tratadas) y el grupo periférico (ratas parkinsonianas tratadas con estimulación periférica). La estimulación superficial de la médula espinal pudo modular la actividad

neural patológica y también la función motora en algunos animales. Sin embargo, debido a las importantes limitaciones del modelo, experimentos adicionales en un modelo bípedo serán necesarios para demostrar el impacto de esta terapia en la función motora. Dado que esta estrategia es menos costosa, más segura y fácil de administrar que otras técnicas de neuromodulación, nuestros hallazgos podrían ser relevantes para la práctica clínica y abrir una nueva área de investigación para el tratamiento de la enfermedad de Parkinson.

2. INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting 1% to 2% of the population over the age of 60 years (Kalia & Lang, 2015; Tysnes & Storstein, 2017). Its worldwide prevalence is estimated at 6.9 million and could be doubled in 2040 (Pringsheim et al., 2014; Dorsey & Bloem, 2018). Pathophysiological findings in PD are related to the degeneration of dopaminergic cells in the substantia nigra pars compacta (SNpc) (Hughes et al., 1992), and result in low striatal dopamine levels and abnormal oscillatory and synchronous neural activity in the basal ganglia and cortex (Williams et al., 2010; Williams et al., 2002). The cardinal clinical manifestations of PD include bradykinesia, rigidity, resting tremor, and balance impairment (Kalia & Lang, 2015).

Available treatment options for PD have critical limitations. For instance, levodopa, the treatment of choice to date, is usually effective in the early stages of the disease, improving PD's motor symptoms (Lewitt, 2008; Lees et al., 2009). Unfortunately, levodopa has proven to be less efficient long term and is accompanied by complications such as motor fluctuations and dyskinesia (Goetz et al., 2005; Lewitt, 2008). Additional therapeutic strategies, such as deep brain stimulation (DBS) of either the subthalamic nucleus (STN) or globus pallidus pars interna (GPi), have become a recognized treatment for PD symptoms (Goetz et al., 2005). Nevertheless, probably because of the highly invasive nature of the device implantation and its need for complex and high-priced technologies, only a minor portion of all patients diagnosed with PD are eligible

candidates for DBS (Morgante et al., 2007). Another therapeutic challenge in PD is the control of axial symptoms, as they respond poorly to both levodopa therapy and DBS (Goetz et al., 2005; Hamani et al., 2005; A. Williams et al., 2010; Grabli et al., 2012; Fasano et al., 2015). In this context, invasive spinal cord stimulation (iSCS) has been proposed as a new therapeutic strategy to improve motor symptoms in PD, especially those that are less responsive to conventional treatment options.

A newer therapeutic option is invasive spinal cord stimulation (iSCS), a less invasive alternative for the treatment of PD with a potential comparable efficacy to DBS (Follett et al., 2010; Agari & Date, 2012). It has been shown to alleviate motor symptoms in rodent and primate models of Parkinson's disease (Fuentes et al., 2009; Santana et al., 2014; Yadav et al., 2014; Shinko et al., 2014; Brys et al., 2016; Kuwahara et al., 2020). Furthermore, several reports of clinical cases suggest that this technique might be beneficial for the motor symptoms in Parkinson's patients, mainly axial and gait impairments (Agari & Date, 2012; Fenelon et al., 2012; Soltani & Lalkhen, 2013; Landi et al., 2013; Mitsuyama et al., 2013; Hassan et al., 2013; Nishioka & Nakajima, 2015; Pinto de Souza et al., 2016; Akiyama et al., 2017; Samotus et al., 2018; de Lima-Pardini et al., 2018; Kobayashi et al., 2018; Mazzone et al., 2019). The mechanisms underlying this therapeutic effect could be related to the desynchronization of neuronal activity within the cortico-basal ganglia circuitry and the global reduction in the coherence between these structures in the so-called beta- frequency (8-30 Hz) (Santana et al., 2014). Despite these results' significance, the technique has not been widely applied since it still requires a surgical procedure and therefore is not exempt from complications and risks associated with surgery and chronic implants.

The goal of this project is to explore the role of non-invasive neuromodulation method that targets the spinal cord on motor symptoms and supraspinal sensory-motor circuits. To this end, we placed two thoracolumbar electrodes on the skin parallel to the spinous process and one electrode on the midline of the abdomen skin surface. We called this method superficial spinal cord stimulation (SSCS). An experimental model of parkinsonism induced by injecting a neurotoxin 6-hydroxydopamine (6-OHDA) in a single brain hemisphere of the rat was used to analyze the motor function and the neural activity in the cortico-basal ganglia-thalamic circuit and motor function. Since this SSCS approach is relatively inexpensive, safe, and simple, it is expected that our current work's positive outcomes could be promptly translated into clinical practice.

3. BACKGROUND

3.1 PARKINSON DISEASE: AN OVERVIEW

3.1.1 History

In 1817 the British physician James Parkinson in his monograph "An essay on the shaking palsy," was the first one describing the main clinical manifestation of Parkinson's disease (Parkinson, 1817). Later, Jean-Martin Charcot, the father of neurology, in the 19th century, completed the clinical description and gave credit to Parkinson by referring to the disease as maladie de Parkinson (Parkinson's disease) (Kempster et al., 2007; Charcot, 2011). More than 100 years after Parkinson's original description by Parkinson, a loss of cells in the substantia nigra was recognized in patients with Parkinson's disease (PD) (Buda et al., 2009), and later dopamine was proposed as the affected neurotransmitter by the nobel prize Arvid Carlsson (Carlsson et al., 1958; Carlsson, 2002).

3.1.2 Epidemiology

Parkinson's disease is the second most frequent neurodegenerative disorder, following Alzheimer's disease, affecting 1% to 2% of the population over the age of 60 years (Kalia & Lang, 2015; Tysnes & Storstein, 2017). The median age of onset is 60 years, and the average length from diagnosis to death is 15 years (Katzenschlager et al., 2008; Lees et al., 2009). Men are about 1.5 times more likely than women to develop

Parkinson's disease; however, this difference is not consistent across different studies (Twelves et al., 2003; Lees et al., 2009).

3.1.3 Physiopathology and electrophysiological findings

Parkinson's disease is a neurodegenerative disorder characterized by a basal ganglia dysfunction resulting from the degeneration of neurons in the dopaminergic nigrostriatal pathway (Hughes et al., 1992) in association with the development of intracytoplasmic protein inclusions termed Lewy Body (LB) and α-synuclein aggregation (Dickson et al., 2009; Lees et al., 2009; Goedert et al., 2012). By the time PD motor symptoms are clinically evident, 50 to 70% of dopaminergic neurons in substantia nigra pars compact (SNpc) are lost (Marsden, 1990; Lang & Lozano, 1998; Dauer & Przedborski, 2003; Ross et al., 2004). This finding suggests it might be a critical preclinical stage in which adaptive mechanism probably exists. Neuronal loss and α-synuclein aggregation occur in many other brain regions, including the locus ceruleus, nucleus basalis of Meynert, pedunculopontine nucleus (PPN), raphe nucleus, the dorsal motor nucleus of the vagus, amygdala, and hypothalamus (Dickson et al., 2009). LB progression has been hypothesized to occur in a stereotyped pattern, affecting the central nervous system in a caudal-to-rostral direction over time (Braak & Braak, 1991; Braak et al., 2003; Kalia & Lang, 2015). This expansion model is interesting because the proposed progression seems to explain PD's clinical course (Braak & Braak, 1991; Braak et al., 2003; Kalia & Lang, 2015). Recent studies suggest that PD pathology is more complex than neurodegeneration due to Lewy pathology

alone, and inflammation, mitochondrial dysfunction, and oxidative stress are also implicated in cell dysfunction and death (Lotharius & Brundin, 2002; Shulman et al., 2011).

Additionally, there is emerging evidence suggesting that PD results from a dysfunction in a multilevel, interconnected network rather than a restricted basal ganglia deficit (de Schipper et al., 2018). Brain recording in animal models and patients with PD have shown the existence of pathologic oscillatory activity in different areas of the sensory-motor network, including cortical and subcortical structures, supporting the view of network dysfunction (Brown et al., 2004; Hutchison et al., 2004; Eusebio & Brown, 2007). Oscillatory activity reflex single neurons, local neuronal populations, and multiple neuronal assemblies distributed across brain regions that often show rhythmic activity over time (Mathalon & Sohal, 2015). In general, any oscillation can be described as a combination of sine waves with different frequencies (cycles per second or hertz) and peak amplitudes (power = amplitude squared). Neural oscillations underlie several physiological processes, such as circadian rhythms, memory, attention, and motion. Additionally, the oscillatory activity can be disrupted or exacerbated by several pathological events (Sarnthein et al., 1998; Hutchison et al., 1998; Varela et al., 2001).

In healthy subjects, before and during voluntary movement, the subthalamic nucleus (STN), globus pallidus pars interna (GPi), and cortex form a long-range functional network that resonates at 60–80 Hz (Brown et al., 2001). This frequency range has shown to be prokinetic (Brown, 2003). By contrast, the frequencies of about 11–30 Hz and 3–10 Hz are considered to be predominantly antikinetic (Brown, 2003; Hutchison

et al., 2004). In contrast, in PD, an excessive synchronization at a lower frequency in the basal ganglia cortical loop has been described (Hutchison et al., 2004). In fact, both animals and human studies have demonstrated an abnormally synchronized oscillatory activity in the beta band (between 8 -30 Hz) at multiple levels in the voluntary sensory-motor circuit (Brown et al., 2004; Priori et al., 2004; Hutchison et al., 2004; Heinz et al., 2004; Kuhn et al., 2004, 2005; Williams et al., 2005; Meissner et al., 2005; Brown & Williams, 2005; Sharott et al., 2005; Silberstein et al., 2005; Foffani et al., 2005; Alonso-Frech et al., 2006; Fogelson et al., 2006; Kühn et al., 2006; Marceglia et al., 2006; Hammond et al., 2007). Therefore, new PD therapies aim to regulate this network dysfunction.

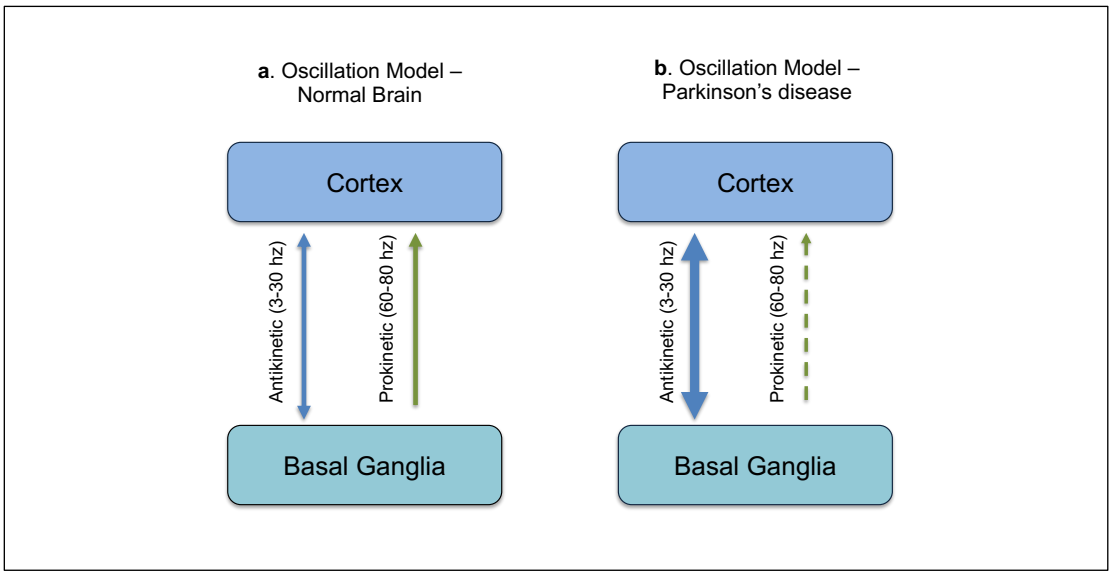

Figure 1. Oscillation model of normal brain and Parkinson's disease.

(A) In a healthy brain exists different frequency bands of oscillations in the basal ganglia–cortical network associated with an akinetic (blue arrow) and prokinetic (green arrow) effects. **(B)** In PD, the striatum's dopamine deficit is related to the presence of pathological oscillations in the loop. Frequencies in the range of 3 to 30 Hz, particularly

in the beta band, arise in the basal ganglia and spread to the cortex, associated with an antikinetic effect. In parallel, oscillations over 60 Hz (that are associated with movement and therefore known as prokinetic) are suppressed or absent in PD (thin dashed green arrow). Adapted from Hutchison, William D., Dostrovsky, J. O., Walters, J. R., Courtemanche, R., Boraud, T., Goldberg, J., & Brown, P. (2004). Neuronal oscillations in the basal ganglia and movement disorders: evidence from the whole animal and human recordings. J Neurosci, 24(42), 9240–9243.

3.2 PARKINSON DISEASE: CLINICAL MANIFESTATIONS AND TREATMENTS

3.2.1 Clinical features

Patients with PD usually present motor and non-motor symptoms. The onset is gradual, and the earliest symptoms might be unnoticed for several years (Lees et al., 2009). From the motor standpoint, PD is characterized by bradykinesia, resting 5-6 Hz tremor, rigidity, and postural and gait disturbance (Lees et al., 2009; Massano & Bhatia, 2012). The later (gait impairment) in conjunction with other symptoms like dysarthria, swallowing, and postural instability are part of a group of clinical manifestations known as axial symptoms (Carne et al., 2005; Van der Marck et al., 2009; Coelho & Ferreira, 2012). These symptoms respond poorly to conventional treatments as levodopa and deep brain stimulation (DBS) (Goetz et al., 2005; Hamani et al., 2005; A. Williams et al., 2010; Grabli et al., 2012; Fasano et al., 2015). They significantly impact the PD patients' quality of life and are a risk factor for institutionalization and mortality (Carne et al., 2005; Coelho & Ferreira, 2012; Bouça-Machado et al., 2020).

A disabling phenomenon that is part of axial symptoms and commonly affect locomotion in PD is the postural instability and gait disorders (PIGD). Cortical regions

like the supplementary motor area (SMA), basal ganglia, and brainstem structures like the pedunculopontine nucleus (PPN) may play a key role in regulating locomotion and can be dysfunctional in PIGD (Takakusaki, 2017). Normal gait requires anticipatory postural adjustments (APA) and compensatory postural adjustments before and after each step (De Lima-Pardini et al., 2017). In PD, the intention to walk may be uncoupled from APA's triggering, resulting in the freezing of gait (FoG) (de Lima-Pardini et al., 2018). FoG is part of PIGS and is characterized by sudden and unpredictable gait failure despite the intention to walk (Pozzi et al., 2019). Patients feel as their feet are glued to the ground when they intend to walk forward (Gao et al., 2020). Different studies showed that FoG might be associated with SMA dysfunction that compromises brain circuits involved in APA control (Jacobs et al., 2009; Bolzoni et al., 2015; Takakusaki, 2017). In turn, this cortical area is connected to the PPN, a brainstem locomotor region that participates in the control of movement initiation and body balance (Aravamuthan et al., 2007; Ballanger et al., 2009). Inputs from ascending lemniscal and extralemniscal pathways to the brainstem and thalamus can modulate SMA and the PPN (Takakusaki, 2017; de Lima-Pardini et al., 2018). PIGS control is critical because it can cause falls and increase morbimortality in PD (Bloem et al., 2004; Delval et al., 2014). However, this phenomenon's treatment remains a challenge in clinical practice (Rogers, 1996; Pötter-Nerger & Volkmann, 2013; Müller et al., 2019).

The non-motor symptoms of PD are diverse, including neuropsychiatric features, sleep disorders, dysautonomia, sensory dysfunctions, pain, fatigue, and cognitive impairment (Silva et al., 2005; Emre et al., 2007; Poewe, 2008; Castelo-Branco et al.,

2009; Chaudhuri & Schapira, 2009; S. Y. Lim et al., 2009; Gallagher et al., 2010). The later, frequently manifested as executive dysfunction, can alter the integration of sensory information and motor planning prosses necessary to dynamics motor tasks like gait (Lord et al., 2010; Barbosa et al., 2016).

3.2.2 Treatments

Parkinson's disease is still an incurable, progressive disease, but available treatments substantially improve quality of life and functional capacity. We will summarize the evidence on levodopa (the gold standard treatment for motor symptoms of PD) and some neuromodulation options.

3.2.2.1 Levodopa (3,4-dihydroxy-l-phenylalanine)

It remains the most effective therapy for controlling PD's motor symptoms (Goetz et al., 2005; Jankovic, 2008). Levodopa, a dopamine precursor, which is indicated to compensate dopamine deficiency, was introduced in 1968 by George Cotzias and coworkers (Cotzias et al., 1969). Although the recognition of levodopa's effectiveness needed several years of research, the drug finally became accepted as the preferred treatment for motor signs and symptoms of PD (Cotzias et al., 1969; Olanow et al., 2004). Usually, motor symptoms improve by 20–70% at the beginning of therapy (Lees et al., 2009). However, not all clinical features of PD are improved by levodopa. Speech, swallowing, and postural instability can improve initially, but axial symptoms are usually less responsive and seem to escape from long-term control (Katzenschlager

& Lees, 2002; Hely et al., 2008). Additionally, patterns of response to levodopa can change over time, and patients can experience motor fluctuations and dyskinesias (Nutt & Holford, 1996; Goetz et al., 2005). These complications are present in about 50% of levodopa-treated patients who have received the medication for more than five years, 80% of patients treated for ten years, and almost all patients with a young-onset disease (Golbe, 1991; Forno, 1996; Fahn, 2000). Consequently, levodopa is associated with many problems, and the development of new therapeutic lines is crucial. During the last years, scientific research in PD has been focused on new strategies that can offer better control of symptoms that are less responsive to current treatment options and, at the same time, avoid the complications that chronic use of levodopa implies. In this context, neuromodulation emerges as a promising technic in the field.

3.2.2.2 Deep brain stimulation

Over the past few decades, after the pioneering work of Irving Cooper and Alim-Luis Benabid in the early 1990s, deep brain stimulation (DBS) has been explored for medication-refractory hypokinetic and hyperkinetic movement disorders, other neurological and psychiatric diseases (Cooper et al., 1980; Benabid et al., 1991).

DBS is a surgical procedure that modulates the neural circuit using electrical current and therefore is part of neuromodulation technics. The procedure involves the placement of microelectrodes in different brain areas through a combination of stereotactic and neuroimaging techniques (Sironi, 2011). In PD, the most commonly used targets are the subthalamic nucleus (STN) or globus pallidus pars interna (GPi)

(Goetz et al., 2005). It modifies the irregular neuronal activity of the target region of the brain (Voges et al., 2007; Benabid et al., 2009; Bötzel & Kraft, 2010). DBS has shown significant improvement in PD's cardinal motor signs, including tremor, bradykinesia, and rigidity, with variable outcomes in medication-refractory gait freezing, postural instability, and gait impairments (Follett et al., 2010; Miocinovic et al., 2013). As an invasive procedure, it is not exempt from surgical complications. Among the most worrisome adverse effects of DBS placement are infection and intracranial hemorrhage (Voges et al., 2006; Sillay et al., 2008; Fenoy & Simpson, 2012; Zrinzo et al., 2012). Finally, another significant limitation of this treatment is the high cost of the therapy. In the United States, DBS therapy cost approximately $90,000 per patient during the first year of treatment (Stroupe et al., 2014).

In conclusion, DBS has emerged as a valuable complement to dopamine replacement therapy in PD's motor treatment (Benabid, 2003). However, possibly because of the highly invasive nature of this surgical procedure and its need for additional complex and costly technologies, only a small fraction of all patients with PD who could benefit from this therapy are eligible for implantation (J Voges et al., 2006; Morgante et al., 2007; Zrinzo et al., 2012; Stroupe et al., 2014). In this context, the proposal of new neuromodulation targets that can offer a less risky and more accessible alternative and, at the same time, can handle unresponsive symptoms of PD patients has become a central goal in the field.

3.2.2.3 Spinal cord stimulation

Shealy introduced the use of invasive spinal Cord Stimulation (iSCS) in 1967 (Shealy et al., 1967). Since then, iSCS has been broadly applied various pain conditions (Barolat, 1995; Taylor et al., 2005). However, in the field of movement disorders, specifically in Parkinson's disease, the use of iSCS is more recent. In 2009, Fuentes et al. were the first to show that high-frequency stimulation at the upper thoracic level could restore locomotion in rodent models of PD (Fuentes et al., 2009). The iSCS has proved to be effective also in primate model of PD, particularly for motor signs known to be challenging to treat with DBS and levodopa, including posture impairment, gait disturbance, and altered speed of locomotion (Santana et al., 2014). Remarkably, the general effect observed in motor symptoms was comparable to the reported long-term reduction in the UPDRS III (Unified Parkinson's Disease Rating Scale) score by DBS (Follett et al., 2010; Santana et al., 2014). These results encouraged various investigators to apply iSCS in PD patients to control motor symptoms, particularly, gait disorders.

Until today, a total of 66 reported patients, including clinical cases and trials, have received iSCS stimulation for treating Parkinson's disease (Thevathasan et al., 2010; Weise et al., 2010; Agari & Date, 2012; Fenelon et al., 2012; Soltani & Lalkhen, 2013; Landi et al., 2013; Mitsuyama et al., 2013; Hassan et al., 2013; Nishioka & Nakajima, 2015; Pinto de Souza et al., 2016; Akiyama et al., 2017; Samotus et al., 2018; de Lima-Pardini et al., 2018; Kobayashi et al., 2018; Mazzone et al., 2019; Prasad et al., 2020).

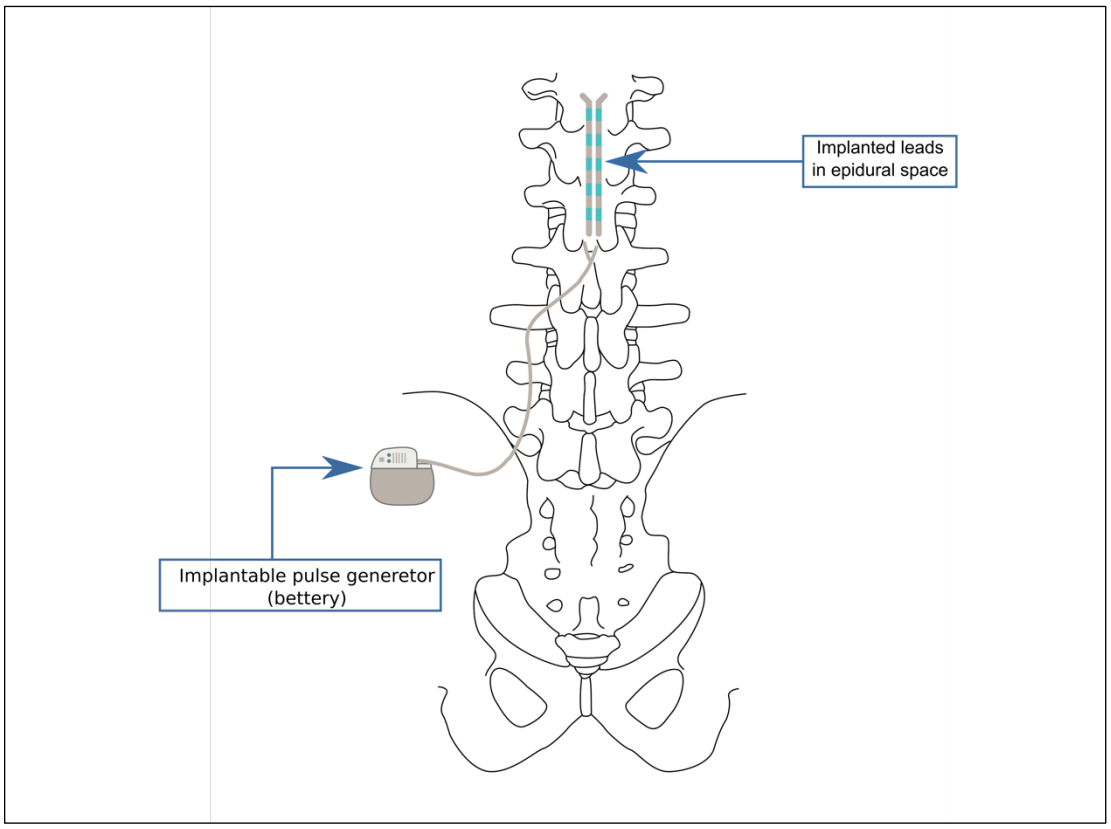

Figure 2. Invasive spinal cord stimulation is implanted in the epidural space and connected to an implantable pulse generator.

Of these, only eight patients did not show any effect (Thevathasan et al., 2010; Prasad et al., 2020), but 58 experienced amelioration of their motor symptoms (Weise et al., 2010; Agari & Date, 2012; Fenelon et al., 2012; Soltani & Lalkhen, 2013; Landi et al., 2013; Mitsuyama et al., 2013; Hassan et al., 2013; Nishioka & Nakajima, 2015; Pinto de Souza et al., 2016; Akiyama et al., 2017; Samotus et al., 2018; de Lima-Pardini et al., 2018; Kobayashi et al., 2018; Mazzone et al., 2019). Of these 66 PD patients treated with iSCS, 37 also presented chronic pain syndromes either not related or secondary to PD, and iSCS also reduced pain in these patients (Weise et al., 2010; Agari & Date, 2012; Fenelon et al., 2012; Landi et al., 2013; Mitsuyama et al., 2013; Soltani &

Lalkhen, 2013; Hassan et al., 2013; Nishioka & Nakajima, 2015; Akiyama et al., 2017; Kobayashi et al., 2018). Remarkably, one of these patients kept improving her motor scores across two years of iSCS (Hassan et al., 2013). Finally, 29 patients without chronic pain syndromes associated improved motor outcomes (Pinto de Souza et al., 2016; Samotus et al., 2018; de Lima-Pardini et al., 2018; Mazzone et al., 2019), especially axial symptoms such as freezing of gait, other gait impairments, or severe posture deficits. Consequently, iSCS seems to yield positive results for PD motor symptoms, particularly those complex to improve with conventional pharmacological treatments or DBS.

The functional recovery observed in animal models was paralleled by a disruption of aberrant low-frequency synchronous oscillations present in the cortex and basal ganglia, leading to the appearance of neuronal activity patterns that resemble the state typically preceding spontaneous initiation of movement (Fuentes et al., 2010; Santana et al., 2014). The mechanism behind this effect can be explained by the modulation of the afferent fibers of the dorsal column, and possibly the activation or excitability change of the medial lemniscal pathway, causing changes in the thalamic-basal ganglia-cortical network. (Fuentes et al., 2010; Santana et al., 2014; de Andrade et al., 2016). Additionally, local spinal circuits might be modulated by iSCS, thus, facilitating the influence of descending control systems, overall improving the motor function (de Andrade et al., 2016).

The progress in motor performance was matched with the maintenance of a higher density of dopaminergic fibers in the striatum and neuronal cell count in the SNc of treated rats (Yadav et al., 2014). The neuroprotective effect can be partially explained

by the upregulation of angiogenic and anti-inflammatory mechanisms (Shinko et al., 2014; Kuwahara et al., 2020). These results indicate that long-term iSCS is associated with functional and structural recovery in a classic animal model of PD, suggesting a potential role in regulating the disease progression in humans.

In summary, consistent data support the promising role of this method as a potential therapy for PD. However, the technique has not been widely applied at the date since it still requires a surgical procedure and therefore is not exempt from complications and risks associated with surgery and chronic implants. Therefore, this project aims to develop a non-invasive method to electrically stimulate dorsal columns in the spinal cord and improve PD's motor symptoms. Because this is a low-cost, safe, and easy-to-implement technique, it could be available in a short time and benefit a large number of patients.

4. HYPOTHESES AND OBJECTIVES

4.1 HYPOTHESIS

4.1.1 General hypothesis

SSCS reduces the abnormal slow oscillatory activity of the sensory-motor circuit and improves motor symptoms in a parkinsonian animal model.

4.1.2 Specific hypotheses

- Specific parameters of SSCS reduce slow oscillatory activity in the beta frequency of the sensory-motor circuit in a unilateral 6-OHDA rat model.

- SSCS that causes changes in cortical activity have improvements over motor symptoms in a unilateral 6-OHDA rat model

4.2 OBJECTIVES

4.2.1 General objective

Analyze the therapeutic effect of SSCS on the oscillatory activity and motor symptoms in a unilateral 6-OHDA rat model.

4.2.2 Specific objectives

In order to explore the therapeutic effect of SSCS, the experiments were divided into three sections.

1. In vivo electrophysiology experiments

Specific objective:

- To identify the optimal SSCS stimulation parameters that cause changes in slow oscillatory cortical and subcortical activity.

Procedural objectives:

- To design and build electrodes for in vivo electrophysiological recording in thalamic nuclei, striatum, ventral medial agranular cortex, primary somatosensory, and primary motor cortices in the left and right hemisphere.
- To design stimulation electrodes arrangements.
- To design different stimulation protocols (using a different combinations of the following variables: duration, intensity, and polarity).

- To probe stimulation protocols in not lesioned rats to observe rat tolerance and discharge any side effects produced by the stimulation.
- To stimulate and record lesioned rats with different electrical stimulation protocols.
- To analyze the effect of the different SSCS protocols on oscillatory brain activity (Local field potential) and determine which protocol causes the most overt cortical activity changes.

2. Behavioral experiments

Specific objective:

- To explore the effect of SSCS on motor symptoms.

Procedural objectives:

- To assess the effect of SSCS using the cylinder test.
- To assess the effect of SSCS using the rotameter test.
- To assess the effect of SSCS using the stepping test.

3. Histological analysis

Specific objective:

- To corroborate the dopaminergic loss in the hemisphere lesioned with 6-OHDA.

Procedural objectives:

- To analyze the density of striatal projections of dopaminergic cells after a 6-OHDA lesion in the lesion and un-lesioned hemisphere.
- To compare the density of striatal projections between groups (SSCS group, sham stimulation group, and peripheral group).

5. MATERIALS AND METHODS

5.1 ANIMALS

Adult male Sprague-Dawley rats weighing 250–350 g at the beginning of the experiment were used. They were housed one per cage in a temperature and humidity-controlled room, exposed to a twelve-hour light/dark cycle with free access to food and water. For the electrophysiological experiments, six rats were analyzed. For the behavioral analysis, a total of 30 rats were used.

5.2 THE 6-OHDA ANIMAL MODEL

All rats were anesthetized with 1–3% isoflurane (Baxter Corp., USA) and placed in a stereotaxic instrument (WPI, USA). Animals received a desipramine injection (25 mg /kg, i.p.; Sigma, Germany) to minimize the uptake of the 6-OHDA by noradrenergic neurons. Two µL of 6-OHDA (4 mg/ml dissolved in 0.1% ascorbate saline (Sigma, USA) was injected into the right striatum with a 28 G Hamilton syringe, at 0.5 µL/min (Shinko et al., 2014). The anteroposterior, mediolateral and dorsoventral lesion coordinates were: +1.0, +/-3.0, -5.0; -0.1, +/ -3.7, -5.0 and -1.2, +/-4.5, -5.0 (Winkler et al., 2002). After the injection, the syringe was left in place for five additional minutes before retracting it slowly (1 mm/min).

5.3 RECORDING ELECTRODES

A particular configuration of microelectrode arrays was specially designed to allow simultaneous recordings of the extracellular activity of large numbers of single neurons, distributed across up to five different structures (ventral posterolateral thalamic nuclei [VPL], dorsal lateral striatum [DLS], ventral medial agranular cortex [Agvm], primary somatosensory [S1] and primary motor cortices [M1]) that participate in the rat sensory-motor system (Nicolelis et al., 1997). Each multielectrode was made of 32 tungsten electrodes (50-µmmicrowire diameter), attached to an Omnetics connector (Omnetics Connector Corp., USA). Two microelectrodes arrays (right and left hemisphere, respectively) were implanted into the target brain areas to aid stereotaxic surgical procedures. First, anesthesia was induced and maintained with 1–3% isoflurane. During anesthesia, the rat was moved to a stereotaxic apparatus and then fixed in a head holder by two ear bars and an incisor fixer to reduce motion (Wang et al., 2011). The head was shaved and then cleaned with a povidone-iodine solution. A single midsagittal incision of the skin was performed to access the surface of the skull. Following the retraction of soft tissue, the periosteal of the surface of the skull was removed, and the bone surface was scrubbed with saline solution until no sign of blood was observed (Wang et al., 2011). The skull's surface was dried, and a series of 2 small craniotomies in each side (left and right) was made with a high-speed dental drill at the stereotaxic coordinates required for the implantation of electrode arrays (Nicolelis et al., 1997). A second series of six craniotomies were made for stainless steel screws to be firmly attached to the skull (Wang et al., 2011). Then, each electrode microarray

was slowly lowered (1 μm/s) into the target brain areas (Wang et al., 2011). The implant was fixated with dental acrylic to stainless screws in the skull (Halje et al., 2012). These screws also functioned as connection points for the electrode ground wire (Halje et al., 2012). Finally, the skin was loosely sealed around the probes, and the animal was transported to a recovery cage. Enrofloxacine (5 mg/kg. s.c. q 24 hr) was administered five days before surgery to prevent infections. Post-operative pain was managed with one dose of ketoprofen (2 mg/kg s.c. q 24 hours) followed by ibuprofen (10 mg/kg oral administration q 24 hours) 3 days. Electrophysiological recordings started eight days after the surgery.

5.4 SPINAL CORD STIMULATION PROCEDURES

Rats were connected to two electrodes (2.5 x 0.5 cm) placed on the skin parallel to the T10-L4 spinous process and one electrode (2.5 x 1 cm) placed on the abdomen skin's midline surface. Electrodes are made of stain. Two-channel stimulator STG4002 (Multichannel system, Germany) was used to deliver stimulation through the surface electrodes. Later, different stimulation protocols were applied for 5 minutes (experiments of electrophysiology) and 30 minutes (behavioral experiments).

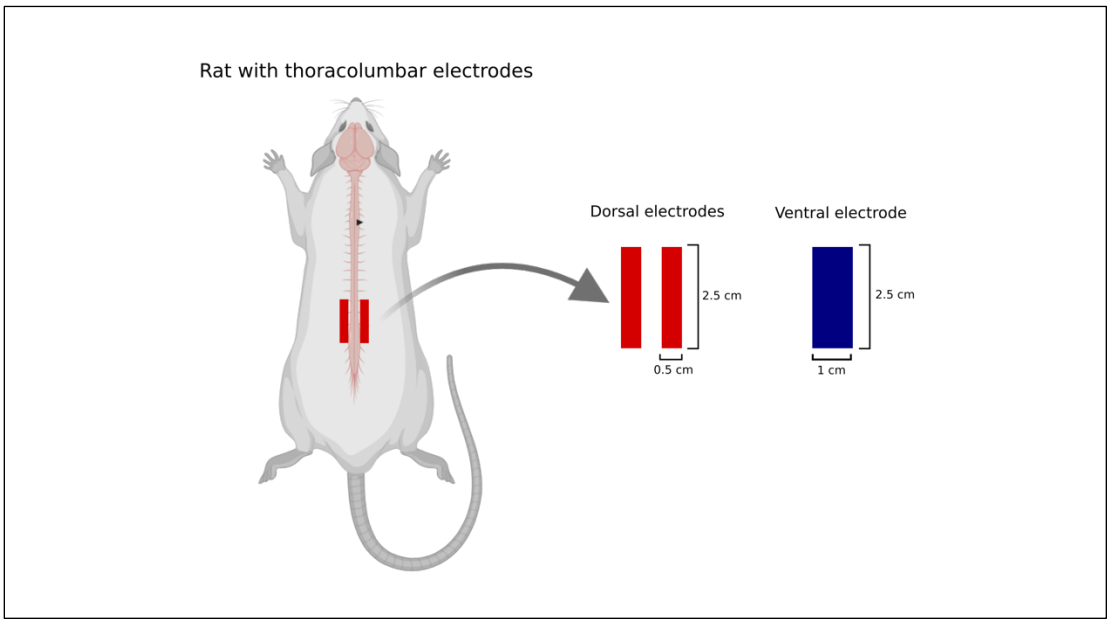

Figure 3. Electrodes design and configuration.

Representation of dorsal (red rectangles) and ventral (blue rectangles) thoracolumbar electrodes and its dimensions.

5.4.1 Direct Current Stimulation Protocols.

We applied cathodal and anodal direct current stimulation with an intensity of:

- 375 µA (0.15 µA /cm2), resulting in a total charge of 0.14 C/cm^2.
- 2500 µA (0.64 µA /cm2), resulting in a total charge of 0.61 C/cm^2.

5.4.2 Alternate- Current Stimulation Protocol

Biphasic charge-balanced square pulses (each pulse with 400 us in duration separated by 100 us) were applied. For each frequency (20, 150, and 300 Hz), we probed an intensity of 375 µA.

5.4.3 Mixed- Current Stimulation Protocol

Consists in applying direct current stimulation and periodic pulses of current, and then apply high-frequency biphasic pulses mounted over the direct current. We tried two types of mixed protocols differentiated by the intensity of anodic current (+375 μA and +2500 μA). In both cases, the direct-current had 300 Hz biphasic pulses.

All the stimulation parameters selected are clearly below the threshold for possible tissue damage (Nitsche et al., 2003; Liebetanz et al., 2009). At the beginning and end of each stimulation protocol, a ramp of 30 seconds was made to achieve the maximum intensity and avoid any discomfort sensation. These specific spine stimulation protocols were chosen because they have demonstrated to elicit motor activity or change cortical activity (Aguilar et al., 2011; Cogiamanian et al., 2012; Santana et al., 2014; Gerasimenko et al., 2015; Faunes et al., 2015; Bocci et al., 2015).

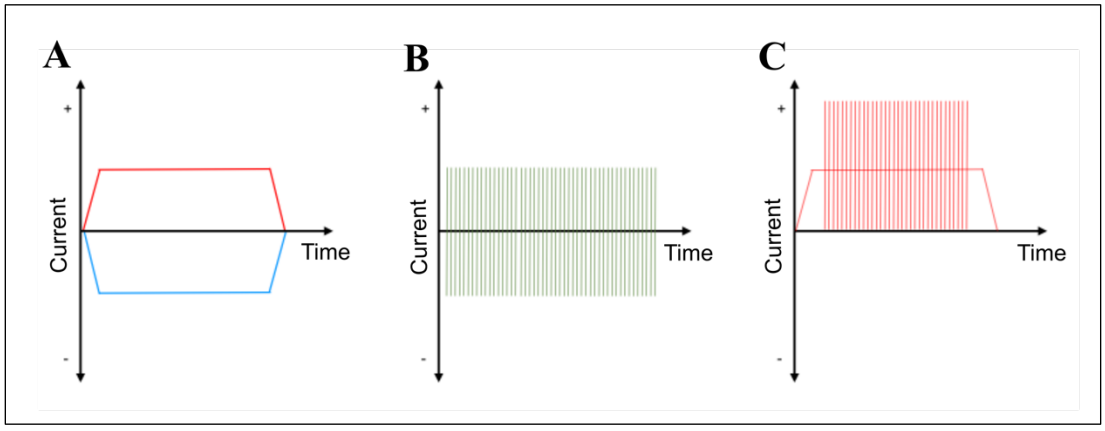

Figure 4. SSCS stimulation protocols.

The protocols can be divided into three types: **(A)** Direct-current stimulation protocols. A red line represents anodic stimulation and a blue line cathodic stimulation. Anodic and cathodic stimulation were tried separately at two different intensities (375 μA or 2500 μA) for cathodic and anodic stimulation. **(B)** Alternate-current stimulation protocols. Green lines represent biphasic charge-balanced square pulses (each pulse with 400 us duration separated by 100 us). We tried 20, 150, and 300 Hz pulses frequencies with an intensity of 375 μA. **(C)** Mixed-stimulation protocols, which consist of apply direct current stimulation followed by periodic pulses of current (red lines). We proved mixed protocols of anodic current (375 μA or 2500 μA) followed by 300 Hz pulses. In total, we analyzed nine different protocols (four of direct-current stimulation, three of alternate-current stimulation, and two of mixed-stimulation).

5.5 SIGNAL ACQUISITION

Local field potentials (LFP) were acquired using a 64-channel RHD2000 Evaluation System (Intan Technologies, CA). LFP was pre-amplified (5000Å~), filtered (0.1–15.000 Hz), and sampled at 30.000 Hz.

5.6 DATA ANALYSIS

Neural activity was analyzed using the local field potentials (LFP). To obtain each channel's local field potentials the signal was low-pass filtered with a cut-off frequency of 150 Hz, down-sampled to 1000 Hz, and high-pass filtered with a cut-off frequency of 0.1 Hz. We did a standardization using a z-score that was calculated without artifacts. Then, we visually detected and eliminated defective channels. This detection was done observing the time series and power spectral. We referenced each channel using the average LFP of all channels. Based on the LFP, spectrograms were constructed by calculating power spectral density (PSD) with an IRASA method (Wen

& Liu, 2016), using a window length of 1 s and a window overlap of 50%. The PSD for each area was calculated using the average signal from the same area's total channels.

To evaluate the parkinsonian state, we compared the PSD of areas from the un-lesioned hemisphere versus areas from the lesioned hemisphere. Next, to assess the effect of SSCS, we compared the LFP of each area in three moments: before, during, and after stimulation. For each moment, we used the same amount of time.

5.7 BEHAVIORAL TESTS

5.7.1 Cylinder Test

The rats were placed in a transparent cylinder (diameter: 20 cm, height: 30 cm) for 5 minutes, and the number of forepaw contacts to the cylinder wall will be counted. The score of the cylinder test in this study was calculated as the percentage of contact of the impaired forelimb against the cylinder compared to the contact made by both limbs (Meredith & Kang, 2006; Shinko et al., 2014). To evaluate the treatment effect, we normalized the data for motor performances observed in the stepping before the 6-OHDA lesion.

We performed the cylinder test 1 day before the 6-OHDA lesion to establish each rat's basal motor performance. Nine days after the 6-OHDA injection, we repeated the test to corroborate that a successful striatal lesion had been done. We evaluated the motor outcomes performing the cylinder test 90 minutes after rats received the stimulation on

the eighth day of the stimulation protocol. To test the long-term effect, we performed the cylinder test again after 24 hours the stimulation was turned off on the eighth day.

5.7.2 Apomorphine-induced rotation test (Rotameter Test).

The rotational behavior was assessed for 30 minutes with the Rota-Count Software, Columbus Instruments, Ohio, USA. The number of U-turns ipsilateral to the lesion was counted to establish a score. To evaluate the treatment effect, we calculated the difference of rotations after treatment with rotations before the 6-OHDA lesion. All rats were tested with apomorphine (0.5 mg/kg, Sigma-Aldrich, USA) (Hudson et al., 1993) before the 6-OHDA injections to establish the basal motor performance. Ten days after the 6-OHDA injection, we repeated the test to corroborate the lesion of the animals. Then, motor performance was examined in all rats 45 minutes after the treatment was applied on the second, sixth, and ninth day of stimulation.

5.7.3 Stepping test

We used a smooth-surfaced 1.10 cm table for the test. During the first three days, the rats were handled by the experimenter to become familiar with the experimenter's grip. Then, the experimenter held the rat with both hind limbs plus one forelimb gently restrained in a fixed position, while allowing the unrestrained forelimb to touch the table and slightly raising the hind part above the surface (Olsson et al., 1995; Phan et al., 2017). The rat was moved sideways along the surface over a distance of 90 cm at an average speed of 18 cm/sec (Phan et al., 2017). The number of adjusting steps was

counted both in forehand (i.e., the rat is moved left when the right paw touches the table, or the rat is moved right when the left paw touches the table) and backhand direction (i.e., the rat is moved right when the right paw touches the table or the rat is moved left when the left paw touches the table) (Phan et al., 2017). Additionally, we measured the number of steps forward with the left and right forelimb. In our experiment, animals have a unilateral 6-0HDA lesion on the right side, so we expect to see a deficit in the contralateral forelimb (left forelimb) and use the right forelimb as a control. Finally, we also calculated the symmetry ratio by dividing the number of steps made by the left forelimb by the total number of steps, which considers the left steps plus the right steps. We calculated this ratio with the left forelimb's steps to the ipsilateral side and contralateral side. To evaluate the treatment effect, we normalized the data for motor performances observed in the stepping before the 6-OHDA lesion.

5.8 TYROSINE-HYDROXYLASE (TH) STAINING AND QUANTIFICATION

Twenty-one days after the 6-OHDA injections, animals were deeply anesthetized with a ketamine (100 mg/Kg) plus xylazine (10 mg/Kg), perfused with 4% paraformaldehyde and then the brains were kept in 30% sucrose until sectioning. During tissue sectioning, 30 μm free-floating sections were obtained from the striatum. Tyrosine hydroxylase (TH) immunohistochemistry was used to confirm the position and extension of striatal lesions and quantify the depletion of dopaminergic fibers in the nigrostriatal pathway. For the TH immunostaining free-floating brain sections were quenched with H2O2 0.3% for 30 minutes at room temperature. Following several

rinses in phosphate buffered saline (PBS), the slices were blocked for one hour and permeabilized with blocking solution (BSA 0.5% + Triton 0.2% in PBS) at room temperature. Then, the slices were incubated with primary antibody [1:1000] (Anti-Tyrosine Hydroxylase, polyclonal antibody, Merck, Cat # Ab152) in the same blocking solution, overnight at 4°C. The next day, after rinses in PBS, the sections were incubated with the secondary biotinylated antibody for two hours at room temperature, rinses in PBS and incubated with Vectastain ABC Kit Rabbit IgG solution (Vector Labs, cat PK-4001) according to the manufacturer's protocol. Finally, the sections were stained with 3,3′-diaminobenzidine (Sigma Cat #D5905) in Tris buffer (Sigma Cat # T5030) for five minutes, following the manufacturer's instructions. The coronal sections were mounted on slides, and the images were digitized using an Epson L355 scanner, 8 bits, 4800 dpi resolution. Before quantification striatum were defined using Paxinos Atlas (Paxinos et al., 2007). Three sections from the striatum were selected from -0.72 to +2.1 anteroposterior segment. Optical density was measured using the ImageJ software. In each section, we measured the optical density of the right and left striatum. Then, a background measurement from the cortex was subtracted from each density measurement in the striatum. The mean of the three sections was calculated for each animal. We compared de average TH + fibers density of the un-lesioned and lesioned hemisphere. We used the denervated striatum percentages (%) for group comparison. The observed density in the intact striatum was equaled to 100%, and the depletion of the denervated striatum was expressed as a % loss of the intact side.

5.9 STATISTICS

Changes in cortical evoked responses were evaluated with two-way repeated measures of ANOVAs (analysis of variance), with TIME as the first factor (before, during, and after SSCS), and registered AREAS as a second factor (VPL, DLS, Agvm, S1, and M1). Behavioral effects of the toxin and the use of SSCS were evaluated using two way ANOVA and repeated measure ANOVA respectively, with TIME as the first factor (before and after 6-OHDA injection) and GROUPS as a second factor (sham, SSCS, and peripheral). Then, we applied Bonferroni multiple comparisons. For group comparisons before and after surgery, we applied a Kruskal-Wallis test. Immunohistochemistry was assessed using Wilcoxon matched-pair signed-rank test and one-way ANOVA. The significance criterion was $p < 0.05$. Mean values were presented with standard error mean (SEM).

5.10 ETHICS

The Institutional Animal Care of Universidad de Chile and Pontificia Universidad Católica de Chile specifically approved all animal procedures in this study.

5.11 EXPERIMENTAL DESIGN

The study was divided into two sections:

5.11.1 In vivo electrophysiology experiments:

This section is focused on the mechanisms of action and the identification of SSCS parameters.

The effect of varying different stimulation parameters (duration, intensity, and polarity) were systematically explored. The measured output was the supraspinal neural activity of different areas (ventral posterolateral thalamic nuclei [VPL], dorsal lateral striatum [DLS], ventral medial agranular cortex [Agvm], primary somatosensory [S1], and primary motor cortices [M1]) recorded by in vivo electrophysiology. The aim was to identify parameters that cause evident activity changes (disruption of aberrant low-frequency oscillations) in any of the mentioned structures. The measured output allows for testing a wide range of parameter values with a low number of animals and in a short time. We then compared the changes in beta band power before, during, and after the stimulation was applied. The protocol that caused the most overt changes in most animals was selected to assess its impact on the motor function.

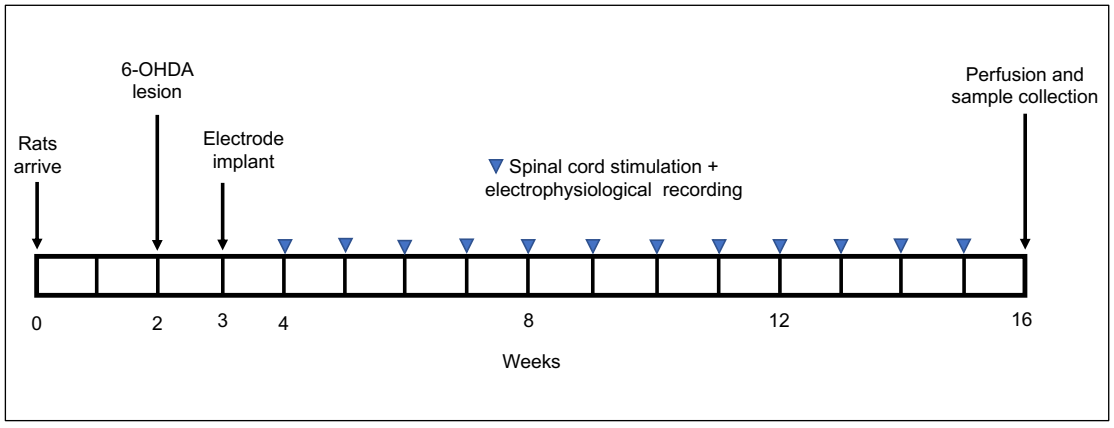

Figure 5. Scheme showing the experimental design for evaluation of the electrophysiological effects of SSCS on the motor-sensory circuit.

One week after the unilateral 6-OHDA lesion, rats were bilaterally implanted with 32 channels microwire array. Electrophysiological recording sessions were conducted regularly for 12 weeks. In each session, we evaluated the motor-sensory circuit's activity before, during, and after stimulation was applied. At the end of the experiment, perfusion and sample collection were done.

5.11.2 Behavioral experiments

This section is focused on exploring the effect of SSCS on motor function. The selected parameters were used to conduct SSCS in unilateral 6-OHDA lesioned rats during 11 daily sessions. Parkinsonian rats received 30 minutes of SSCS, and then their motor performance was tested using the cylinder (90 minutes and 24 hours after stimulation), rotation test (45 minutes after stimulation), and the stepping test (5 minutes after stimulation) (Fig. 6). We compared the effect between 3 groups:

- SSCS group: Rats treated with SSCS (T10-L4).
- Sham group: Rats were connected to the electrodes but did not receive current.
- Peripheral stimulation group: Animals stimulated in peripheral dermatomes that correspond to the stimulation level (T10-L4).

At the end of the last stimulation session, animals were perfused and tyrosine hydroxylase (TH) immunostaining performed in brain sections to confirm the 6-OHDA lesion in the nigrostriatal dopaminergic system by assessing the conditions of their striatal projections.

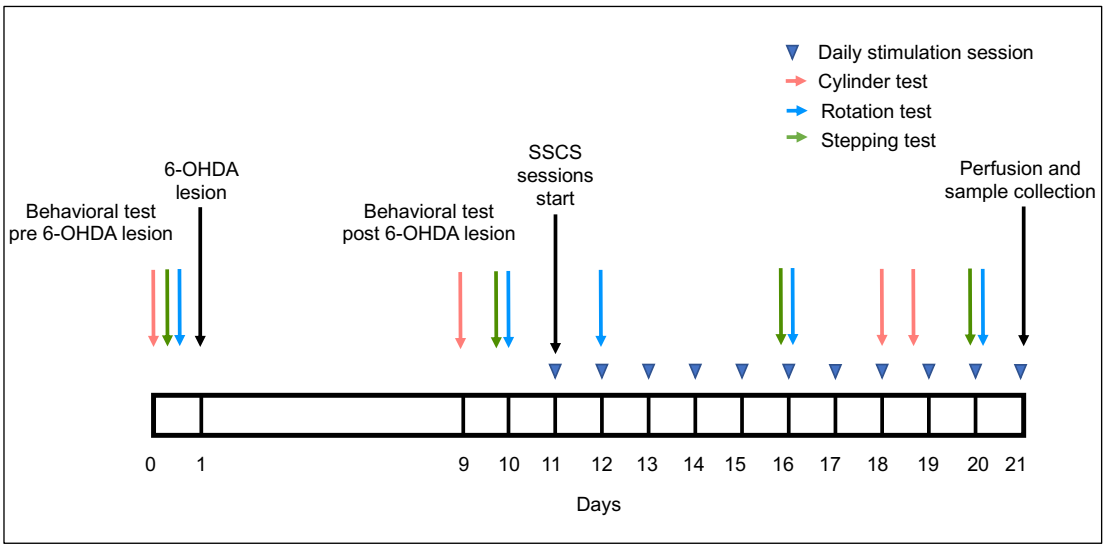

Figure 6. Scheme showing the overall experiment design to assess the motor effect of SSCS.

Behavioral tests were performed before the 6-OHDA lesion to establish the baseline motor performance and after the 6-OHDA lesion to corroborate a successful lesion. Then, stimulation sessions started on the 11th day and continued per 11 days. Each stimulation session lasted 30 minutes. The cylinder test was done on the eighth day after 90 minutes and repeated 24 hours after the stimulation was turned off. We also evaluated the motor performance using the apomorphine rotation test, which was performed 45 minutes after the SSCS was turned off the second, sixth, and tenth day of stimulation. The stepping test was done 5 minutes after SSCS was turned off the sixth and tenth day. After the last session of stimulation (11th day), perfusion and sample collection was done.

6. RESULTS

This section aims to select an SSCS protocol that can decrease aberrant beta activity in the sensory-motor circuit and then assess its impact on parkinsonian rats' motor function. Therefore, we evaluated the effect of different protocols and chose the one that causes the most overt changes in most animals. Later, we analyzed these protocols' influence on motor function using the stepping test, apomorphine-induced rotation test, and the cylinder test.

6.1 ELECTROPHYSIOLOGY RESULTS

We conjectured that SSCS could produce a modulation in the beta activity of the sensory-motor circuit that characterized parkinsonian states. In order to examine this question, we first designed a total of nine SSCS protocols based on literature evidence on cortical or motor activity changes. These protocols were divided into three different types: direct current stimulation, alternate current stimulation, and mixed stimulation (Fig.3).

Using adult Sprague Dawley male rats with unilateral 6-OHDA lesion (n=6), we evaluated SSCS protocols' effect in the sensory-motor circuit's electrophysiological activity. We used in vivo extracellular electrophysiological recording of awake animals from five different areas (ventral posterolateral thalamic nuclei [VPL], dorsolateral striatum [DLS], ventral medial agranular cortex [Agvm], primary somatosensory [S1], and primary motor cortices [M1]), using as control the uninjured hemisphere.

First, we confirmed the existence of beta oscillatory neural activity (8-30 Hz) that is characteristic of parkinsonism (Williams et al., 2002; Santana et al., 2014) in the local field potential (LFP) of all the areas of the circuit registered (Fig. 7). Then, we compared the parkinsonian beta power in the LFP before (pre), during (on), and after (post) SSCS for each protocol.

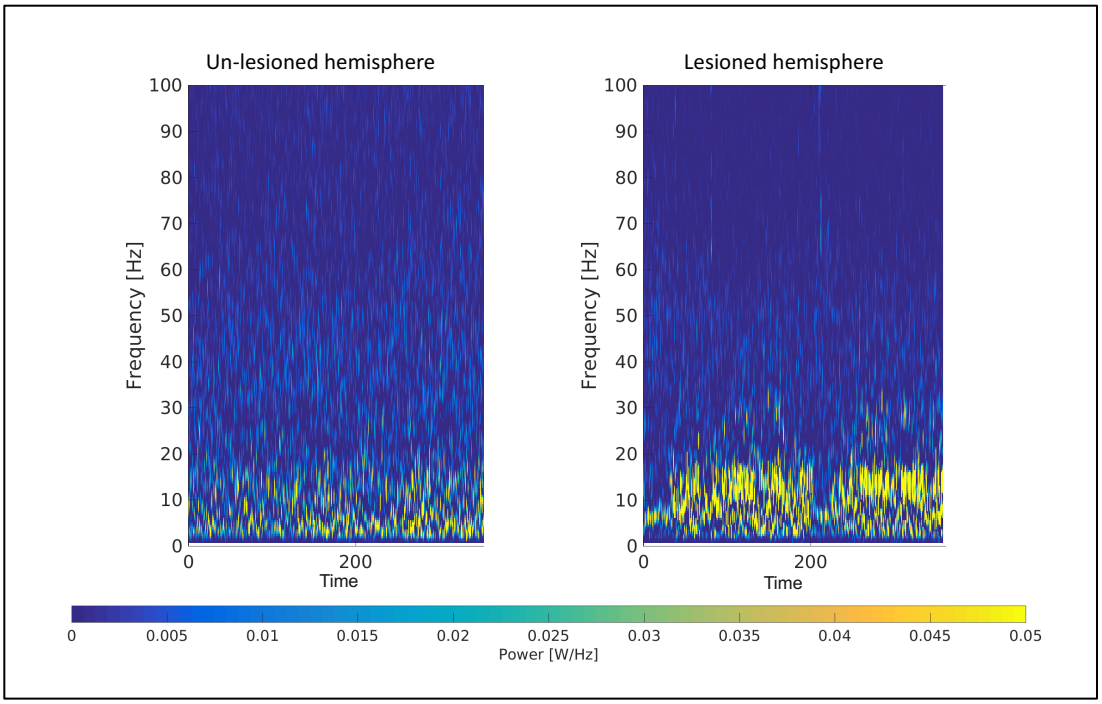

Figure 7. Unilateral 6-OHDA produced an augmented oscillatory activity in the beta band of the lesioned hemisphere.

Example of a representative spectrogram from S1 LFP of the un-lesioned (left) and lesioned (right) brain hemisphere in a 6-OHDA lesioned rat. The color bar denotes power density expressed as W/Hz.

Then, our goal was to select the most effective protocol decreasing beta power, measured by the on/pre ratio, defined by the beta power during (on) stimulation divided by the beta power before stimulation (pre). A ratio below 1 indicates a decrease in beta

power with SSCS. We calculated an average on/pre for each protocol condition. Additionally, we also measured the average ratio between beta power after (post) stimulation divided by the beta power before (pre) stimulation to evaluate if the effects continue or not overtime. Tables 1 summarizes the results of different protocols.

6.1.1 Alternate current stimulation protocols did not consistently decrease the parkinsonian beta activity in the sensory-motor circuit.

We analyzed the effect of three different SSCS alternate current protocols on the beta activity in the sensory-motor circuit. Alternant current protocols have shown positive outcomes controlling motor symptoms in invasive spinal cord stimulation of PD patients (Agari & Date, 2012; Fenelon et al., 2012; Soltani & Lalkhen, 2013; Landi et al., 2013; Mitsuyama et al., 2013; Hassan et al., 2013; Nishioka & Nakajima, 2015; Pinto de Souza et al., 2016; Akiyama et al., 2017; Samotus et al., 2018; de Lima-Pardini et al., 2018; Kobayashi et al., 2018; Mazzone et al., 2019), but has not been analyzed no-invasively. Furthermore, this kind of protocol has shown a modulatory role of oscillatory activity in the nervous system (Moreno-Duarte et al., 2014; Teo et al., 2017). We expected to find a protocol that could consistently reduce beta activity in the sensory-motor circuit. However, none of the SSCS alternate current protocols could consistently decrease beta oscillations in the LFP during or after stimulation was applied (Table 1) with average on/pre ratios < 1. Therefore the 20 Hz (on/pre average ratio: 1.45 ± 0.77, post/pre average ratio: 1.53 ± 0.9), 150 Hz (on/pre average ratio: 1.04 ± 0.24, post/pre average ratio: 1.14 ± 0.45), and 300 Hz (on/pre average ratio: 1.07

± 0.33, post/pre average ratio: 1.34 ± 0.44) protocols were not eligible for behavioral experiments (Table 1). Hence, our alternate SSCS did not reduce the abnormal slow oscillatory activity of the sensory-motor circuit consistently.

6.1.2 Some direct current stimulation protocols showed a discrete effect on parkinsonian beta activity in the sensory-motor circuit.

Direct current protocols have shown an effect altering neuronal excitability and motor behaviors in animal and humans studies (Moreno-Duarte et al., 2014; Ganguly et al., 2020). However, the application of non-invasive direct current protocol in the spinal cord for PD treatment has not been studied before. Consequently, we want to evaluate the impact of four non-invasive direct current protocols in 6-OHDA lesioned rats' beta activity.

In the case of direct-current stimulation protocols, +375 μA anodic protocol presented a discrete short-term effect showing a decrease in the parkinsonian beta power measured in the LFP, reflected in the on/pre average ratio <1 (+375 μA protocol: 0.99 ± 0.28). The +2500 μA did not show a significant effect (on/pre average: 1.03 ± 0.09) (Table 1).

The effects of cathodic protocols were also variable, -375 μA did not present and average ratio <1 (1.03 ± 0.09), and the -2500 μA protocol revealed an on/pre average ratio <1 (0.87 ± 0.26). This short-term effect observed in the -2500 cathodic protocols was not consistent across animals (Table 1).

None of the direct current protocols displayed an effect after SSCS was turned off, showing post/pre average ratio > 1 in all the cases (+375 μA protocol: 1.34 ± 0.6, +2500 μA protocol: 1.11 ± 0.1, -3750 μA: 1.64 ± 0.37, -2500 μA: 1.1 ± 0.46).

On the basis of these findings, direct current SSCS did not show a robust effect modulating the aberrant beta activity in the sensory-motor circuit.

6.1.3 The +2500 μA /300 Hz mixed-stimulation protocol consistently decreased the parkinsonian beta activity in the sensory-motor circuit during and after SSCS.

Finally, we tested the impact of the mixed protocols in the beta activity of the 6-OHDA lesioned rats' sensory-motor circuit. The mixed-stimulation protocols consist of applying direct current stimulation and periodic pulses of current (Fig. 4 C). The rationale of using this protocol is first to modify the cell excitability by bringing its membrane potential closer to its excitation threshold (Nitsche & Paulus, 2000; Wassermann & Grafman, 2005), and then apply high-frequency biphasic pulses mounted over the direct current, which has shown positive outcomes in PD patients with invasive spinal cord stimulation. We tried two types of mixed protocols differentiated by the intensity of anodic current (+375 μA and +2500 μA). In both cases, the direct-current had 300 Hz biphasic pulses.

The +375 μA/ 300 Hz protocol did not show a positive effect in the short-term neither in the long-term, with an average on/pre ratio, and post/pre ratio >1 (1.08 ± 0.14 and 1.17 ± 0.13 respectively). However, the +2500 μA/300Hz mixed protocol caused an acute power reduction on the beta power, indicated by the on/pre average ratio <1 (0.44

± 0.35). Additionally, this protocol showed a long-lasting effect determined by a post/pre average ratio <1 (0.12 ± 0.03). Consistently, the short and long-term beta reduction occurred in all animals.

Fig. 8 shows an example of the spectrograms and the power spectral densities (PSDs) from different areas of a parkinsonian rat before (pre-stim), during (on-stim), and after (post-stim) +2500μA/300Hz protocol has been applied. SSCS reduced the LFP power in the parkinsonian beta range (8-30 Hz) during the stimulation period and after it (Fig. 8 A-B). This effect was consistent in the three recorded rats, and, remarkably, in all the areas of the cortico-basal ganglia-thalamic circuit recorded [$F(2,6) = 7.93$, $p < 0.05$] (Fig. 9).

These findings demonstrated that this particular SSCS protocol (+2500μA/300Hz) reduces the abnormal slow oscillatory activity of all the areas registered in the sensory-motor circuit. This result supported our hypothesis and showed that SSCS could achieve a supraspinal effect. Interestingly, we observed a long last effect in all the areas recorded and a modulation in the Agvm cortex. The latter is homologous of human's supplementary motor area, which plays an important role in controlling axial symptoms but has not been studied before in the context of spinal cord stimulation mechanisms in Parkinson's disease.

In summary, the +2500μA/300Hz SSCS protocol modulated the aberrant oscillatory activity, supporting our hypothesis. For this reason, we selected this protocol to evaluate its effect on 6-OHDA lesioned rats' motor function.

Protocol (intensity and frequencies)	Rat	On/pre Ratio	On/pre Ratio Average	On/pre Ratio SD	Post/pre Ratio	Post/pre Ratio Average	Post/pre Ratio SD	Consistency Ratio Pre/On
20 Hz	rat 02	0.69	1.45	0.77	0.56	1.53	0.99	No
	rat 04	2.22			2.54			
	rat 06	1.44			1.49			
150 Hz	rat 01	0.96	1.04	0.24	0.72	1.14	0.45	No
	rat 02	0.85			1.21			
	rat 04	1.39			1.62			
	rat 05	0.96			1.01			
300 Hz	rat 01	1.27	1.07	0.33	1.96	1.34	0.34	No
	rat 02	0.70			1.53			
	rat 03	1.23			1.30			
	rat 04	0.74			0.69			
	rat 06	1.41			1.20			
+375 µA	rat 01	1.16	0.99	0.28	2.57	1.34	0.64	No
	rat 02	1.44			1.11			
	rat 03	0.85			0.88			
	rat 04	0.64			1.45			
	rat 05	0.88			0.87			
	rat 06	0.99			1.16			
+2500 µA	rat 01	0.97	1.03	0.09	1.18	1.11	0.10	No
	rat 02	1.09			1.04			
-375 µA	rat 01	1.11	1.15	0.19	1.26	1.64	0.37	No
	rat 02	0.92			1.42			
	rat 03	1.02			1.48			
	rat 04	1.31			2.32			
	rat 05	1.42			1.68			
	rat 06	1.13			1.68			

Protocol	Rat	On/pre Ratio	On/pre Ratio Average	On/pre Ratio SD	Post/pre Ratio	Post/pre Ratio Average	Post/pre Ratio SD	Consistency Ratio Pre/On
-2500 µA	rat 01	1.18	0.88	0.26	1.63	1.10	0.46	No
	rat 02	0.78			0.87			
	rat 03	0.68			0.80			
+375µA/ 300Hz	rat 01	0.98	1.08	0.14	1.05	1.17	0.13	No
	rat 02	1.03			1.14			
	rat 03	1.24			1.31			
+2500µA/ 300Hz	rat 01	0.18	0.44	0.35	0.09	0.12	0.03	Yes
	rat 02	0.30			0.15			
	rat 05	0.84			0.14			

Table 1. Different SSCS protocols electrophysiological effects.

A total of nine protocols were evaluated in terms of the changes produced in the beta LFP power. We evaluated the short-term effect (on/pre ratio), long-term effect (post/pre ratio), and the consistency across animals of the changes produced in the short- term. The protocol with better results in on/pre ratio, post/pre ratio, and consistency in on/pre ratio was the +2500 µA/300Hz protocol (indicated by a red rectangle).

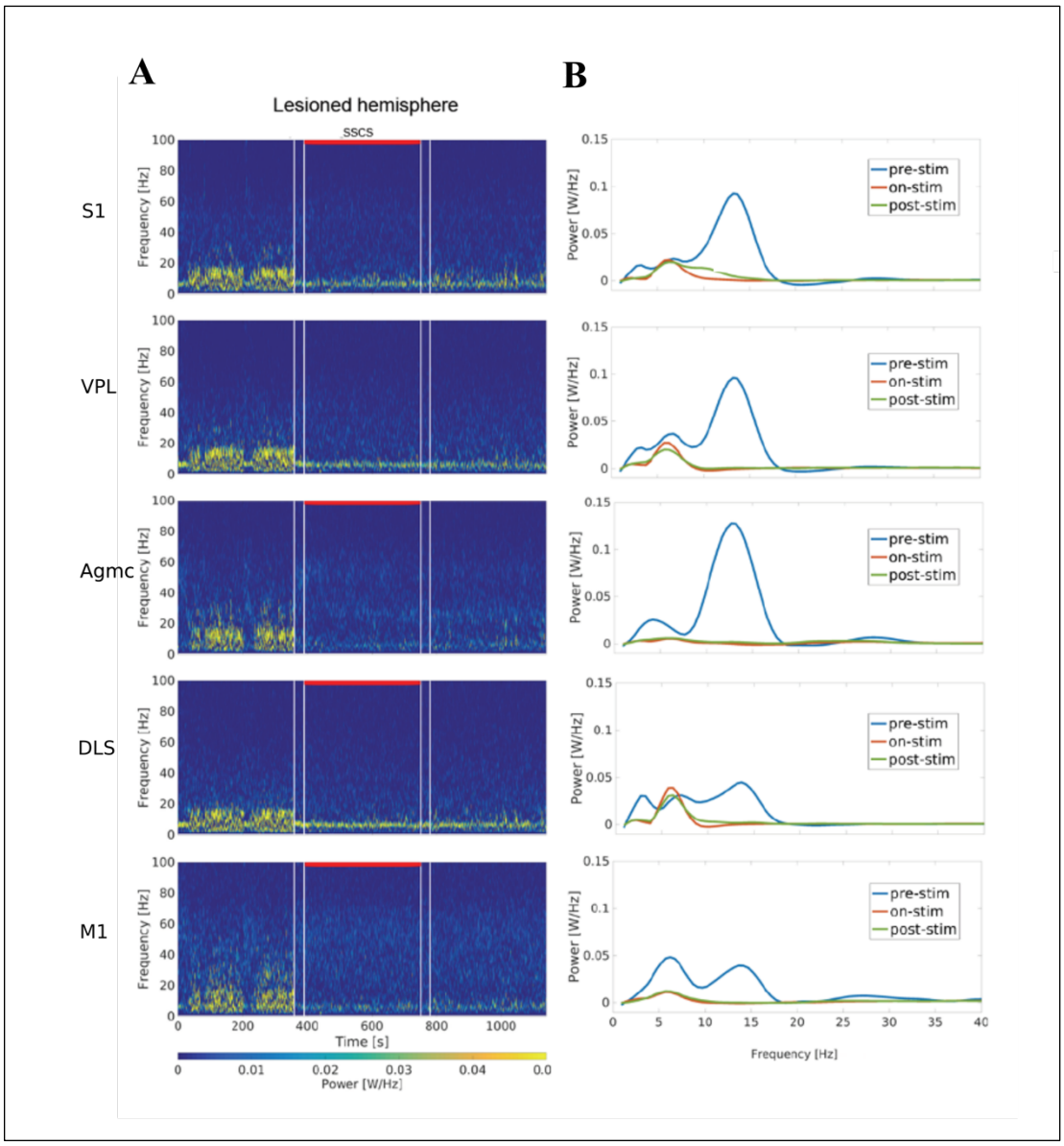

Figure 8. SSCS alters oscillatory patterns in the sensory-motor circuits

(A) Example of spectrograms that reflects parallel changes in LFP power of different areas of the cortico-basal ganglia-thalamic circuit in the lesioned hemisphere (right side). Note the immediate reduction of the parkinsonian beta band after SSCS was applied (red line, stimulation protocol: +2500 μA/300Hz; color codes denote power density expressed as W/Hz) **(B)** Average power spectral density during pre (blue line), on (red line) and post (green line) stimulation period. A reduction in beta-band PSD of the on and post-stimulation period was observed in all the structures.

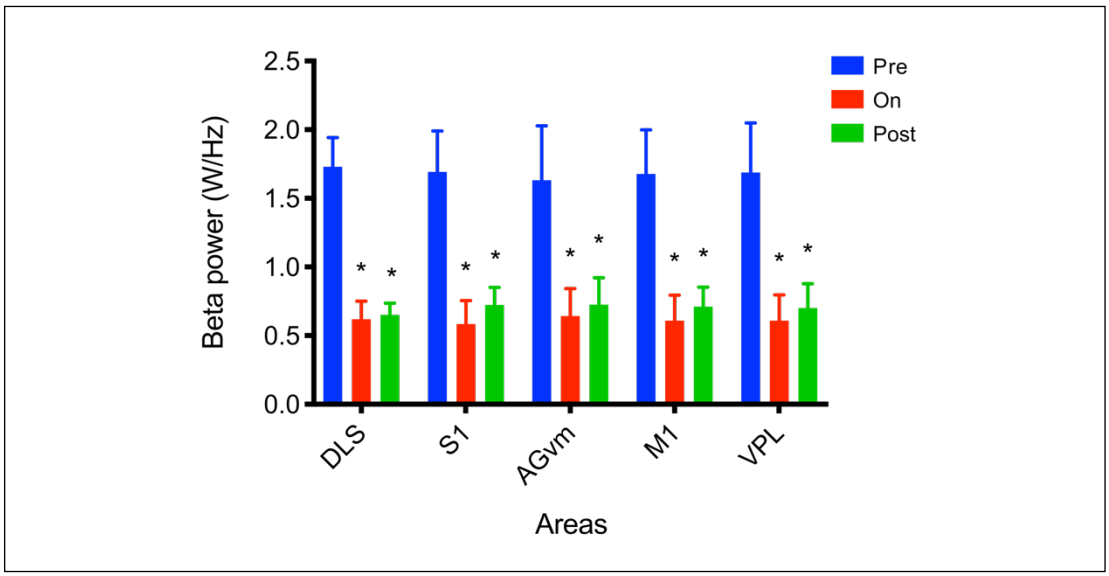

Figure 9. The +2500 µA/300Hz SSCS protocol reduces the PSD of the beta band.

After SSCS, the basal LFP, the PSD activity in the beta band (pre: blue bar) of all rats decreased during (on: red bars) and after (post: green bars) the application of the protocol. (∗ represents a significant difference ($p < 0.05$) with the blue bar (own precondition).

6.2 BEHAVIORAL RESULTS

To assess the therapeutic effect of SSCS in parkinsonian rats, we first corroborated the behavioral impact of the unilateral 6-OHDA model using the apomorphine-induced rotation test, the cylinder test, and the stepping test. The apomorphine-induced rotation test reflects the lesioned striatum's hypersensitivity by measuring 180º U-turns contralateral to the lesion. In this test, 6-OHDA lesioned rats show a higher U-turns number. The cylinder test evaluates forelimb use symmetry (Meredith & Kang, 2006; Decressac et al., 2012) and is a sensitive measure of the degree of unilateral dopamine

loss (Meredith & Kang, 2006). The stepping test intends to monitor limb akinesia (Olsson et al., 1995).

Then, in line with our general objective, we studied the effect of the selected protocol of SSCS (+2500μA / 300Hz) on motor function, using the stepping test (5 minutes after stimulation session), apomorphine-induced rotation test (45 minutes after the stimulation session), and the cylinder test (90 minutes and 24 hours after the stimulation session). We compared the motor performance of the SSCS group (6-OHDA lesioned rats treated with SSCS), sham group (6-OHDA lesioned rats with sham stimulation), and peripheral group (6-OHDA lesioned rats with peripheral stimulation) (see experiment design overview on the material and method sections for a complete description of the experiment design).

6.2.1 The 6-OHDA lesion deteriorates the motor performance in rats.

Before we began with the stimulation protocol application, we evaluated the unilateral 6-OHDA lesion's motor effect in all the rats. In comparison to the basal levels (before the 6-OHDA administration), all the lesioned animals presented impairment in their motor performance in the apomorphine-induced rotation test, cylinder test, and the stepping test after the toxin was applied (Fig. 10).

A repeated measure ANOVA indicated that differences in the apomorphine-induced rotation test (before and after 6-OHDA lesion) were significant [$F(1,19) = 45.21$, $p < 0.0001$]. Post hoc tests using the Bonferroni correction reveled that this difference was significant for sham (before lesion: 22.25 ± 5.11; after lesion 274.4 ± 66.88; p =

0.003), treatment (before lesion: 17.57 ± 6.8; after lesion: 252.1 ± 65.7; p = 0.005) and peripheral group (before lesion: 17.86 ± 6.01; after lesion: 313.6 ± 76.19; p = 0.002). Kruskal-Wallis test did not show differences between groups before surgery (H(2) = 0.71, p = 0.7) neither after surgery (H(2) = 0.28, p = 0.87) (Fig. 10 A).

In the cylinder test a repeated measure ANOVA, showed a statically significant difference on the left forelimb usages before and after 6-OHDA lesion [F (1,20) = 162.6, p < 0.0001]. Post hoc tests using the Bonferroni correction reveled that this change was significant for sham (before lesion: 48.35 ± 3.07; after lesion: 5.65 ± 2.1; p < 0.0001), SSCS group (before lesion: 54.82 ± 5.63; after lesion : 8.193 ± 4.09; p < 0.0001) and peripheral group (before lesion: 53.41 ± 5.01; after lesion: 8.14 ± 2.69; p < 0.0001). Kruskal-Wallis test did not show differences between groups before surgery (H(2) = 0.42, p = 0.82) neither after surgery (H(2) = 0.15, p = 0.93) (Fig. 10 B).

In the stepping test a repeated measures ANOVA determined that after the 6-OHDA lesion the performance of the left forelimb (contralateral to the lesioned hemisphere) was significantly impaired [F (1,20) = 35.8, p < 0.0001]. Post hoc tests using the Bonferroni correction reveled that this difference was significant for sham (before surgery: 9.75 ± 0.63; after surgery: 5.56 ± 1.4, p = 0.01), treatment (before surgery: 9.31 ± 1.16; after surgery: 5.53 ± 0.81; p = 0.03) and peripheral group (before surgery: 11.64 ± 0.93; after surgery: 5.71 ± 0.84; p = 0.001). Kruskal-wallis test did not show differences between groups before surgery (H(2) = 2.86, p = 0.24) neither after surgery (H(2) = 0.04, p = 0.98) (Fig. 10 C).

We concluded that the 6-OHDA lesion elicited a statistically significant reduction in motor function in the rats that belong to the three groups, and therefore we could

continue with our essential question that aims to determine the therapeutical effect of SSCS.

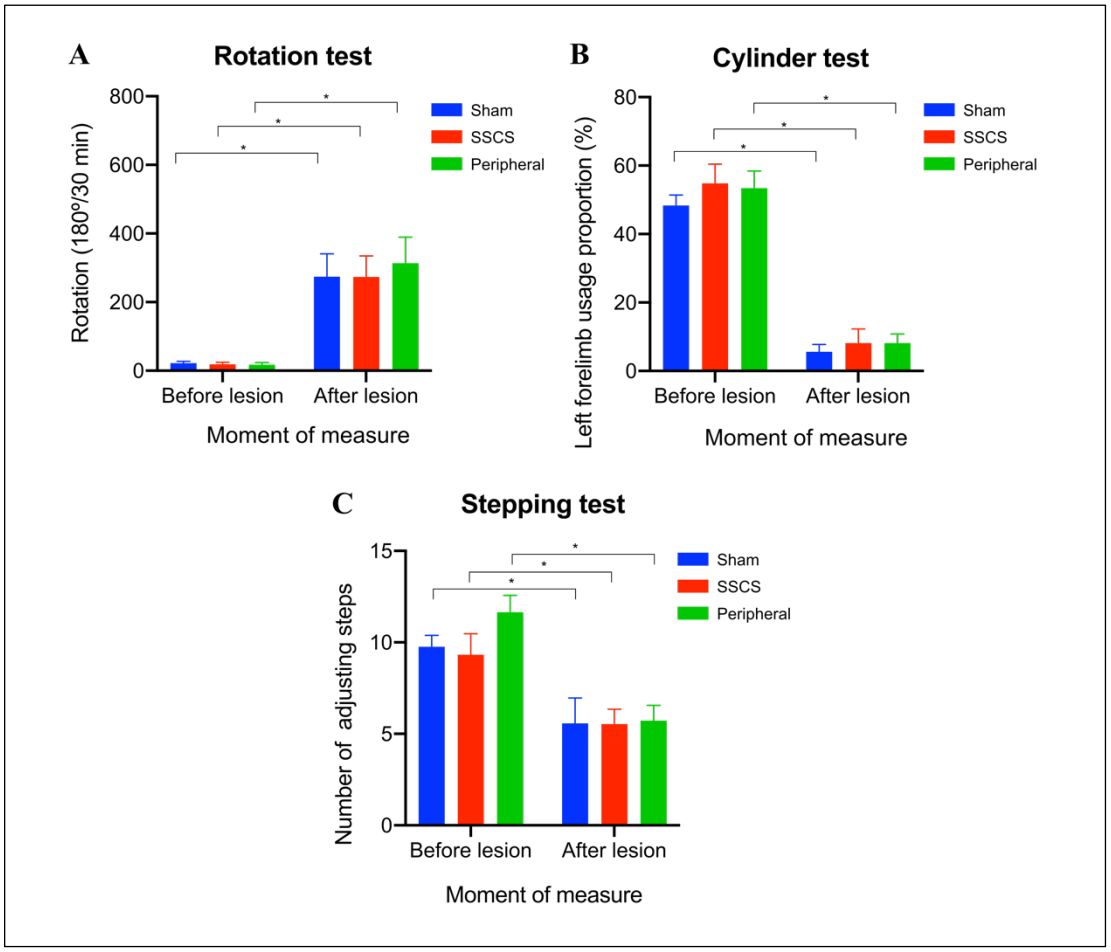

Figure 10. The unilateral 6-OHDA lesion impacts motor performance in the apomorphine-induced rotation test, cylinder test, and stepping test.

(A) Average parkinsonian rats' performance in the apomorphine-induced rotation test (sham group = 8, SSCS group = 7, peripheral group = 7). We calculated the number of 180º U-turns ipsilateral to the lesion for 30 minutes. After the 6-OHDA lesion, rats in the sham group (blue bars), treated group (red bars), and peripheral group (green bars) showed a significant increase in the number of ipsilateral 180º U-turns (*$p < 0.05$, $n = 22$). There were no significant differences in the number of rotations between the three groups before neither after the 6-OHDA lesion. **(B)** The average performance of parkinsonian rats in the cylinder test (sham group = 8, SSCS group = 7, peripheral

group = 8). The cylinder test score was calculated as the percentage of contact of the impaired forelimb (left forelimb) against the cylinder compared to the contact made by both forelimbs. After the 6-OHDA lesion, rats in the three groups (sham group = 8, SSCS group = 7, peripheral group = 8) showed a significant reduction in the uses of the left forelimb in comparison with the evaluation that was done before the surgery (*$p < 0.05$, n = 23). There were no significant differences between groups in the left forelimb usage proportion before neither after the 6-OHDA lesion. **(C)** Parkinsonian rats' average performance in the stepping test (sham group= 8, treatment group = 8, peripheral group = 7). The number of left forelimb adjusted steps (contralateral to the lesioned hemisphere) were counted for each rat before and after the lesion. A significant decrease in adjusted steps was observed in the three groups (*$p < 0.05$, n = 23). No significant differences between groups were observed before neither after the 6-OHDA lesion.

6.2.2 SSCS seems to improve motor performance in the short-term.

To study the immediate effect of SSCS on motor function, we applied the selected protocol daily and compared the motor performance in the stepping test and apomorphine-induced rotations test between groups; after 5 and 45 minutes, respectively, the stimulation was turned off.

In the stepping test case, we analyzed the number of steps with the left forelimb (to the ipsilateral side and contralateral side) and a symmetry ratio (to the ipsilateral side and contralateral side) after two and ten days of treatment. We observed higher motor performance values in the group treated with SSCS versus the sham and peripheral groups (Fig. 12).

The number of steps (normalized by stepping number before 6-OHDA lesion) with the left forelimb to the ipsilateral side were higher in the SSCS group (day 2: 0.74 ± 0.08; day 10: 0.70 ± 0.09) in comparison with the sham group (day 6: 0.48 ± 0.11; day 10: 0.49 ± 0.08) and peripheral group (day 6: 0.45 ± 0.1; day 10: 0.51 ± 0.06) (Fig. 12 A).

In the ipsilateral task a repeated measured ANOVA showed that these differences between groups were not statistically significant [F (2,20) = 3.2, p = 0.06]. In the case of the contralateral side the SSCS group did not demonstrate statistically differences either [F (2,20) = 2.4, p = 0.11], but the same trend showing a higher number of adjusted steps in the SSCS group was observed (SSCS group: day 6: 0.32 ± 0.13; day 10: 0.57 ± 0.22; sham group: day 6: 0.17 ± 0.07; day 10: 0.23 ± 0.14; peripheral group: day 6: 0.2 ± 0.22; day 10: 0.2 ± 0.09) (Fig. 12 B). The symmetry ratio to the ipsilateral side was also superior in the SSCS group (day 6: 0.88 ± 0.07; day 10: 0.96 ± 0.07) versus the sham group (day 6: 0.68 ± 0.13; day 10: 0.72 ± 0.09) and the peripheral group (day 6: 0.65 ± 0.13; day 10: 0.77 ± 0.06) (Fig. 12 C). The symmetry ratio to the contralateral side also increased in the SSCS group (day 6: 0.54 ± 0.21; day 10: 0.95 ± 0.28) in comparison with the sham group (day 6: 0.17 ± 0.07; day 10: 0.23 ± 0.14) and the peripheral group (day 6: 0.34 ± 0.11; day 10: 0.44 ± 0.19) (Fig. 12 D). A repeated measure ANOVA test showed that the symmetry was not statistically significant for the ipsilateral symmetry ratio [F (2,20) = 2.42, p = 0.12], neither for the contralateral symmetry ratio [F (2,20) = 3.4, p = 0.05]. We did not see differences between right and left side in the walking forward task.

The apomorphine-induced rotations were evaluated after two, six, and ten days of stimulation for each group. As in the stepping test, the SSCS group showed an average better performance than the sham and peripheral groups. After two, six and ten days of stimulation the rats treated with SSCS presented lower apomorphine-induced rotations differences (between baseline and after treatment sessions) (SSCS group: day 2: 207 ± 73.74; day 6: 316 ± 95.46; day 10: 227.7 ± 59.48) in comparison with the sham group

(day 2: 309.8 ± 63.14; day 6: 411.6 ± 63.14; day 10: 419 ± 97.06) and peripheral group (day 2: 310.3 ± 73.36; day 6: 339.1 ± 60.36; day 10: 490.4 ± 117.1). However, a repeated measure ANOVA indicated that these differences were not significant [F= (2,25) = 1.63, p = 0.22] (Fig. 11).

The results observed in the stepping and apomorphine-induced rotation test did not show statistically significant differences between groups. However, the same positive trend was observed in all the evaluations that have been done, showing an average lower number of rotations, a better symmetry ratio, and a higher number of steps with SSCS usages. Furthermore, in the stepping test case, the ipsilateral adjusted step number and contralateral symmetry ratio showed a p-value close to the significance. Therefore, the potential benefit of this neuromodulator treatment that we postulated cannot be rejected, and future experiments should consider a higher number of animals and particular experiment design modifications to clarify groups difference.

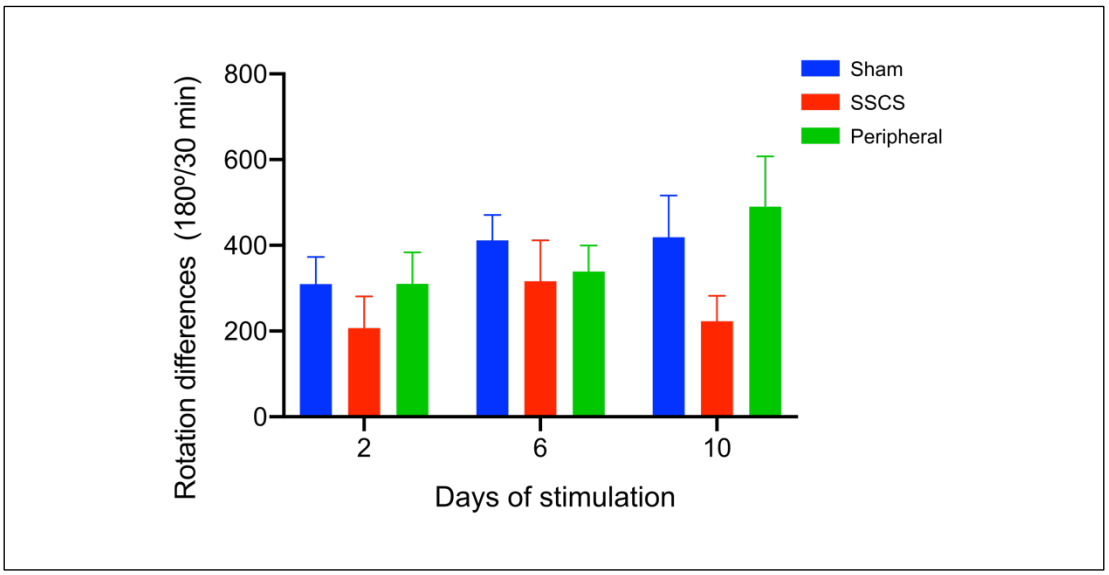

Figure 11. Rats treated with SSCS seems to have a better performance in the apomorphine-induced rotation test.

In the apomorphine-induced rotation test, rats of the treated group (red bars) showed a better performance in comparison with the sham group (blue bars) (second, sixth and 10^{th} days of stimulation) and peripheral group (green bars) (second and 10^{th} days of stimulation). However, these differences were not statistically significant (sham group = 11, SSCS group = 10, peripheral group = 7).

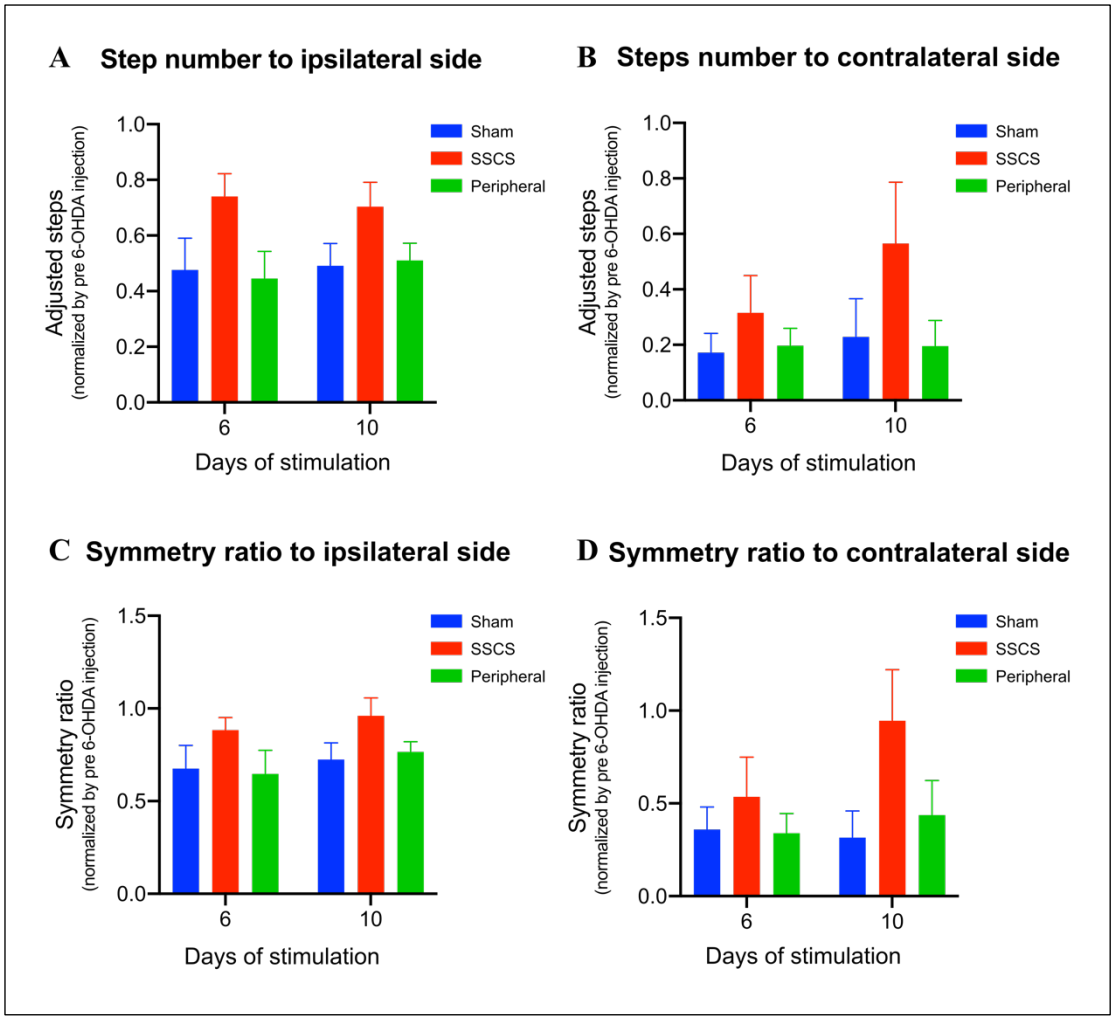

Figure 12. Motor performance in the stepping test after SSCS seems to improve.

(A) and (B) In the stepping test, the rats of the SSCS group (red bars) presented a higher number of ipsilateral (a) and contralateral (b) adjusted steps with the left forelimb in comparison with the sham group (blue bars) (sixth and 10th days of stimulation) and peripheral group (green bars) (sixth and 10th days of stimulation for the ipsilateral task and 10th day for the contralateral). **(C) and (D)** The symmetry ratios for the ipsilateral and contralateral side also showed a higher value with the SSCS treatment. These differences were not statistically different (sham group = 8, SSCS group = 8, peripheral group = 7).

6.2.3 SSCS does not show a long-lasting motor effect.

We also evaluate the motor impact of SSCS after a more extended period had passed since the stimulation was turned off. To achieve this purpose, we use the cylinder test on the eight days of stimulation; after 90 minutes and 24 hours, the SSCS, sham, or peripheral stimulation was applied.

The SSCS group reported a higher number of left forelimb usages after 90 minutes of stimulation (0.38 ± 0.33) in comparison with the sham group (0.09 ± 0.03) and peripheral group (0.06 ± 0.05). A repeated measure ANOVA did not show that these differences were significant [$F(2,20) = 0.53$, $p = 0.6$]. Moreover, the higher value observed in the SSCS group at the 90 minutes evaluation could be due to the 100% usages percentage observed in one treated rat, which raises the total group average. The evaluation after 24 hours did not show significant differences between groups neither (sham group: 0.16 ± 0.08; SSCS group: 0.1± 0.06; peripheral group: 0.06 ± 0.05) (Fig. 13). These findings did not resemble the outcomes observed in the stepping and apomorphine-rotation test, excluding a possible long-lasting effect over 90 minutes.

In summary, we could find a SSCS protocol that decreased aberrant slow oscillatory activity in parkinsonian rats lesioned with 6-OHDA. The motor effect of SSCS therapy was not conclusive. However, we observed a positive trend in the motor outcomes after the acute assessment of this treatment. Moreover, we did not observe any side effects in all the evaluations we performed, supporting this therapy's safety profile. These findings encourage us to study this Parkinson's disease neuromodulator treatment more deeply.

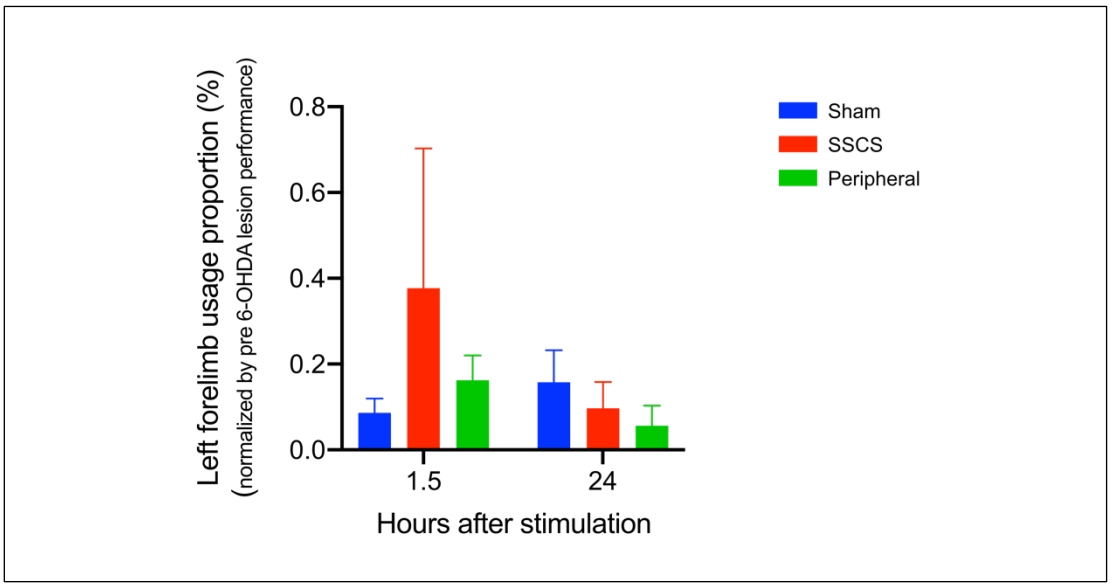

Figure 13. Motor performance in the cylinder test does not change after SSCS.

In the cylinder test, rats of the SSCS group (red bars), the sham group (blue bars), and peripheral group (green bars) did not show feasible differences at 90 minutes nor 24 hours after the stimulation was applied (sham group = 8, SSCS group = 7, peripheral group = 8).

6.3 HISTOLOGICAL ANALYSIS RESULTS

Twenty-one days after the 6-OHDA lesion rats were perfused a tyrosine hydroxylase (TH) analysis was performed for each rat of the behavioral experiment to corroborate dopaminergic cell injury. Wilcoxon matched-pair signed-rank test, showed a statistically significant difference on the % density of TH-positive (TH +) fibers in the lesioned striatum (5.74 ± 1.12) in comparison with the un-lesioned striatum (51.12 ± 2.87) ($Z = -190$, $p < 0.0001$) (Fig. 14 A). A one-way ANOVA did not show differences in striatal TH-positive fiber loss between groups [$F(2,16) = 1$, $p = 0.39$] (Fig. 14 B). These results confirmed that the 6-OHDA injection produces significant damage to the

dopaminergic terminals in the lesioned striatum compared to the un-lesioned side. The SSCS, sham, and peripheral group did not show significant differences in their striatal fibers lost, suggesting that the therapy did not affect the axons lost either regeneration, at least when it is applied for this limited period of time.

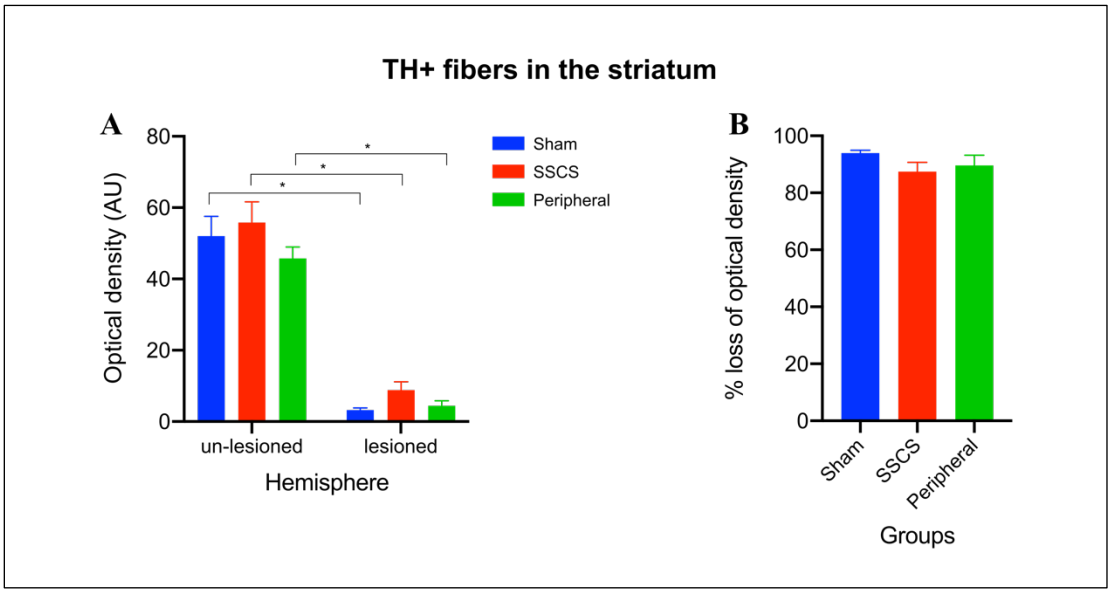

Figure 14. Tyrosine hydroxylase immunostaining in the striatum shows differences between hemispheres.

(A) A significant difference of TH + fibers density was observed in the lesioned hemisphere of 6-OHDA rats (*$p < 0.05$, n = 19) (sham group = 8, SSCS=7, peripheral = 8). **(B)** The different groups (sham (blue), SSCS (red) and peripheral (green)) did not show differences in TH + fiber loss in the lesioned striatum (sham group = 8, SSCS group=7, peripheral group = 8).

7. DISCUSSION

To our knowledge, this is the first study to explore the role of non-invasive spinal cord stimulation on the beta oscillatory activity and motor-impaired motor function that characterized Parkinson's disease (PD). We found that a specific protocol of SSCS can modulate aberrant beta oscillatory activity in different areas of the sensory-motor circuit during and after stimulation. The effect of SSCS on motor function did not show significant changes after the stimulation was applied. However, we observed a repetitive trend to improve motor performance measured by the stepping and apomorphine-induced rotation test after SSCS.

7.1 ELECTROPHYSIOLOGICAL EFFECTS OF SSCS

In line with our hypothesis, we observed a reduction in the power spectral density of beta-band with the application of SSCS in different areas of the sensory-motor circuit (ventral posterolateral thalamic nuclei, dorsal striatum, ventral medial agranular cortex, primary somatosensory and primary motor cortices). This reduction reflects that SSCS has a supraspinal effect that can modulate aberrant oscillatory activity associated with parkinsonian states (Brown, 2003; Wichmann & DeLong, 2006; Hammond et al., 2007; Fuentes et al., 2010; Santana et al., 2014). Interestingly, the effect remains also when the stimulation was turned off. Since this is the first study that analyzes non-invasive spinal cord stimulation on PD, we cannot compare our findings with previous evidence. However, evidence in invasive SCS studies agrees with our results, showing a

reduction in the of beta band frequency with the treatment on different cortical and subcortical areas of the motor-sensory circuit in mouse, rats, and non-human primates (Fuentes et al., 2010; Santana et al., 2014; Brys et al., 2017). Additionally, in humans without PD, non-invasive spinal cord stimulation has shown a supraspinal effect in the motor evoked potential (MEP) that lasts when the stimulation stops (Cogiamanian et al., 2008; C. Y. Lim & Shin, 2011; Bocci et al., 2014; Knikou, 2014; Bocci et al., 2015; Dongés et al., 2017; Albuquerque et al., 2018; Powell et al., 2018; Murray & Knikou, 2019; Benavides et al., 2020). We did not investigate the duration of the SSCS after-effect on the oscillatory activity, which might be an important point to consider in this therapy's clinical aspect. Further studies will be necessary to characterize the relation between the duration of SSCS and its after-effect.

Interestingly, we observed modulation in the activity of the ventral medial agranular cortex (the homologous of the supplementary motor area [SMA]). This area, which participates in the control of axial symptoms (Jacobs et al., 2009; Nutt et al., 2011), has an inappropriate function in Parkinson's patients (Yu et al., 2013; Pollok et al., 2013; Matar et al., 2019). None of the previous studies using invasive SCS have analyzed the agranular medial cortex or SMA implication. Therefore, our finding opens a new edge in understanding the network involved in the modulatory effect of SSCS.

7.2 MOTOR EFFECTS OF SSCS

Regarding motor function, an effect of SSCS cannot be a priori excluded. We observed that animals treated with SSCS had on average better mobility and symmetry in the stepping test and a lower rotation index in the apomorphine-induced rotation test; however, these differences were not statically significant. We cannot compare with previous results since this is the first study using electric non-invasive spinal cord stimulation in parkinsonian animals or patients with Parkinson's disease. Nevertheless, we can hypothesize that different factors might explain the lack of significance. First, we may overestimate the power of the therapeutic effect, and probably a higher number of animals are needed to observe significant differences between groups. Based on our result, we calculate that a number between 60 and 130 animals are optimal to reach significant differences. Second, after we applied SSCS, we observed variable motor outcomes across animals. Invasive spinal cord stimulation has also shown this variability in motor effects in animal models and human studies (Shinko et al., 2014; Brys et al., 2016; Samotus et al., 2018; Prasad et al., 2020). These differences are not well understood but might be due to stimulation parameters (pulse width, frequency, the intensity of stimuli, and electrodes position) or intra-subject variability. Parkinson's disease is a complex entity where each patient has a different combination of environmental triggers and genetic susceptibility that can lead to a heterogeneous clinical profile (Emborg, 2004), explaining the variability of the therapy results. The 6-OHDA animal model also has individual variability due to each subject's different sensitivity to the toxin (Emborg, 2004). In this context, recent studies of iSCS in

patients with PD have chosen to make the stimulation parameters more flexible and personalized (Samotus et al., 2018). In our case, the settings were always the same (not flexible) based on the efficacy of the preliminary electrophysiological results. Third, the usage of the beta oscillatory activity as a marker is arguable. The evidence that associates beta oscillatory activity with Parkinson's disease is robust (Brown & Williams, 2005; Alonso-Frech et al., 2006; Kuhn et al., 2006; Weinberger et al., 2006); however, these findings indicate a correlation and not causality. Recent studies suggest that the parkinsonian beta oscillations can be mixed with physiological beta oscillations (Deffains et al., 2018). Effective stimulation protocols should try to target specifically pathological beta oscillation and preserve the physiological ones. The discrimination between both can be challenging; apparently, the duration of the periods of beta oscillations in the subthalamic (STN) nuclei can be a reliable metric of pathological beta activity (Deffains et al., 2018; Deffains & Bergman, 2019). In our design, we did not record the STN, and therefore, we cannot do this differentiation. Finally, we always analyze the therapy's impact after the treatment was applied due to methodological aspects. The effect of the treatment may decrease after the stimulation was turned off. In fact, the only motor test where we did not observe this positive trend was the cylinder test, which was applied later. Therefore, the discrepancy with the other results (the stepping and apomorphine-induced rotation tests) could be due to each test's timing. In the case of the stepping and rotation test, both were applied earlier (5 minutes and 45 minutes after stimulation was applied respectively) in comparison with the cylinder test, which was applied 90 minutes and 24 hours after the stimulation was turned off. Previous studies using direct current non-invasive spinal cord stimulation in humans

have shown that changes in the motor system also persist after the stimulation is turned off (Cogiamanian et al., 2008; Winkler et al., 2010; C. Y. Lim & Shin, 2011; Lamy et al., 2012; Bocci et al., 2014, 2015; Albuquerque et al., 2018; Benavides et al., 2020); however, these effect decays in time after the first hour (Lim & Shin, 2011). In consequence, future studies should consider evaluating a higher number of animals, using flexible parameters to optimize the motor impact, or a chosen more specific biomarker, for example, pathological beta activity detected in the STN, and ideally evaluate the impact of the SSCS during the stimulation rather than after it.

7.3 UNDERLYING MECHANISMS OF SSCS

How the modulation of beta activity can influence motor function remains speculative. We believe that the spatial extent of the high-power spectral density of beta in all the areas we record generates a higher noise/signal ratio in the system and leads to impaired motor processing. We hypothesized that the selected SSCS protocol that decreases this pathological beta activity would decrease this noise level, facilitate motor information processing, and consequently improve motor function. However, the behavioral response to SSCS was not as robust as we expected. Therefore, we cannot dismiss that possibly other factors, in addition to the modulation of this aberrant synchronic activity, might have a role. For instance, recent evidence has shown that the intensity of stimulation might play a critical role in defining the positive or negative response to the usage of iSCS in PD patients. When the stimulation exceeds an intensity threshold that determines paresthesia's appearance, significant clinical effects have been

observed (Samotus et al., 2018); in contrast to protocols where stimuli are under the thresholds to produce paresthesia and no changes in the motor symptoms have been observed (Prasad et al., 2020). Paresthesia may be operating as sensory cues that optimize the processing and integration of sensory-motor information known to be damaged in Parkinson's disease (Conte et al., 2013). In line with this, other sensory stimuli (visual and auditory) have been seen to achieve control over motor symptoms of Parkinson's disease that belong to the category of so-called axial symptoms (Buated et al., 2012; McAuley et al., 2009; Spildooren et al., 2010; Barthel et al., 2018). In summary, beta modulation is likely to play an important role, but also the stimulation of the proprioceptive system itself may be acting as a key to help this system to process motor commands more efficiently. Our experiments did not consider the evaluation of paresthesia to set the stimulation's intensity, and this point should be considered in future study designs.

One of the most promising features of iSCS is the impact of the treatment on the refractory axial symptoms, the mechanism that underlays this effect remains unknown. Our findings demonstrate that the agranular medial cortex (the homologous of SMA), which is involved in the physiopathology of axial symptoms (Jacobs et al., 2009; John G. Nutt et al., 2011), is modulated by SSCS. This finding suggests that the control of symptoms like freezing of gait (FoG), postural, or gait impairment may be regulated by modulation of this particular area. The SMA contributes to human locomotion by allowing task switching, updating sequencing motor plans, and its role in internally cued movement (Nachev et al., 2008; Takakusaki, 2017; Pozzi et al., 2019). It also participates in the generation of anticipatory postural adjustment (APA), which is

critical to normal gait. In PD patients with FoG the APA are impaired. A recent study has shown that iSCS improves APA responses and FoG in PD patients, however, the areas involved in this regulation have not been investigated in this context (de Lima-Pardini et al., 2018). In addition to SMA, the pedunculopontine nucleus (PPN), situated in the upper pons and dorsal tegmentum of the midbrain, may also play a role in the control of FoG and APA. This brainstem nucleus is relevant to the control of gait (Pahapill & Lozano, 2000). In PD, axial symptoms seem to be related to dysfunction of the PPN (Garcia-Rill, 1986; Pahapill & Lozano, 2000; Grabli et al., 2012). Interestingly, neuroanatomical studies in humans revealed that the PPN has connections with the basal ganglia, spinal cord, and also the SMA (Matsumura et al., 2000; Aravamuthan et al., 2007). Analyzing the role of the PPN was beyond our goals. However, due to the modulation we observed in SMA and its anatomical connection with PPN, further studies should explore deeply this circuit and its implication on the control of axial symptoms by spinal cord stimulation.

7.4 STUDY LIMITATIONS

The 6-OHDA rodent model has significant limitations: it implies an acute rather a progressive lesion, and second, some clinical manifestations like rigidity and coordinated movement are difficult to assess or mimic (Sedelis et al., 2001; Beal, 2001; Tillerson et al., 2002; Meredith & Kang, 2006). Toxin rodent PD models produce a selective dopaminergic cell dysfunction, and the modeling of symptoms such as unpredictable freezing, which includes deficit outside the nigrostriatal system, might

not be present (Meredith & Kang, 2006). Also, we used a unilateral 6-OHDA lesion that is useful to evaluate motor function symmetry but did not resemble the bilateral motor impairment that PD patients usually present.

In our protocol, the daily stimulation period was limited; recent studies in animal models of parkinsonism suggest that continuous stimulation (prolonged for 8 to 24 hours) would have a more significant behavioral and neuroprotective impact (Kuwahara et al., 2020).

Therefore, considering the safety profile of SSCS and the limitations of the 6-OHDA toxin model, this non-invasive neuromodulator treatment's therapeutical benefit should be tested in the future in PD patients, allowing a better approach to evaluate axial symptoms.

8. CONCLUSION

Overall, this study's results suggest that SSCS can modulate supraspinal activity attenuating oscillatory activity associated with PD. This modulation does not guarantee clinical efficacy, but our results provide interesting findings that encourage exploring this Parkinson's disease treatment more deeply. Since we did not observe any side effects and this approach is simple, inexpensive, and non-invasive, this new method might be an important contribution to clinical practice. Future studies should be considered to evaluate SSCS in humans where paresthesia can be reported, and according to this finding and motor outcomes, we can select specific settings of stimulations in a graded manner based on individual state and responsiveness. Human studies also allow us to evaluate the impact of ongoing, continued SSCS focusing on axial symptoms like FoG and gait impairment that have reported remarkable results with invasive spinal cord stimulation and are unmet need for conventional treatments.

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