Non-invasive Neuromodulation in MPTP-induced Parkinson's Mice Model.

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NON-INVASIVE NEUROMODULATION IN MPTP-INDUCED PARKINSON'S MICE MODEL.

A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science

In
Biomedical Sciences

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We hereby recommend that the dissertation prepared under our supervision by Nayab Nawaz (NUST2013362092MSMME62413F) titled: **Non-invasive Neuromodulation In MPTP-induced Parkinson's Mice Model** be accepted in partial fulfillment of the requirements for the award of MS degree with ___ Grade.

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I dedicate my thesis to my parents and my brothers (Najaf Nawaz and Nashit Hassan) for their immense support, motivation & love.

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Nayab Nawaz.

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LIST OF ACRONYMS

%	Percent
°C	Centigrade
mA	Milli amperes
PD	Parkinson's Disease
DA	Dopaminergic Neurons
MPTP	1-methyl-4-phenyl-1,2,3,6-tertrahydropyridine
tDCS	Transcranial direct current stimulation
EEG	Electroencephalography
IP	Intraperitoneal
B	Beta
Hz	Hertz
min	minute
S	second
G	Gram

ABSTRACT

Transcranial direct current stimulation (tDCS) is connected with change in NMDA receptor movement either increase or decrease in their activity and change in cortical blood stream. Therefore, repeated tDCS of the brain with Parkinson's disease (PD) will induce the functional and histological changes as well as changes in EEG activity. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (30 mg/kg; i.p injection) was used for inducing PD in mice model. Twelve Balb/c mice were used in this study, which were allocated to 4 groups namely the control group (n=3), tDCS group (n=3) receiving tDCS treatment only, MPTP group (n=3) given MPTP injection of 30 mg/kg, and MPTP plus tDCS group (n=3) having both MPTP injection and tDCS treatment. Schedule for both MPTP and tDCS-treatment schedule comprised of 5-days. Grid walking test, and swim test scores were checked at different days postoperatively. Cup electrodes were used for tDCS treatment as well as EEG recordings. EEG of each mice was taken on the 5^{th} day. After the experiments, mice were sacrificed for the evaluation of histological changes (changes of neuronal cells). The results shows that the MPTP plus tDCS group showed improvement of grid walking and swim test scores at 5^{th} day of tDCS treatment postoperatively. Significant increase in β-activity was seen in MPTP group whereas after the tDCS treatment significant decrease in β-activity towards the normal condition was seen in MPTP plus tDCS group. During histological analysis, well preserved neurons were seen in MPTP plus tDCS group. From these results, it could be inferred that repeated tDCS have a preventive effect on dopaminergic neurons in MPTP-induced PD mice model.

Chapter 1

Introduction

Chapter 1

INTRODUCTION

Parkinson's disease (PD) refers to a group of neurodegenerative condition affecting several brain parts which includes pigmented nuclei in midbrain and brainstem, the olfactory tubercle, the cerebral cortex, and elements of the peripheral nervous system (Braak et al., 2006). PD affects 1 in 100 people over the age of 60 (Tanner and Ben-Shlomo, 1998, Van Den Eeden et al., 2003) and the number of affected individuals is set to rise dramatically owing to increasing life expectancy (Dorsey et al., 2007). Motor impairments are thought to be the earliest and most striking physical disabilities that are together known as 'parkinsonism'. These impairments include akinesia (paucity), bradykinesia (slowness of movement), rigidity (muscle stiffness), and tremor in rest position (Galvan and Wichmann, 2008).

Due to its debilitating nature, an enormous social and economic burden is placed on society. Worldwide, it is estimated that 6.3 million people have PD with no differentiation for race and culture. The age of onset is usually over 60, but it is estimated that one in ten are diagnosed before the age of 50, and it can affect people in their 40's and younger (Khandhar and Marks, 2007).

One of the major pathophysiology of PD includes a progressive degeneration of the dopaminergic (DA) neurons situated in the midbrain substantia nigra pars compacta (SNpc) afferent fibers that venture to the striatum (STR) (Kish et al., 1992). At first sustained DA neuron would be able to make up for this loss. Symptoms of PD regularly show when more

or less than 60% of the SNpc neurons have been compromised (German et al., 1989, Przedborski et al., 2001).

Another troublesome symptom of Parkinson's is mild cognitive impairment. Many people with Parkinson's are surprised to find that they feel distracted or disorganized, or have difficulty planning and carrying through tasks. It may be harder to focus in situations that divide their attention, like a group conversation. When facing a task or situation on their own, a person with PD may feel overwhelmed by having to make choices. They may also have difficulty remembering information, or have trouble finding the right words when speaking. For some people these changes are merely annoying, for others they interfere with work or with managing household affairs. To some degree, cognitive impairment affects most people with Parkinson's. The same brain changes that lead to motor symptoms can also result in slowness in memory and thinking. Stress, medication, and depression can also contribute to these changes (Verbaan et al., 2007).

Traditionally, Parkinson's disease (PD) has been known as a movement disorder, characterized by such symptoms as tremor and slowness of movement. Increasingly, it is becoming recognized also for its non-motor characteristics, including cognitive difficulties. Non-motor symptoms can vary substantially from patient to patient and can include the following: drooling; change in taste and smell; choking and swallowing difficulties; nausea and vomiting; constipation; uncontrolled loss of stool; bladder dysfunction; unexplained changes in weight; dementia and cognitive impairment; hallucinations; depression and anxiety; sexual dysfunction, orthostatic hypotension; excessive daytime sleepiness; insomnia; REM sleep behavior disorder; restless leg

syndrome; leg swelling; excessive sweating; double vision; delusions and impulse control disorders (Poewe, 2008).

For the past several decades, animal models of PD have come in a variety of forms. Typically, they can be divided into those using environmental or synthetic neurotoxins or those utilizing the in vivo expression of PD-related mutations (genetic).

Of the neurotoxic models, compounds that produce both reversible (reserpine) and irreversible (MPTP, 6-OHDA, paraquat, rotenone) effects have been used effectively; however recent studies have focused more on irreversible toxins to produce PD-related pathology and symptomatology. Therefore, the neurotoxins covered in this paper will focus on those that produce an irreversible effect. Neurotoxin-based models produced by 6-hydroxydopamine (6-OHDA) and 1-methyl-1,2,3,6-tetrahydropyridine (MPTP) administration are the most widely used toxic models, while paraquat and rotenone are more recent additions to the stable of toxic agents used to model PD. A common feature of all toxin-induced models is their ability to produce an oxidative stress and to cause cell death in DA neuronal populations that reflect what is seen in PD. Oxidative stress results from increased production of extremely reactive free radicals, including reactive oxidative species (ROS) and peroxynitrite. ROS may be formed during a number of cellular processes, including mitochondrial oxidative respiration and metabolism. There are some drawbacks to the use of these models such as the time factor in these models versus the time factor in the human condition, but these do not negate the value of neurotoxin-based animal models in the study of PD.

Despite numerous toxins and neurological insults that damages the basal ganglia and the substantia nigra result in neurological ailments which incorporate parkinsonian features, one toxin named 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), seems to target particularly those neurons that are included in PD. MPTP has been utilized to create animal models for testing new treatments in human disease. Investigation of the components of MPTP toxicity have likewise given experiences with respect to the possible pathogenesis of PD (Tillerson and Miller, 2003).

There are several techniques to assess changes in brain activity such as positron emission tomography (PET), magnetic resonance imaging (fMRI), measures of brain magnetic activity (Magnetoencephalography - MEG) and measures of brain electrical activity (EEG). Beta band oscillations have been associated with both cognitive and motor functions in normal animals and human subjects and have been hypothesized to play a key role in the maintenance of the current behavioral state (Kamarajan et al., 2004). The normal levels of beta oscillations and synchronization along the cortico-basal ganglia pathway in the normal state undergo a dramatic increase during parkinsonism. The oscillations decrease drastically during different treatments of parkinsonism such as dopaminergic medication or high frequency DBS.

Recent research has highlighted the potential of non-invasive brain stimulation, such as transcranial direct current stimulation (tDCS), to complement and enhance neuroplasticity and learning in patients with neurological disorders and older individuals (Broeder et al., 2015). TDCS is a technique that elicits constant weak electric currents through the scalp via two electrodes (anode and cathode), which has been shown to

modulate excitability in cortical and subcortical tissue (Bindman et al., 1964, Nitsche and Paulus, 2000a, Nonnekes et al., 2014a, Radman et al., 2009a). A possible beneficial effect of tDCS stimulation specific for PD patients could be the induction of dopamine release in the caudate nucleus via the glutamatergic corticostriatal pathways as was shown in animal studies (Li et al., 2011, Lu et al., 2015, Strafella et al., 2001, Tanaka et al., 2013). Recently, it was suggested that tDCS may also have a neuroprotective role in PD by reducing the oxidative damage of dopaminergic neurons (Lu et al., 2015).

1.1. Research objectives

Since its first description, PD has gone from a rarely reported disorder to one of the most common disabling diseases among older adults. Many studies have shown a link between PD and prevention of its symptoms through certain electric current treatments. A lot about the effect of transcranial direct current stimulation has yet to be identified.

The hypothesis of the study is "Transcranial direct current stimulation (tDCS) could cause neuroprotection of dopaminergic neurons by increasing the level of dopamine in substantia nigra of MPTP induced Parkinson's mice".

Main objective of the study is to determine the relation between Parkinson's and DA neurons neuroprotection through tDCS treatment in cortex region of brain i.e. determining the therapeutic effect of tDCS on MPTP- induced mice.

Chapter 2

Literature review

Chapter 2

LITERATURE REVIEW

2.1. Parkinson's Disease

Parkinson's disease (PD) was first therapeutically depicted as a neurological disorder by James Parkinson in 1817, however fragments of Parkinsonism can be found in earlier descriptions. Parkinson's opening portrayal has the key essentials: "Involuntary tremulous motion, with diminished muscular power, in parts not in real life and even when supported; with an affinity to bend the trunk forward, and to go from a walking to a running pace: the senses and intellects being uninjured"(Parkinson, 2002).

PD is a progressive neurodegenerative condition coming about because of the death of the dopamine containing cells of the substantia niagra. There is no consistently reliable test that can recognize PD from other conditions that have comparable clinical symptoms. The diagnosis is primarily a clinical one in the view of the history and examination (Dauer and Przedborski, 2003).

The earliest and most striking physical disabilities resulting because of these changes are motor impairments that, together, are called 'parkinsonism'. Individuals with PD classically give the symptoms and signs connected with parkinsonism, namely hypokinesia (i.e. poverty of movement), bradykinesia (i.e. slowness of movement), rigidity and rest tremor. Parkinsonism can likewise be caused by drugs and less normal conditions such a multiple cerebral infarction, and degenerative conditions for example progressive sypranuclear palsy (PSP) and multiple system atrophy (MSA) (Daley, 2013).

Despite the fact PD is predominantly a movement disorder, other impairments including psychiatric problems such as depression and dementia. Autonomic disturbances and pain may later guarantee, and the condition advances to bring about significant disability and handicap with impaired quality of life for the affected individual. Family and carers might likewise be affected indirectly. PD is a common, chronic, progressive neurological condition, assessed to influence 100–180 individuals per 100,000 of the population (somewhere around 6 and 11 people per 6000 of the general population in the UK) and has a yearly rate of 4–20 for every 100,000. There is a rising prevalence with age and a higher prevalence and rate of PD in males (Conditions, 2006).

2.2. MPTP-induced Parkinsonism

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin which is precursor to MPP+, which causes permanent symptoms of PD. MPTP is basically a lipid soluble neurotoxin which penetrates the blood brain barrier entering the brain cells. Administration of MPTP causes a specific loss of SNpc neurons that reiterates the DA neuronal loss seen in idiopathic PD (Smeyne and Jackson-Lewis, 2005, Hare et al., 2013).

In the brain, MPTP is metabolized by glial cells utilizing the MAO-B enzyme (Ransom et al., 1987), which results in a temperamental metabolite, MPDP that further metabolizes to generate the corresponding pyridium species, MPP+. (Brooks et al., 1989). MPP+ is then discharged from the glial cells and enters neurons by means of the dopamine transporter (DAT) where it meddles with Complex I respiration in the electron transport chain of the mitochondria (Cui et al., 2009) and creates more neuronal harm through the initiation of reactive microglia (Gao et al., 2003) and resulting generation of free radicals

(as shown in figure 1) (McGeer and McGeer, 2008). With one-week of post administration of MPTP a noteable loss of DA neurons in the SNpc is clear, alongside with a significant reduction of DA generation in the terminal field within the striatum. In this manner, MPTP administration actuates a DA neuron loss that mirrors the loss found in end-stage PD (Jackson-Lewis et al., 1995).

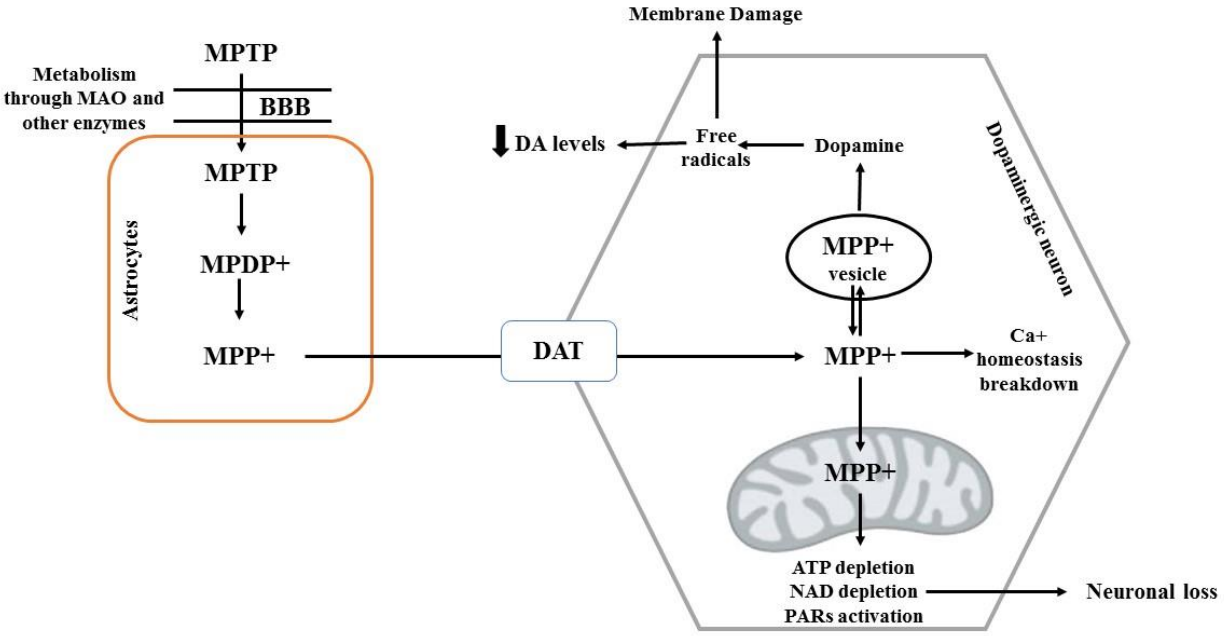


Figure 1: Schematic representation of mechanism of MPTP toxicity. BBB- Blood brain barrier; MPTP- 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; MAO- Monoamine oxidase; MPDP+- 1-methyl-4-phenyl-2, 3-dihydropyridium; MPP+- 1-methyl-4-phenylpyridinium; ATP- Adenine triphosphate; NAD- Nicotinamide adenine dinucleotide; PARs- Protein activated receptors; DA- Dopamine; DAT- Dopamine transporter (Modified from (Smeyne and Jackson-Lewis, 2005)).

2.3. Motor Deficits in PD

PD is an irreversible, progressive neurodegenerative disorder, characterized clinically by four major symptoms: resting tremor, bradykinesia, postural instability and rigidity. These motor symptoms do not typically develop until 50-60% of nigral neurons have been lost and 80-85% of the dopamine substance of the striatum has been exhausted. In addition to the severe motor deficits connected with Parkinson's disease, an assortment

of cognitive and emotional impairments additionally might manifest during disease course (Rodriguez-Oroz et al., 2009).

In an investigation of recently diagnosed PD patients, 24% of patients showed cognitive dysfunction, contrast with 4% of controls (Muslimović et al., 2005). Braak and colleagues (2005) reported that 33% of patients with sporadic PD at stage 3 of the Braak neuropathological staging already demonstrated noteworthy cognitive decrease, despite the fact that alpha-synuclein accumulations are not yet present in the cortex during this early pathological stage. This number rose to 66% of the cohort at stage 4, was greater than 90% at stage 5 and reached 100% at stage 6 (Braak et al., 2005). Population-based longitudinal studies have proposed that some level of cognitive impairment is present in 80% of patients inside of 12 to 20 years after motor symptom development (Aarsland et al., 2001, Hely et al., 2008).

2.4. Loss of dopaminergic neurons in PD

In Parkinson's disease (PD), degeneration of dopamine-producing neuronal cells starts leaving less of the chemical i.e. dopamine. This causes "short circuiting" of movement control center of brain, overstimulating the target neurons. This leads towards the muscle tremor symptomatic of Parkinson's (Youdim and Riederer, 1997).

The degeneration of dopaminergic SNc neurons and their projections in case of PD is gradually evolving process which may take years to generate. These SNc projections begin to detoriate earlier as compared to the striatum associative or limbic portions. In relation to this slow degeneration time course, the motor signs and symptoms of PD develop before non-motor signs and systems (Dickson, 2007).

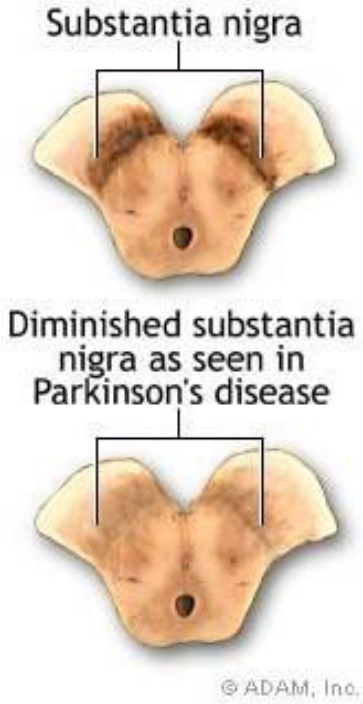


Figure 2: Substantia nigra and Parkinson's disease. (Adapted from PubMed Health).

Prominent secondary morphological changes are initiated by the loss of dopamine in basal ganglia. One change that may have pathophysiological importance is the decrease of thickness and sensitivity of dendritic spines on MSNs, especially in the putamen, which might considerably interfere corticostriatal transmission (Villalba et al., 2006, Zaja-Milatovic et al., 2005). According to the recent studies, MSNs with D2 receptors related to the indirect pathway may be preferentially influenced by the spine loss which might involve in the dysregulation of Ca+ channels (Day et al., 2006).

The DA receptors in subcellular areas of the striatum might also change. Therefore, as compared to normal condition, extent of D1- receptors bound to the plasma membrane is prominent, whereas proportion in the cytoplasm is smaller, in Parkinsonism (Guigoni et al., 2007). At the level of direct pathway terminations in GPi or SNr, this is not the situation

in MPTP-treated animals (Kliem et al., 2009). The subcellular distribution of striatal D2-receptors seems to be only influenced by MPTP-treatment (Guigoni et al., 2007).

The motor symptoms of PD result in response idiopathic cell death of the dopaminergic neurons of SNc. This further causes the depigmentation of the SNc observed in PD patients. The basal ganglia pathways are basically influenced in PD. The DA consumption is the most serious there in PD, as DA neuron of the SNc extends mostly towards the dorsolateral putamen (Alexander, 2004). Not every single nigrostriatal neuron is as affected, however, as DA neurons of the VTA are left for the most part in place with significantly less DA consumption at their projection site in caudate. SNc signaling deficiency prompts the difficulty in the start and execution of movement seen in PD, as well as the muscle rigidity and resting tremors, as SNc is thought to responsible for altering basal ganglia output in coordination. The subthalamic nucleus (SN), the target for deep brain stimulation (DBS) and transcranial direct current stimulation (TDCS) treatment, normally serves to modulate the action of the GPi and SNr and also levels of dopamine responsible for the inhibition of unwanted movements. The SNc DA neurons serve to restrain this pathway, thus inhibiting the motor neurons (Galvan and Wichmann, 2008).

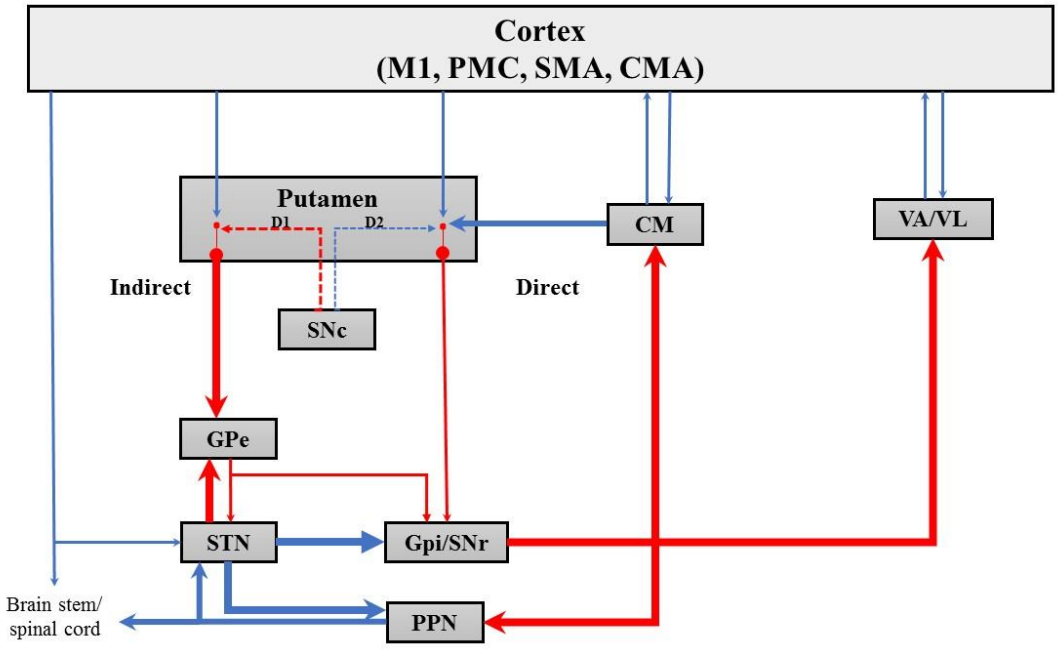


Figure 3: Schematic diagram of the direct (Dir.) and indirect (Indir.) pathways of the basal ganglia motor circuits in parkinsonian state. Red arrows indicate inhibitory projections, and blue arrows indicate excitatory projections. The changes in the thickness of the arrows in the parkinsonian state indicate the proposed increase (larger arrow) or decrease (thinner arrow) in firing-rate activity of specific connections. The dashed arrows used to label the dopaminergic projection from the SNc to the putamen in parkinsonism indicate partial lesion of that system in this condition. Note that many connections have been purposefully omitted from this diagram. CM, centromedian nucleus; CMA, cingulate motor area; GPe, globus pallidus, external segment; GPi, globus pallidus, internal segment; M1, primary motor cortex; PMC, pre-motor cortex; PPN, pedunculopontine nucleus; SMA, supplementary motor area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; VA/VL, ventral anterior/ventral lateral nucleus (modified from (Galvan and Wichmann, 2008)).

2.5. Basal Ganglia (BG) oscillations and PD pathophysiology

Up till now two hypotheses have been proposed in relation to PD pathophysiology and BG oscillations that are the "firing rate model" and the "firing pattern model".

2.5.1. Firing rate model:

The firing rate model initially proposed that Dopamine consumption causes reduction in toxic excitation to striatal neurons proceeding towards the internal segment of the globus pallidus (GPi) known as direct pathway and toxic inhibition to striatal neurons progressing towards external segment of the globus pallidus (GPe) known as indirect pathway (Gerfen et al., 1990, Mallet et al., 2006). Both of these pathways are thought to increase average firing rates of GPi and SNPr neurons. This enhanced activity in BG output causes downregulated activity in the thalamic and cortical neurons, which ultimately results in akinesia (Figure 4). A number of studies in relation to the original one also confirmed same changes in the activity (Filion, 1991, Bergman et al., 1994, Boraud et al., 1996, Boraud et al., 1998, Heimer et al., 2002, Wichmann et al., 2002, Soares et al., 2004). According to the recent optogenetic study, facilitation of striatal direct pathway in PD mice alleviates akinesia and facilitation of striatal indirect pathway neurons in normal mice also leads to akinesia (Kravitz et al., 2010).

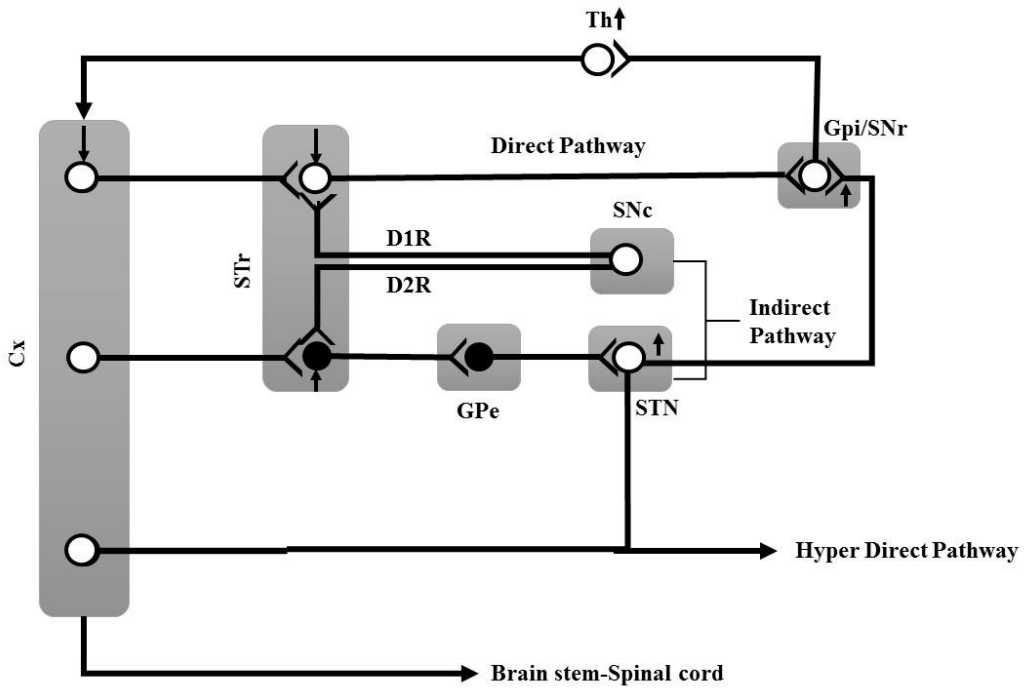


Figure 4: "Firing rate" model explaining the pathophysiology of Parkinson's disease. Open and filled symbols represent excitatory and inhibitory neurons, respectively. Cx, cerebral cortex; D1R, D2R, dopamine D1 and D2 receptors; GPe and GPi, external and internal segments of the globus pallidus; SNc and SNr, substantia nigra pars compacta and reticulata; STN, subthalamic nucleus; Str, striatum; Th, thalamus. Modified from (DeLong, 1990).

2.5.2. Firing Pattern Model

The 'firing pattern model" emphasis on the oscillatory and synchronized activity of the brain. These activities are frequently observed in BG of patients having movement disorders as well as animal models that may cause detoriation of information processing with in the BG (Bergman et al., 1998). Recording of unit activity and local field potentials from PD patients and animal models have shown oscillatory and synchronized activity in GPe, GPi, and STN (Figure 5) (Bergman et al., 1994, Heimer et al., 2002, Wichmann and Soares, 2006, Heimer et al., 2006). The tremor (4-9Hz) and β (10-30Hz) bands are included in frequency bands. The β-band oscillatory activity is thought to be the primary cause of akinesia, since suppression of β-band oscillations have been seen in the treatment of akinesia with drugs. Recent studies have also reported β-band synchronized activity in STN activity with freezing gate cycle in PD patients (Moshel et al., 2013, Toledo et al., 2014).

Deep brain stimulation (DBS), an accepted therapeutic option of PD, is thought to improve motor symptoms through the activation of afferent fibers, changes in oscillatory activity and decoupling STN-GPi oscillations (Hashimoto et al., 2003, Vitek, 2008, Moran et al., 2012). As compared to the course of MPTP-treatment of primates, appearance of oscillatory activity in response to PD motor symptoms, seems to be in contradiction with firing pattern model (Leblois et al., 2007).

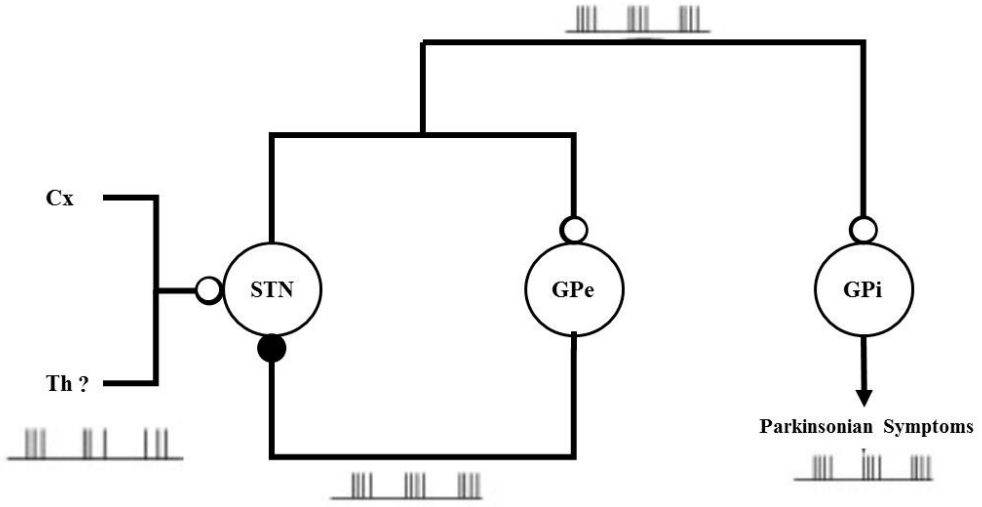


Figure 5: "Firing pattern" model explaining the pathophysiology of Parkinson's disease. Open and filled symbols represent excitatory and inhibitory neurons, respectively. Cx, cerebral cortex; GPe and GPi, external and internal segments of the globus pallidus; STN, subthalamic nucleus; Th, thalamus. Modified from (Tachibana et al., 2011).

2.6. Therapeutic strategies in PD

Despite the fact that there is no lasting cure available for PD, during late years, there have been various alternatives for the effective treatment of PD including both pharmacotherapy and neurosurgery based methodologies. These strategies are aimed at enhancing the motor symptoms thereby bettering the life of the patient without no real side effects. In recent years, various pharmacological approaches, which in the recent past has increased significantly along these lines giving several therapeutic options for PD. While the definitive target of PD treatment is stopping/ delaying progression of the illness, most of the present treatments have not conclusively exhibited neuroprotective effects in PD patients.

A large number of the pharmacological treatments for PD concentrates on renewing the lost dopamine in the brain, but treatments with other mechanistic methodologies have likewise been misused in the treatment of early PD. Most of the current treatments are effective in symptomatic relief in early PD, however numerous patients develop motor complications with long-term treatment. Shockingly, PD medications do not successfully handle tremor, postural instability and cognitive deficits. Because of these serious lacunae, there has been huge momentum to create more up-to-date treatments involving neuroprotective, disease modifying, restorative, possibly curative drugs with lesser side effects.

In case of PD, since dopamine Substitution is the most reasonable option, levodopa (L-dopa) has been the most effective medication since 40-50 years (Miyasaki et al., 2002). L-Dopa treatment was initially produced by Birkmayer and Hornykiewicz in the 1960s

(Birkmayer and Hornykiewicz, 2001). Despite its prosperity, the efficacy of L-dopa has been limited because of several problems including over the top stimulation of the dopamine receptors altering their normal performance. During early PD, L-dopa treatment is primarily effective against bradykinesia and rigidity however not against different symptoms such as postural instability, speech issues and gait defect. In the later stages of PD, L-dopa is absolutely ineffective against speech volume, freezing of gait, balance control and swallowing problems (Almeida and Hyson, 2008).

Several pharmacological agents that target different mechanisms in PD pathology are being sought as major aspect of L-dopa saving methodologies. These incorporate monoamine oxidase B (MAO-B) inhibitors, dopamine receptor agonists, COMT inhibitors and so on. Recently, anti-glutamatergic medications such as Riluzole have also been utilized as neuroprotective agents in neurodegenerative diseases. These agents have been hypothesized to slow the hyperactive subthalamic nucleus that might contribute to nigral neurodegeneration. Riluzole has been exhibited to be neuroprotective in an in vivo PD model (Boireau et al., 1994a, Boireau et al., 1994b).

Surgery for Parkinson's disease has made some amazing progress since it was initially developed over 50 years ago. The newest form of this surgery, deep brain stimulation (DBS), was developed in the 1990s and is presently a standard treatment. Around the world, about 30,000 individuals have had deep brain stimulation (Pereira et al., 2007). During deep brain stimulation surgery, electrodes are embedded into the focused brain region using MRI and neurophysiological mapping to ensure that they are implanted in the correct spot. A device called an impulse generator or IPG (similar to a pacemaker)

is inserted under the collarbone to give an electrical impulse to a part of the brain included in motor function. The individual who undergo the surgery are given a controller, which permits them to check the battery and to turn the device on or off. An IPG battery goes on for around three to five years and is generally simple to supplant under local anesthesia (Benabid, 2003).

Deep brain stimulation is not a cure for Parkinson's, and it does not slow disease progression. Like all brain surgery, deep brain stimulation surgery conveys a little risk of infection, stroke, or bleeding. A small number of individuals with Parkinson's have experienced cognitive decline after this surgery. All things considered, for many people, it can significantly soothe few indications and enhance quality of life. Studies show benefits lasting no less than five years (Machado et al., 2006).

2.7. Transcranial direct current stimulation

Non-invasive stimulation with tDCS and TMS is able to improve neuroplasticity processes at least in healthy elderly (Zimerman and Hummel, 2010). Previous efficient reviews have shown that repetitive TMS (rTMS) can enhance motor function in PD (Elahi et al., 2009, Chou et al., 2015, Fregni et al., 2005, Zanjani et al., 2015). Also, it was recently showed that the use of TMS over the right posterior parietal cortex after focused visuomotor training improved the retention of a recently gained motor skill in PD for no less than 24 h (Moisello et al., 2015). Although both TMS and tDCS can possibly adjust to modulate cortical excitability, bringing about immediate and long-term effects, tDCS is considered to have more therapeutic potential as it is safer and secure, less costly and more user-friendly (Yokoi and Sumiyoshi, 2015). TDCS evokes constant weak electric currents

which modulates excitability by activating alterations of neuronal resting membrane potentials in cortical and subcortical tissue (Nitsche and Paulus, 2000b, Nonnekes et al., 2014b, Radman et al., 2009b). Moreover, different mechanisms for example dynamic modulation of synaptic efficacy and the induction of the release of neurotransmitters might be included as well (Parasuraman and McKinley, 2014, Stagg et al., 2009, Tanaka et al., 2013). A possible beneficial impact of tDCS stimulation particular for PD patients could be the impelling of dopamine discharge in the caudate nucleus through the glutamatergic cortico striatal pathways as was shown in animal studies (Li et al., 2011, Lu et al., 2015, Strafella et al., 2001). Recently, it was recommended that tDCS might likewise have a neuroprotective part in PD by reducing the oxidative damage of dopaminergic neurons (Lu et al., 2015). Besides, it was found that tDCS regulates functional connectivity of the cortico-striatal and thalamo-cortical circuits in the human brain (Polanía et al., 2011).

2.8. Neuroprotective role of tDCS

Transcranial direct current stimulation (tDCS) is a noninvasive tool that alters cortical excitability. Preliminary observations suggest that this approach can indeed influence a number of cellular and molecular pathways that may be disease relevant. However, the mechanisms of action underlying its beneficial effects are largely unknown and need to be better understood to allow this therapy to be used optimally.

tDCS has recently been used as a functional intervention technique for the treatment of psychiatric and neurological diseases. The tDCS effects are based on the polarity of the electrode, stimulus location, intensity and duration, as well as the timing of application depending on the pathophysiological feature of a disease. It has been reported that anodal

tDCS increases neuronal excitability, whereas cathodal tDCS reduces it (Nitsche and Paulus, 2000a). The tDCS of the parietal cortex can modulate working memory performance (Heimrath et al., 2012) and increase extracellular DA levels in the rat striatum (Tanaka et al., 2013). These effects of tDCS suggest that tDCS may be beneficial for the cognitive function and behavioral tasks in PD patients (Boggio et al., 2006). However, the studies of tDCS on PD animal model are limited.

The mechanism for the neuronal protection of tDCS may be also related to a role of brain-derived neurotrophic factor (BDNF). It was recently reported that tDCS promotes synaptic plasticity via a mechanism involving BDNF secretion (Fritsch et al., 2010). It is known that BDNF promote neuronal survival (Massa et al., 2010), partially via a mechanism involved in antioxidative stress (Duman et al., 1997, Chan et al., 2010). It has been reported that cathodal tDCS has neuroprotective effects on the immature rat hippocampus after pilocarpine-induced SE, including reduced sprouting and subsequent improvements in cognitive performance. Such treatment might also have an antiepileptic effect (Kamida et al., 2011).

Non-invasive stimulation with tDCS is able to enhance neuroplasticity processes at least in healthy elderly (Zimerman and Hummel, 2010). The long-term neuroplasticity effects of tDCS on M1 were proposed to be based on several processes that accompany motor learning such as LTP via modulating intracellular signals by increasing the net calcium influx into the targeted cortical neurons after stimulation (Karabanov et al., 2012). In addition, tDCS may adjust resting membrane potentials mediated by changes in N-

methyl-d-aspartate-receptor activation and GABAergic inhibition (Liebetanz et al., 2002, Paulus et al., 2008, Stagg et al., 2009, Tanaka et al., 2013).

De Xivry and Shadmehr (2014) proposed three polarity-dependent key principles underlying the effects of tDCS on motor control and learning: (i) the alteration of neuronal firing rates (i.e. the increase by anodal and decrease by cathodal stimulation), (ii) the strengthening and stabilization of newly formed associations in the cerebral cortex by anodal polarization and (iii) the formation of new and/or preferred firing pattern of neurons in memory after anodal stimulation. The authors stated that the first principle may be responsible for the direct effects of tDCS on motor performance. The second and third principle on the other hand could be linked to the acquisition and consolidation phases of motor learning and particularly relevant for use in combination with behavioral interventions in PD (De Xivry and Shadmehr, 2014). Thus, tDCS has the potential to influence synaptic plasticity, which may enhance training-induced learning in PD.

TDCS was also shown to modulate cognitive function in healthy young and older subjects (Fertonani et al., 2014, Harty et al., 2014). As in motor learning, tDCS was suggested to influence cognitive networks by altering cortical excitability in key cognitive regions which are potentially penetrable such as the dorsolateral prefrontal cortex (DLPFC) (Miniussi et al., 2013). In PD, this area has been implicated in executive function impairment through the dopaminergic dysfunction of the striatofrontal network and top-down attentional dysfunction through alterations in the cholinergic frontoparietal circuits (Gratwicke et al., 2015). Indirectly, by improving cognitive function, it was suggested that motor control is likely to be affected as well (De Xivry and Shadmehr, 2014).

Though increased excitability of cortical areas by tDCS may induce spontaneous compensatory neural activity and result in direct symptomatic benefits for patients with PD, the exact relationship between alterations in neuroplasticity and clinical motor and cognitive symptoms is still unclear (Bologna et al., 2016).

Although the results of tDCS interventions in PD are still preliminary, they encourage further in-depth studies to define its role in the treatment of the disease. For tDCS to become a relevant clinical tool in PD, it must show to have positive, durable and lasting effects on cortex excitability and activities of daily living.

Chapter 3

Materials and Methods

Chapter 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine hydrochloride (MPTP hydrochloride) was obtained from Med Chem (Product catalogue# HY-15608). All other chemicals were purchased from Sigma-Aldrich (USA) unless indicated otherwise.

3.2 Animals

BALB/c mice were bred and housed in animal house of Atta ur Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST). Mice were kept in cages at constant temperature (25±2 ºC) and natural light-dark cycle (12-12 hours). Animals were given distilled water *ad libitum* and fed with standard diet consisting of (%): crude protein 30, crude fat 9, crude fiber 4 and moisture 10.4. Male mice (n=12) weighing 35-45 g and 4-6 months of age were used in experiments.

3.3 Ethics Statement

All experiments performed were in compliance with the rulings of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011). The protocol was approved from the Internal Review Board (IRB), SMME, NUST.

3.4 Study Design:

A 5 days long plan was formulated to generate a Parkinson's disease like mouse model by injecting MPTP and investigate the effect of transcranial direct current

stimulation on neurogenesis in this mouse model. Behavioral tests were performed for all 5 days and also on different days respectively in accordance with behavioral tests protocol, following which the animals were decapitated for histological studies (Figure 5).

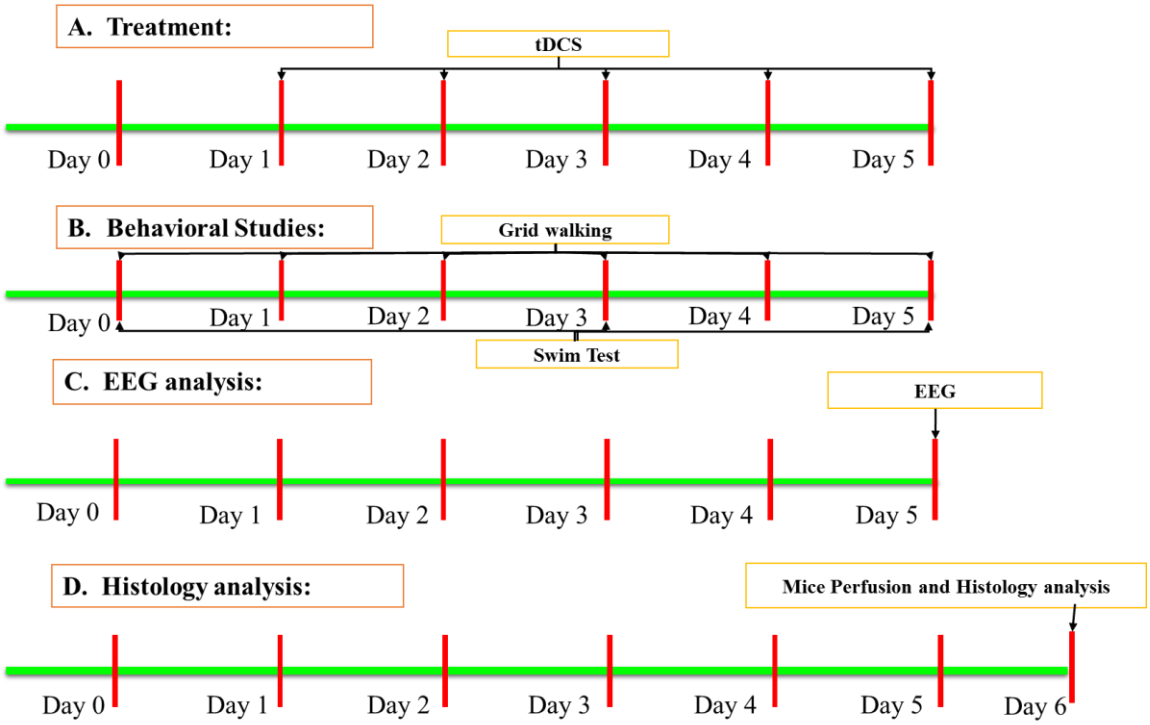


Figure 6: Experimental Plan for the study. (A) Balb/c male mice of 4-6 months of age received MPTP for 5 days as well as TDCS treatment. **(B)** For all 5 days' animals were analyzed for grid walking test (each trial of 1 min at least. Animals were also analyzed for swim test before the treatment day as well as on day 3 and day 5. **(C)** On day 5, EEG analysis of animals was done. **(D)** Mice were sacrificed and transcardially perfused on day 6 and their brains were processed for staining.

3.4.1 Animal Groups for Study:

Animals were randomly divided into four groups. Each group had a total of 3 animals of 4-6 months of age. Details of all the groups are as follows.

Table 1: Experimental design. Untreated Balb/c mice were used as the control. Other groups comprised of tDCS, MPTP (PD), and MPTP+tDCS (tDCS treated) treated mice.

Sr No.	Groups	No. of animals	IP injection	No. of days
1.	Control	3	Vehicle	5
2.	tDCS	3	Vehicle	5
3.	PD mice	3	MPTP= 30mg/kg	5
4.	tDCS treated mice	3	MPTP= 30mg/kg	5

3.5. MPTP-induced mice model of Parkinson's disease

MPTP induced mice model was developed using peritoneal (IP) injections of MPTP hydrochloride. For IP injections BD Ultra-Fine II short needles (30 Gauge x 8 mm) were used. Mice were weighed daily and IP dose of 30 mg/kg body weight was given to the experimental mice everyday between 10 am-12 am. The cage lid was removed carefully to avoid excessive disturbance to the animals. The mouse to be injected was restrained smoothly by grasping its tail into forefinger and thumb. The mouse was lifted from the floor of the cage onto the cage lid maintaining a firm grip on the tail. Using the forefinger

and the thumb of second hand, loose skin was drawn up form over the shoulders and held securely to restrict movement of the mouse's head. Maintaining the grip on the scruff and the base of the tail, the mouse was lifted and turned over so that the body was supported on the palm of the hand. "Two man" procedure was employed in which one investigator restrained the mouse by the scruff with one hand and slightly extended the body of the animal by holding tail with the other hand. The body was tilted so that the head was facing downwards and the abdomen was exposed. The second investigator inserted the needle into the abdomen at about 30˚ angle to minimize the penetration into the abdominal organs. IP injection was made into the right or left lower quadrant of the mouse (Figure.7). After the injection, the needle was withdrawn and the mouse was put back into its cage and released.

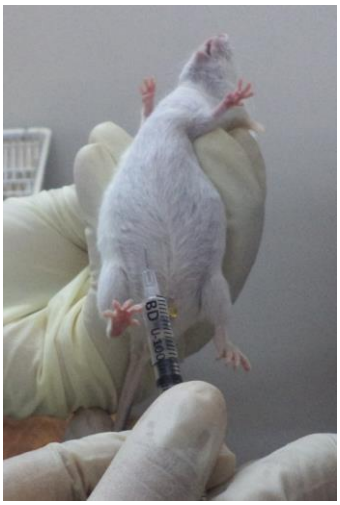


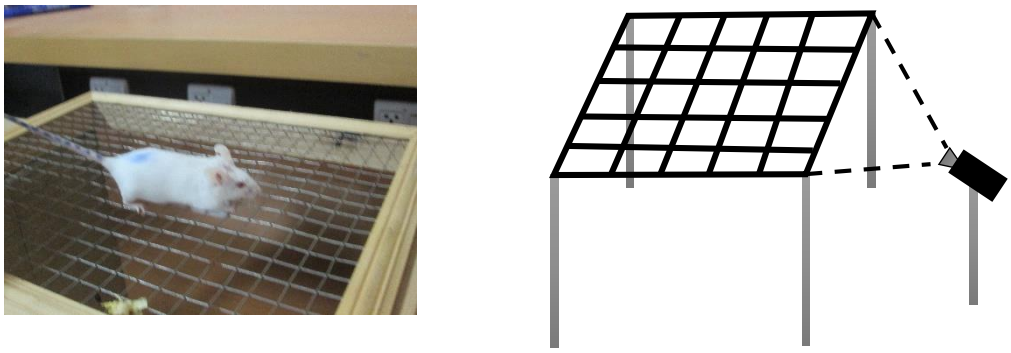
Figure 7: Two man method for intraperitoneal injection.

3.6. Behavior Studies

3.5.1. Grid Walking Test

3.5.1.1. Apparatus

An elevated metal square grid (41×41 cm2, with each grid cell 3.5×3.5 cm2; height: 41 cm) was used (Fig. 1A). The grid apparatus was located in a sound attenuated room with dim lighting. After each trial, 70% ethanol was used to clean the apparatus. A camera recorder was located below the apparatus with an angle of about 20–40 degrees (Fig. 1B). Behaviors on the grid were recorded and were analyzed later.



(A) **(B)**

Figure 8: The grid walking test setup. **(A)** The grid-walking test apparatus, **(B)** the relative location between the apparatus and the camera. The camera, was located in front of the grid apparatus and lower than the grid with an angle. Video recorded by the camera was required to capture the whole extent of the grid in order to count foot-faults.

3.5.1.2. Procedure

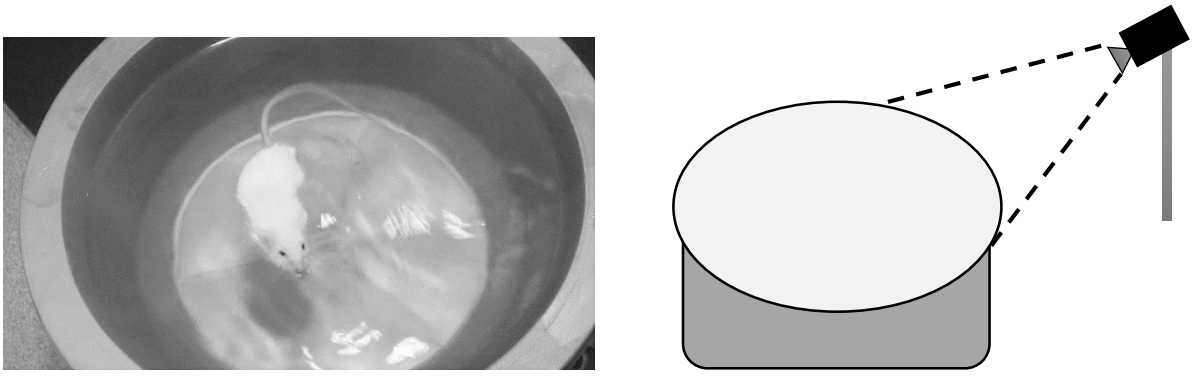
The grid-walking test assesses spontaneous motor deficits and limb movements involved in precise stepping, coordination, and accurate paw placement (Chao et al., 2012). One foot-miss was counted when the hind limb paw protrudes through the grid. Each mouse was placed at one end of the grid and monitored or videotaped from the side as they walk across the grid. The number of forelimb and hind limb placement errors as the animal traverses the grid was scored. An error is counted whenever a limb misses a bar and extends downward through the plane of the bars.

Each mouse was acclimatized for 1 minute to the grid. The total number of paired steps (placement of both forelimbs) was counted. During this period, the number of foot-fault errors in which the animals misplaced a forelimb such that it fell through the grid was monitored, and the total number of errors for each forelimb was recorded.

3.5.2 Swim Test

3.5.2.1. Apparatus

Swim-test was carried out on different days after MPTP treatment in water tubs (40 cm length×25 cm width×16 cm height). The depth of water was kept at 12 cm and the temperature was maintained at 27±2 ◦C. A camera recorder was located above the apparatus at a certain angle (Figure 9).



(A) **(B)**

Figure 9: The swim test setup. (A) The swim test apparatus, **(B)** the relative location between the apparatus and the camera. The camera, was located in front of the swim tub and slightly above than the tub with an angle. Video recorded by the camera was required to capture the whole extent of the swim test in order to count swim scores.

3.5.2.2. Procedure

On the 1st day of the swim test, mice were placed in clear tub filled to 12 cm with 27 ± 2°C water. After 1 min 30 sec of swimming, the mice were removed from the water, and wiped dry immediately after the experiment using a dry towel and returned to cages kept at 27±2 ◦C. Swim-score scales were: 0, hind part sinks with head floating; 1, occasional swimming using hind limbs while floating on one side; 2, occasional floating/swimming only; 3, continuous swimming (Figure 10) (Donnan et al., 1987, Muralikrishnan and Mohanakumar, 1998).

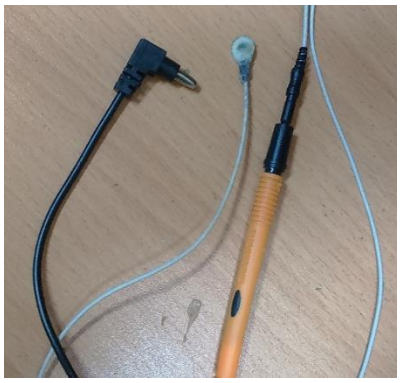
Figure 10: Swim-score scales for assessing swim activity in mice.

Swim test sessions were videotaped from the side of the tub and scored. Mice were rated at 5-s intervals throughout the duration of the retest session; at each 30-s interval, the predominant behavior was assigned to one of four categories mentioned earlier.

3.6. Transcranial direct current stimulation (tDCS) treatment

3.6.1. Apparatus

Anodal tDCS was applied using a constant-current isolated stimulator in Powerlab (ADInstruments, Australia) to deliver a current of 0.1 mA for 15 min. The electrode was 1 cm diameter, cup-shaped and filled with conductive gel. The contact area of active electrode was 0.785 cm2. However, the ground electrode used was square with 3×3 cm2 rubber pad.



(A)

(B)

(C)

Figure 11: Electrodes and their positioning for experiment. (A) Active electrode**, (B)** Ground electrode**, (C)** Fixation points of both electrodes.

3.6.2. Procedure

First, the surgical instruments were prepared by sterilizing them. Mouse was placed in the dissection tray. Head of the mouse was shaved and then positions for electrodes were marked using permanent marker. To simulate clinical studies in mice, the active electrode was attached transcranially to a mouse and fixed with glass ionomer cement. The active electrode was positioned 3 mm to the left and 2 mm in front of the interaural line. However, the counter electrode was attached to the trunk and surrounded by surgical tape to avoid displacement.

After electrodes fixation to the mouse head, they were further connected to the isolated stimulator in Powerlab intermediate teaching system (AD instrument; PTB4262). A constant current of 0.1mA was given to mice for about 15min. The whole tDCS experiment was carried out in faraday's cage. After experiment completion mouse was returned back to the cage. All the mice in tDCS group and MPTP+tDCS group were given the same current treatment with the same procedure followed.

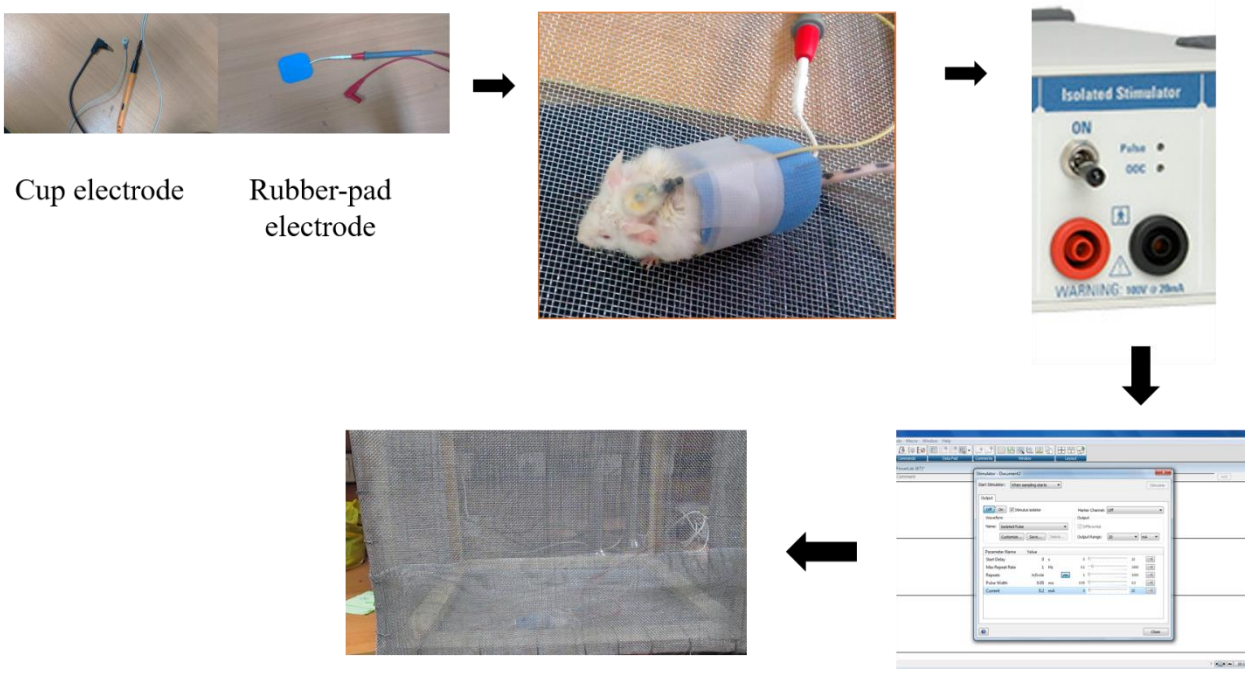


Figure 12: Experimental procedure for Transcranial direct current (tDCS) stimulation.

3.7. Electro encephalography (EEG) testing

3.7.1. Electrodes and placement

The active electrode was 1 cm diameter, cup-shaped and filled with conductive gel and was glued with glass ionomer cement on the skin's surface of the animal's head. The electrode placement was made by selecting four imaginary quadrants in an animal (Figure 13), and each one was fixed on each side of the head (bipolar register) after trichotomy. One electrode was glued with adhesive tape on the tail, as ground.

Figure 13: Electrodes placement for EEG-analysis.

3.7.2. Procedure

First, the surgical instruments were prepared by sterilizing them. Mouse was placed in the dissection tray. Head of the mouse was shaved and then positions for electrodes were marked using permanent marker (As shown in Figure 13). Electrodes were then filled with conductive gel and fixed on those marks. Positive and negative electrodes were placed on the head whereas Ground/ Reference electrode was placed on the tail of the mouse. Fixation of electrodes was done using glass ionomer cement to make sure that electrodes do not

move. Then these electrodes were attached to the BioAmp wire of the Powerlab device (Figure 14). After all this EEG signal was recorded using LabChart (v3.7) Software.

After all of the electrodes were in place, they were connected to the amplifier (module one of EEG equipment). The EEG activity was recorded with 120Hz of sampling frequency immediately after the electrodes placement for approximately 3 hours in awake freely moving rats. All experiments were performed at the same time of day (10:00 to 12:00 am).

3.7.3. Data analysis

The EEG recorded were analyzed by using digital signal processing techniques implemented in Matlab® software (MathWorks, Inc., MA, USA). In order to measure the system accuracy by comparing with literature results, the digital signal collected was analyzed by using Fourier transform (FT). Finally, Mean FFT values in specifics band frequencies (beta) was calculated and expressed as mean±S.E.M, analyzed by ANOVA followed by Tukey test, considering level of $P<0.05$. The precision of one measurement was defined by comparing it with the mean of N measurement as described by NORTHROP (2005).

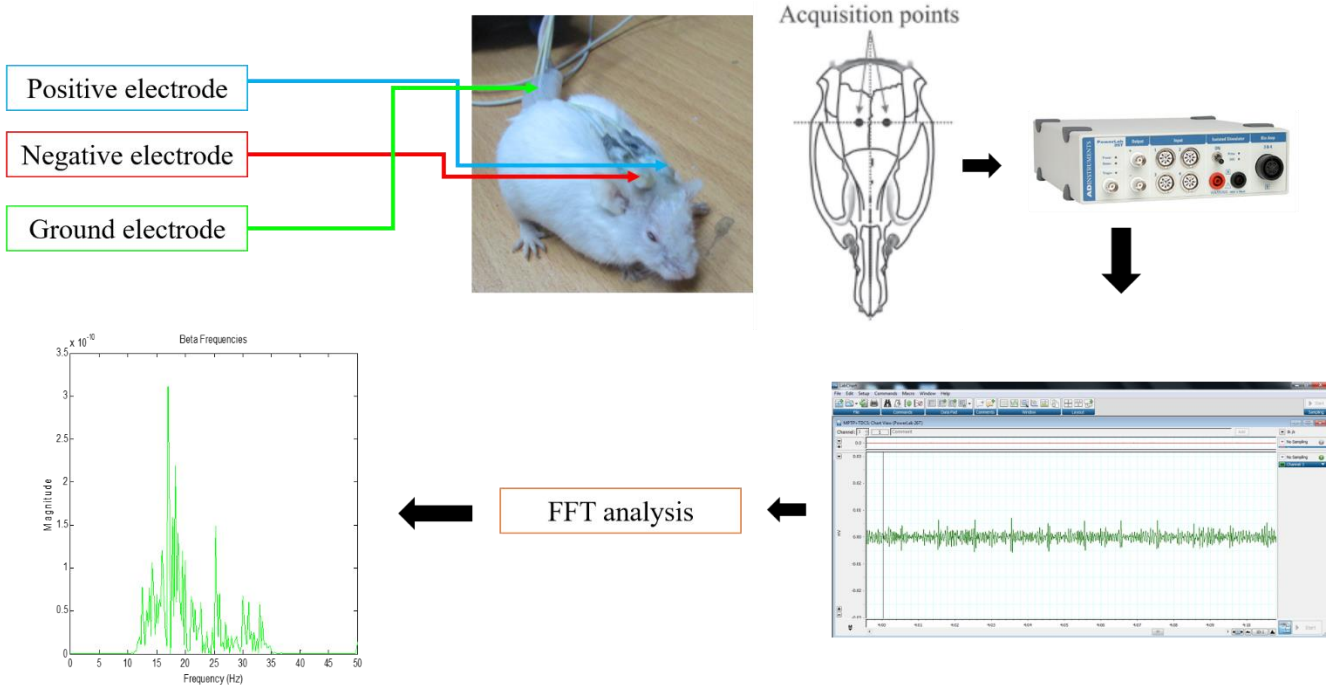


Figure 14: Experimental procedure for EEG examination.

3.8. Histological Examination of Brain Regional Tissues

3.8.1. Tissue Perfusion/Fixation for Histological Assessment

Heart perfusion was performed in accordance with the protocol of (Gage et al., 2012). Briefly, mice were weighed and then deeply anaesthetized by using Ketamine (300µl/50g i.p.). A midline incision was made and a sternotomy was performed to expose the heart. A steady and slow flow of normal saline was allowed at about 5ml/minute in to left ventricle by inserting a needle to about 5mm depth while holding the heart at fixed position with the forceps. An incision was made in the right atrium to allow blood to flow out. After about 80ml of normal saline injection, 100 ml of 4% paraformaldehyde solution is injected through left ventricle and brain was excised. The brain tissue was then placed in 4% paraformaldehyde for 24hrs at 4˚C before being processed further for paraffin processing and embedding. After 24 hrs in 4% paraformaldehyde, the brain tissue was dehydrated through a series of alcohols (isopropanol), 70% (1hr), 95% (1hr), and 100% (1hr) before paraffin infiltration. The brain tissues were then placed in xylene (4 hrs) and paraffin embedding was performed by keeping the tissue in molten paraffin (4 hrs at 60°C). It was then left to solidify (4^{o}C) in mold (block formation) prior to cutting.

3.8.2 Cresyl Violet Staining

Tissue sections (4 microns) mounted on slides were deparaffinized in xylene for 10 minutes before being rehydrated by 70% isopropanol (10 minutes), and washed with dd H2O (5 minutes). Cresyl violet stain was poured over the tissues sections and left for proper staining for 4 minutes. The sections were then washed with dd H2O and 70 % acid alcohol (2 minutes) and later dried for 2 hours before being mounted with cover slips. The slides

were visualized by inverted microscope (Labomed, USA) at 10X and 40 X resolutions. The images were captured by Pixel Pro™ image analysis software (Labomed, USA).

3.9. Statistical Analysis

Statistical analysis was done using Statistica 13.0. One-way ANOVA was used for finding significant differences between two FFT-means in the EEG signal. The data for behavioral studies were statistically evaluated for significance employing Two-way ANOVA followed by Tukey's multiple comparisons test. Results are given as mean±S.E.M. values. Values of $p \leq 0.05$ were considered significant.

Chapter 4

Results

Chapter 4

RESULTS

4.1. Effect of transcranial direct current stimulation on motor coordination in mice

Transcranial direct current stimulation (tDCS) was investigated for its effect on motor coordination using Grid walking test as well as Swim test. The assessment of motor coordination was done by measuring the percentage foot faults and swim score.

The percentage of foot faults within the control, tDCS, MPTP and MPTP plus tDCS groups were found to be statistically significant ($p<0.05$) indicating that every group of mice behaved differently during the tDCS treatment (Figure 15). After treating with MPTP (i.p injection) and tDCS (0.1 mA constant current), significant decrease in percentage foot faults ($p<0.05$) of motor coordination was seen in MPTP plus tDCS when compared with MPTP alone by measuring foot faults of mice in grid walking testing with no significant difference among control, tDCS and MPTP plus tDCS groups.

The swim score within the control, tDCS, MPTP and MPTP plus tDCS groups were not found to be statistically significant ($p>0.05$) indicating that every group of mice behaved same before the tDCS treatment (Figure 16). On 3rd day of after treating them with tDCS as well MPTP (i.p injection), significant difference ($p<0.05$) was seen among MPTP alone and MPTP plus tDCS group in swim scores of mice during swim test (Figure 17). The comparison of percentage of swim score on 5th day after treatment with tDCS of control with MPTP and MPTP plus tDCS with MPTP alone showed statistically significant

($p<0.05$) depicting that tDCS had helped MPTP mice to regain their motor coordination (Figure 18). However, no significant difference was seen between control, tDCS and MPTP plus tDCS group ($p>0.05$).

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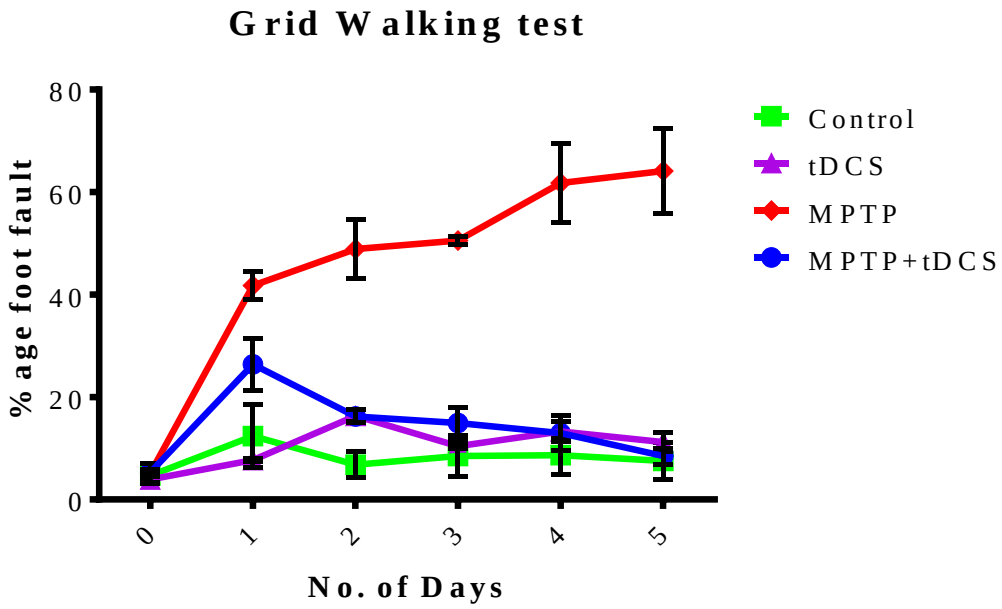


Figure 15: Comparison of after treatment percentage foot faults of grid walking test between control, tDCS, MPTP alone (MPTP) and tDCS plus MPTP (MPTP+tDCS) mice (n=3). The values in the Y-axis represent the percentage foot faults over 1-minute period when a mice traversed the grid. The difference in the mean percentage foot faults between them for grid walking was statistically significant ($p<0.05$). Error bars represent Mean±S.E.M (One-way ANOVA test).

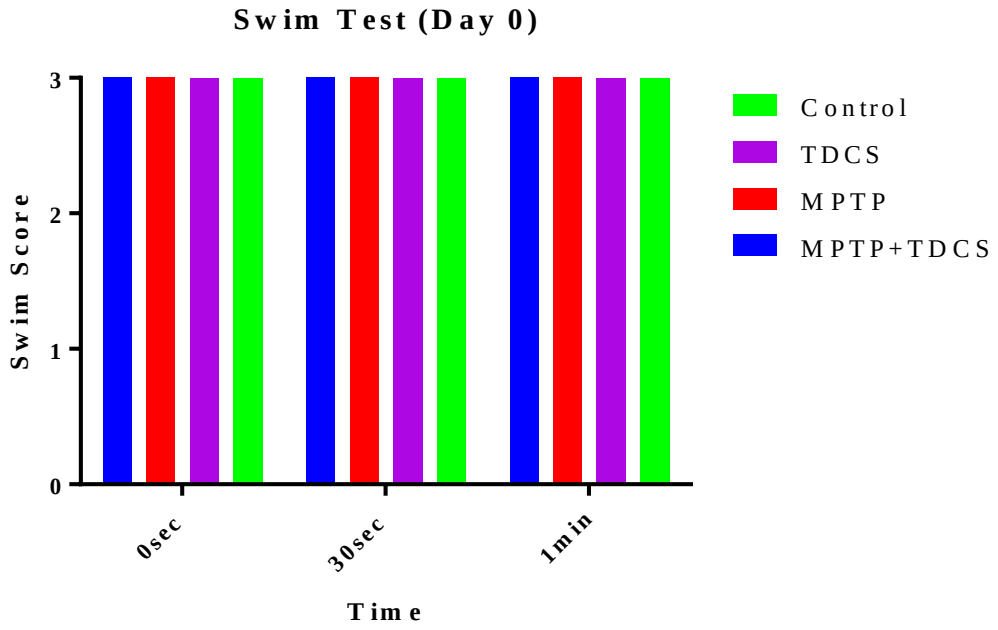


Figure 16: Comparison of before treatment swim scores of swim test between control, tDCS, MPTP alone (MPTP) and tDCS plus MPTP (MPTP+tDCS) mice (n=3). The difference in the mean swim scores between them for fear conditioning was not statistically significant (p=0.7583) Error bars represent Mean±S.E.M (One-way ANOVA test).

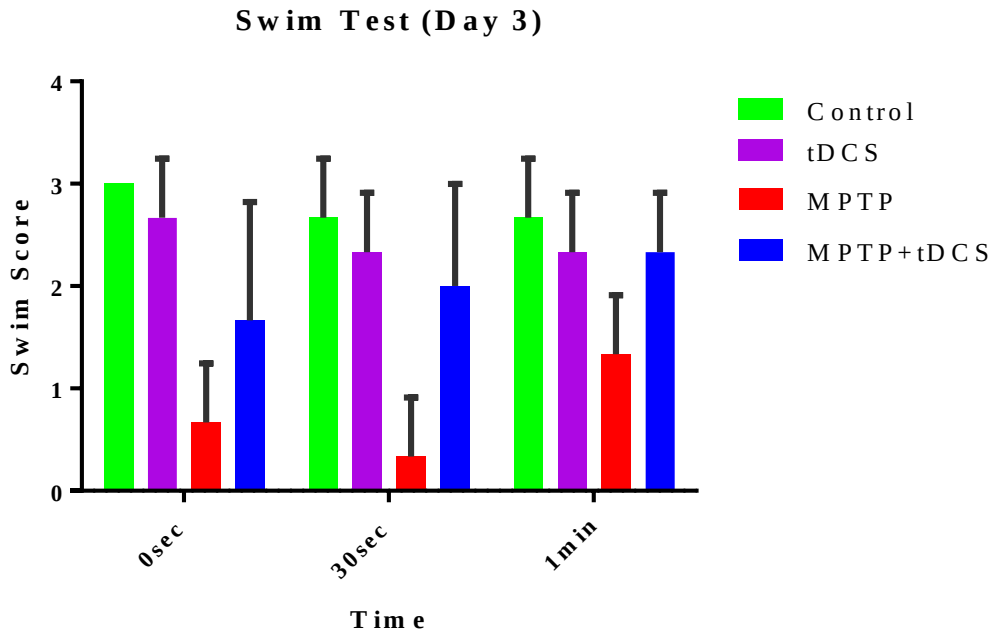


Figure 17: Comparison of after treatment on 3rd day swim scores of swim test between control, tDCS, MPTP alone (MPTP) and tDCS plus MPTP (MPTP+tDCS) mice (n=3). The difference in the mean swim scores between them for swim test was statistically significant ($p<0.05$). Error bars represent Mean±S.E.M (One-way ANOVA test).

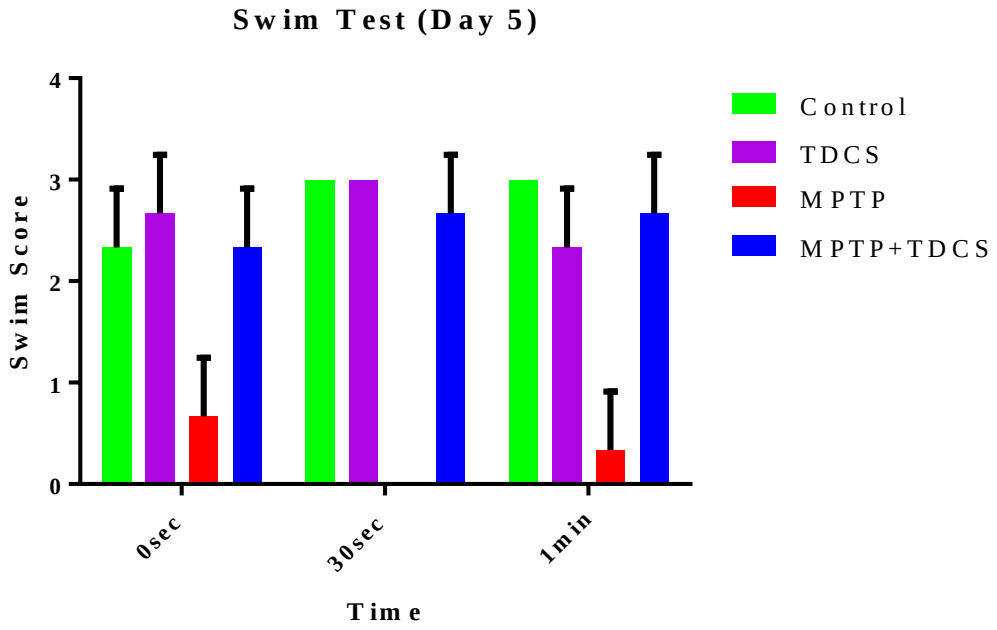


Figure 18: Comparison of after treatment on 5th day swim scores of swim test between control, tDCS, MPTP alone (MPTP) and tDCS plus MPTP (MPTP+tDCS) mice (n=3). The difference in the mean swim scores between them for swim test was statistically significant ($p<0.05$). Error bars represent Mean±S.E.M (One-way ANOVA test).

4.2. Effect of Transcranial direct current stimulation (tDCS) on beta oscillation in MPTP-induced Parkinson's diseases

Transcranial direct current stimulation (tDCS) was also investigated for its effect on beta (β) oscillation in mice brain using constant current of 0.1 mA stimulation treatment. The assessment of changes in β-oscillations was done by measuring the electroencephalography (EEG) of each mice.

EEG of each mice was carried out using cup electrodes and confirmation of EEG was done through further signal processing analysis done in MATLAB. Understanding the effect of tDCS on EEG level was of prime importance. Therefore, in this study EEG was studied in the cortex region of the brain providing the electrophysiological basis of change in electrical activity of brain due to tDCS treatment in Parkinson's. tDCS for its effect on beta waves in cortex region of PD mice was studied.

Normal β-activity was shown in both control and tDCS group (Figure 19 and 20). However, in MPTP-group increase in β-activity was seen as compared to the control group. In case of MPTP plus tDCS group, the β-activity was somewhat restored to normal when compared with the control one (Figure 21). The frequency domain of EEG signals recorded are shown in these figures. It is observed, that there is a prevalence of frequencies ranging from 13Hz to 35Hz, in agreement with previous literature reports. In case of MPTP plus tDCS group, increase in high β-waves have been seen after the treatment (Figure 22).

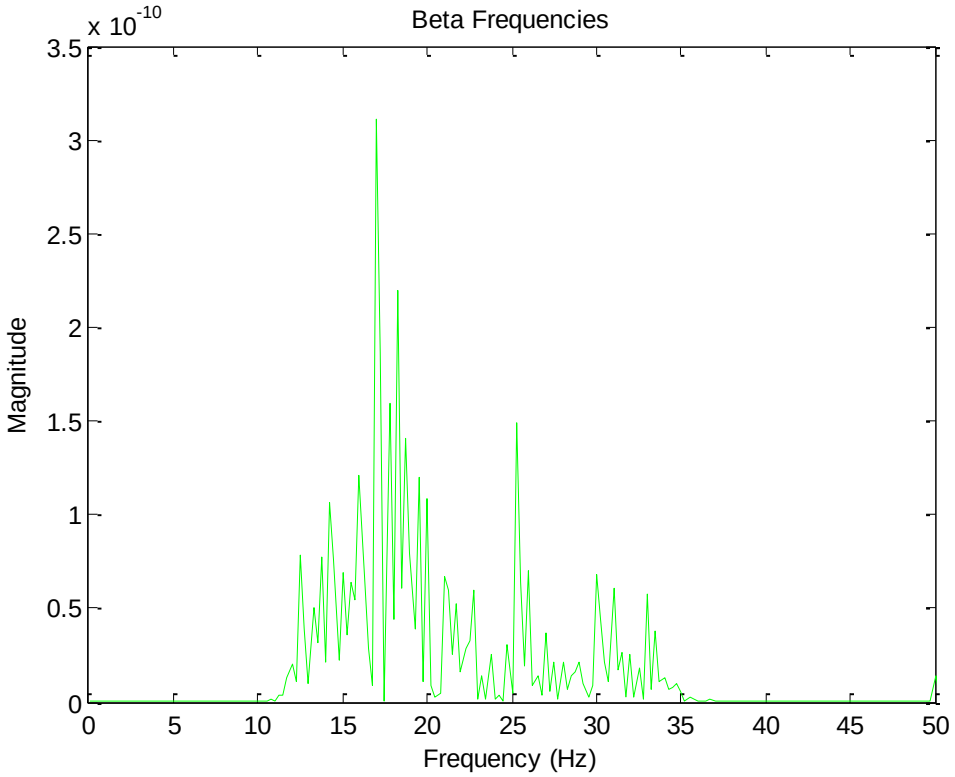


Figure 19: Effect of tDCS on the EEG recorded in the cortex area in control mice.

Here normal presence of beta waves was seen (n=3).

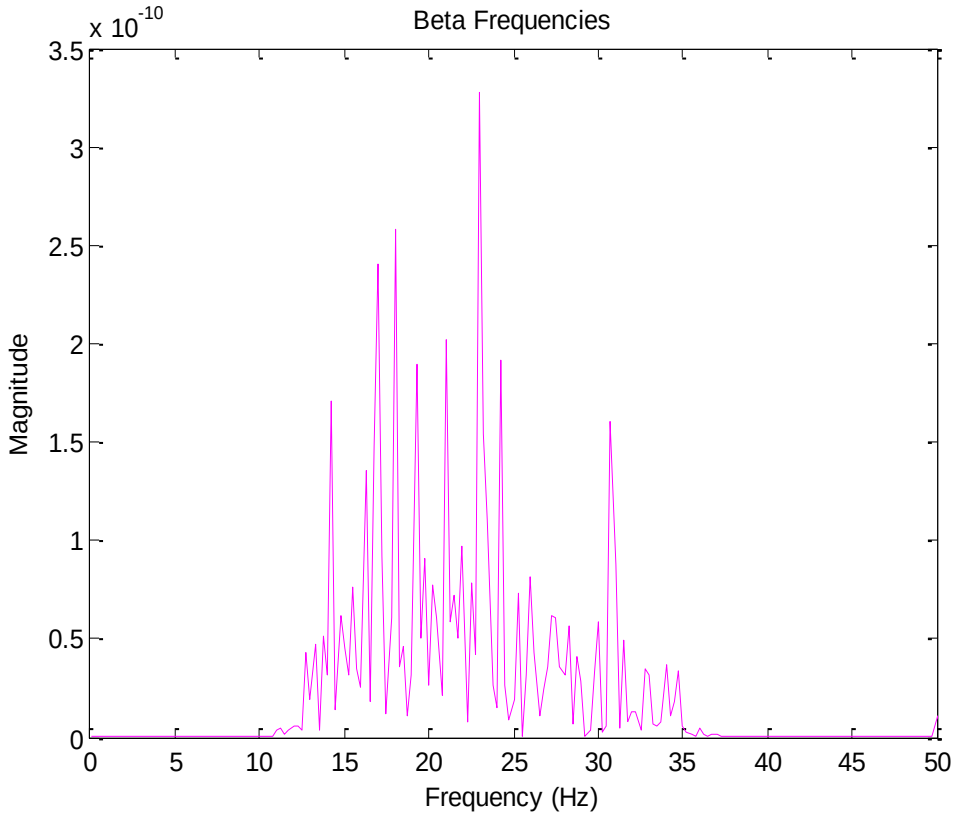


Figure 20: Effect of tDCS on the EEG recorded in the cortex area in only tDCS treated mice group. In this case also normal presence of beta waves was seen (n=3).

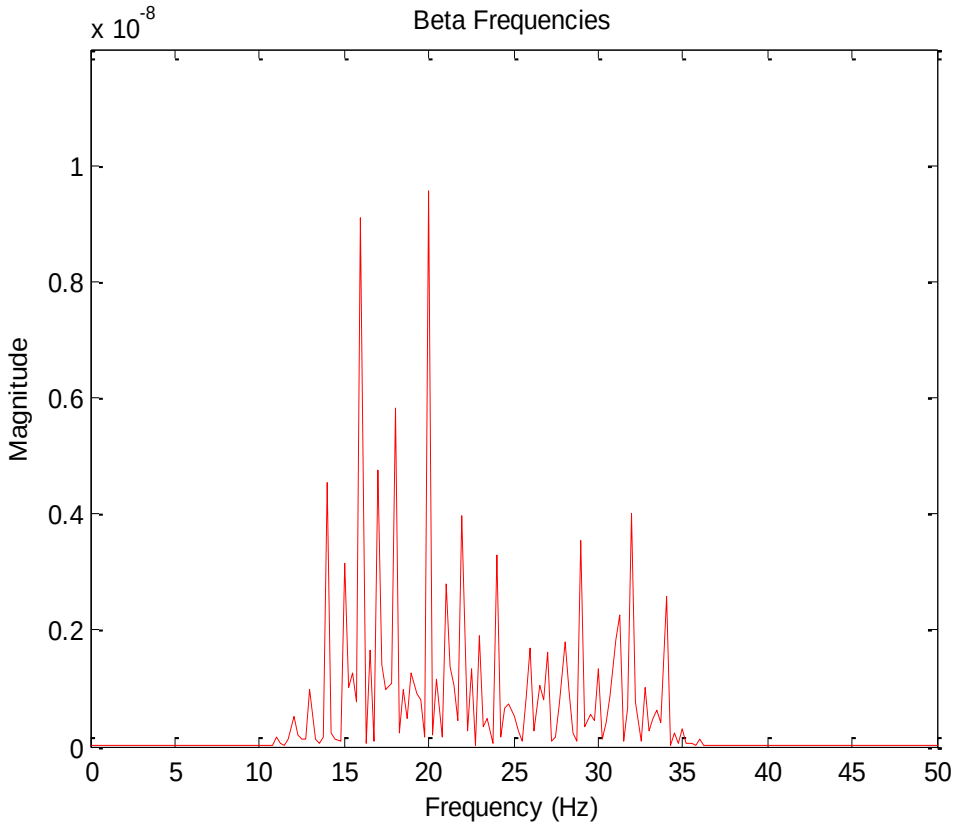


Figure 21: EEG recorded in the cortex area in PD mice (MPTP-treated). EEG of mice treated with MPTP shows the elevation in beta waves as compared to the control one (n=3).

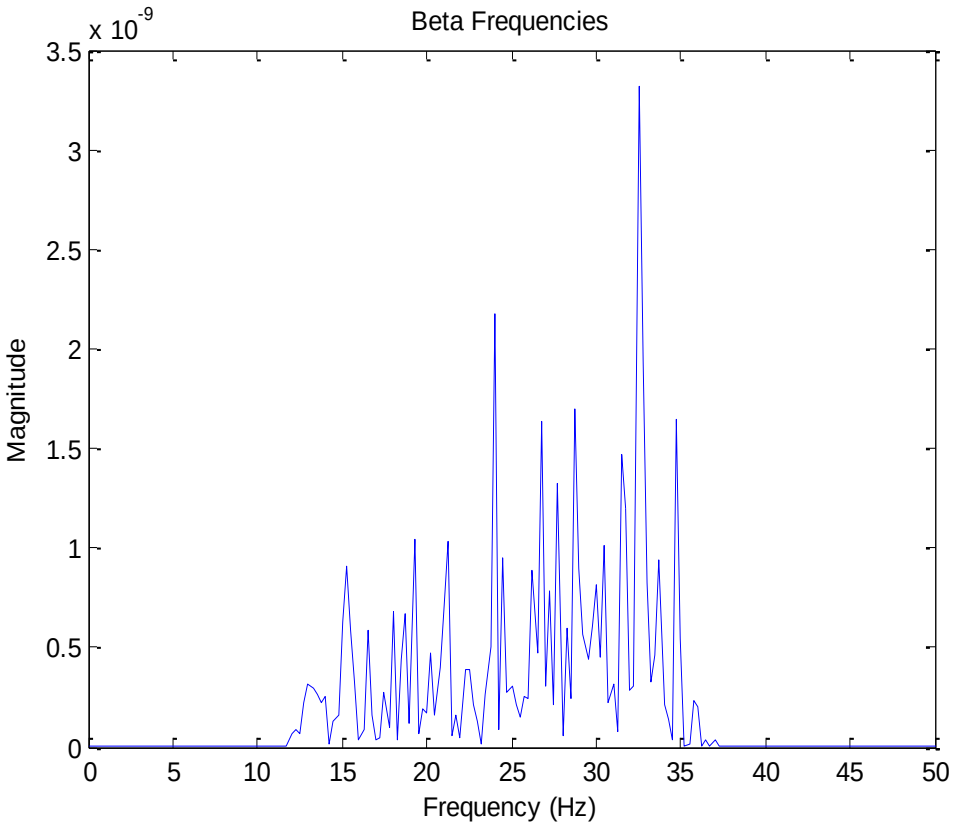


Figure 22: Effect of tDCS on the EEG recorded in the cortex area in MPTP plus tDCS treated mice group. EEG of mice treated with MPTP as well as tDCS shows the restoration of beta waves to the normal when compared to the control one (n=3).

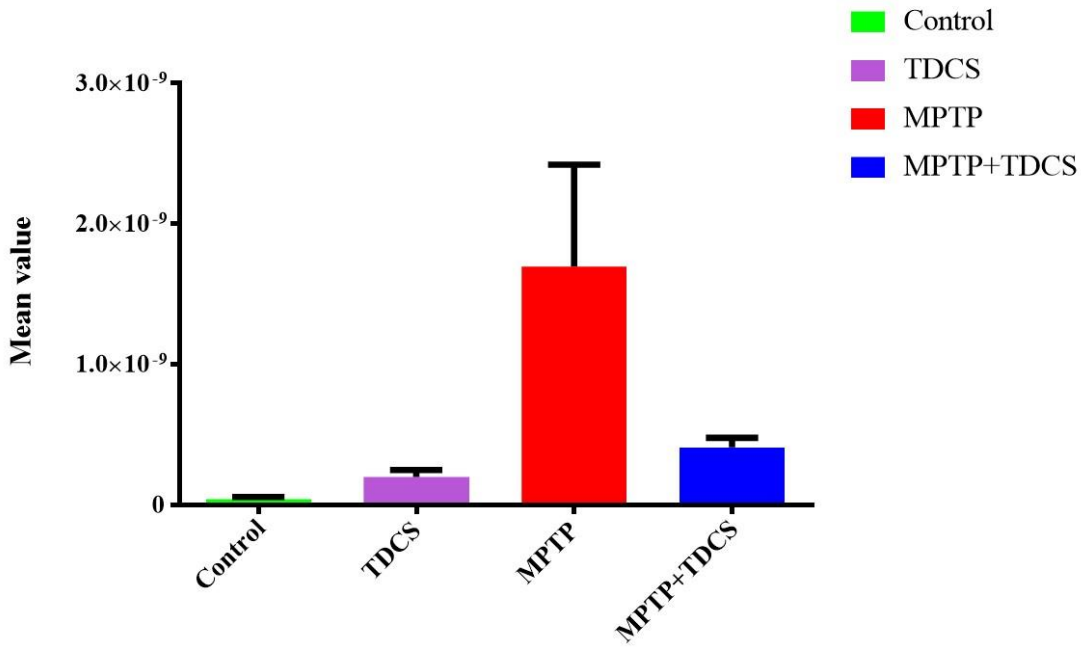
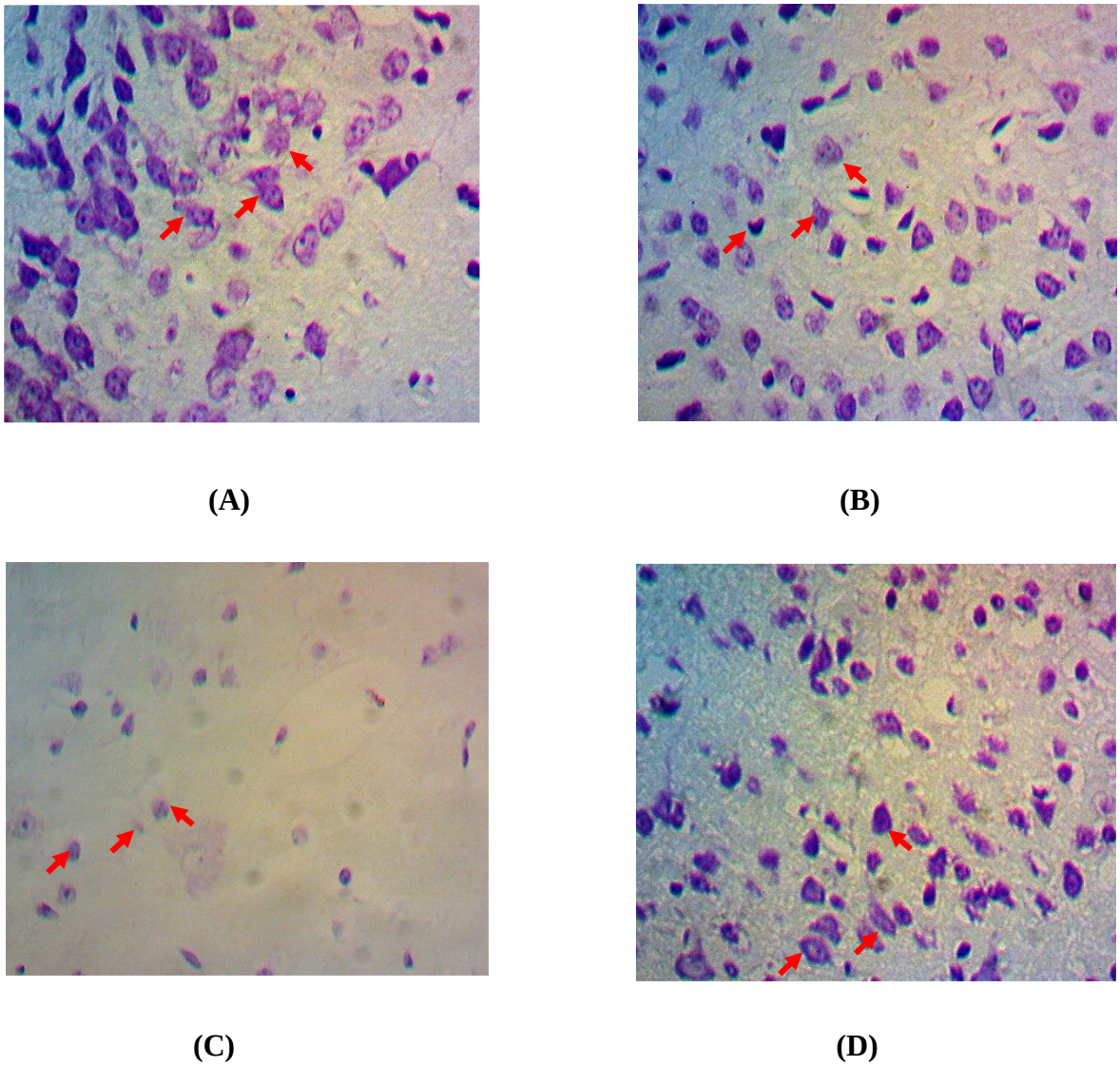


Figure 23: FFT- mean value analysis of change in β-activity after tDCS treatment. No significant difference was seen among control, tDCS and MPTP+tDCS group. However, significant increase in FFT- mean of β-activity can be seen in MPTP-treated group when compared with control ($p<0.005$; Tukey's multiple comparisons test). Significant decrease in β-activity can be seen in MPTP+tDCS group when compared with MPTP group ($p<0.005$; Tukey's multiple comparisons test).

4.3. Effect of Transcranial direct current stimulation (tDCS) on histological features in MPTP-induced Parkinson's diseases

Histopathological assessment of cortex of all the study groups was performed to observe morphological changes that occurred in affected region (cortex). Light microscopic analysis of each group was done. The Cresyl violet staining revealed a marked reduction in Nissl substances in MPTP treated group as compared to control. tDCS treatment to the MPTP plus tDCS mice group showed an increase in number of Nissl bodies as compared to MPTP group. Number of cell bodies in tDCS treated group is similar to control.



(A) **(B)**

(C) **(D)**

Figure 24: Cresyl Violet stained sections of Cortex: (A) Healthy neurons with intact nucleolus and predominant Nissl bodies **(B)** tDCS treated group. **(C)** MPTP treated group **(D)** MPTP+tDCS treated group. Original magnifications 40X.

Chapter 5

Discussion

Chapter 5

DISCUSSION

This study was done to reveal the functional, EEG (beta-activity) and histological changes after tDCS using the mice PD model (MPTP-induced). Repeated transcranial anodal stimulation improved motor function (according to Grid walking test and the swim test) in MPTP-induced PD mice model. Histologically, this had reduced neuronal deterioration.

Transcranial direct current stimulation has not been widely studied in mice, although it has been examined with respect to anticonvulsant effects(Liebetanz et al., 2006b) and the propagation velocity of cortical spreading depression, which represents cortical excitability (Liebetanz et al., 2006a). In accordance with the Liebetanz' study, 0.1 mA stimulus intensity was selected because this intensity and total charges would not injure the mice brain (Liebetanz et al., 2009).

5.1. Enhancement of Behavioral activity

The grid-walking test provides a suitable and sensitive behavioral assessment for testing the sensorimotor function of parkinsonian animal, especially when the extent of the lesion is moderate. Impairments in forelimb and digit use are the most obvious symptoms after nigro-striatal dopamine loss in rodents, especially when they are linked to sensorimotor integration (Aldridge and Berridge, 1998 and Schallert and Woodlee, 2003).

In current study, improvements in Grid walking test after the tDCS treatment might be due to the modulation Ca+ channels as well as NMDA receptors because of increase in

simple movement activities due to the tDCS treatment (Ding et al., 2004). Improvement in motor symptoms can be due to the activation of afferent fibers, changes in oscillatory activity and decoupling STN-GPi oscillations (Hashimoto et al., 2003, Vitek, 2008, Moran et al., 2012).

tDCS treatment reduced the number of slips of the contralateral forelimb following severe unilateral dopamine depletion, which validates the applicability of this test upon pharmacological treatment. Furthermore, animals with dopaminergic lesions showed an increase of contralateral foot-slips in the grid-walking test. Our results suggest that the grid-walking test is sensitive to behavioral deficits in animal model of PD, and, thus, may provide a sensitive procedure to assess motor changes and within modulation in animals with a lower degree of dopamine depletion.

The swim-score decreased as the dose of MPTP increased. The results of current study indicate that swim ability is directly proportional to decrease in DA neurons after MPTP-treatment, and suggest that swim-test could be used as a major technique to monitor motor dysfunction in experimental animals. Increase in swimming ability after tDCS-treatment could be due to its activity on brain dopamine system, as it has been reported that tDCS causes increase of dopamine levels in cortical area of mice brain (Tanaka et al., 2013).

Repeated tDCS has been associated with a significant motor function improvement in stroke patients and the effect lasted for 2 weeks after the treatment (Boggio et al., 2007). However, the events made in the injured brain are unknown and the mechanism of the motor recovery is uncertain.

5.2. Increase in β-activity after MPTP-treatment

As compared to the course of MPTP-treatment of primates, appearance of oscillatory activity in response to PD motor symptoms, seems to be in contradiction with firing pattern model (Leblois et al., 2007). Changes in oscillatory brain activity play an important role in the formation of perception and memory and thus are essential for higher cognitive functions (Herrmann et al., 2010, Herrmann et al., 2004).

In current study, increase in β-activity in MPTP group can be due to the reduction in toxic excitation to striatal neurons proceeding towards the internal segment of the globus pallidus (GPi) toxic inhibition to striatal neurons progressing towards external segment of the globus pallidus (GPe) (Gerfen et al., 1990, Mallet et al., 2006). Both of these pathways are thought to increase average firing rates of GPi and SNPr neurons. A number of studies in relation to the original one also confirmed same changes in the activity (Filion, 1991, Bergman et al., 1994, Heimer et al., 2006, Wichmann and Soares, 2006).

5.3. Decrease in β-activity after tDCS-treatment

Compared to TMS, which is another non-invasive brain stimulation technique, tDCS is considered much more suitable for therapeutic purposes mainly because of its low cost and relative portability (Maeoka et al., 2012).

In current study, decrease in β-activity after tDCS treatment in MPTP plus tDCS group and shift to higher β-frequencies can be due to the increase motor activity due to hyperpolarization after the treatment as supported by the previous studies (Stein and Bar-Gad, 2013, Little and Brown, 2014, Williams, 2015). Taken together, the data for tDCS hold promise for the treatment of diseases affecting the central nervous system. However,

a significant amount of fundamental research still needs to be done to support the therapeutic usefulness of tDCS. Furthermore, stimulation dose response curves also need to be performed to identify the most effective conditions and thus optimize the therapy, as stimulation parameters are critical in determining outcome.

5.4. Histological Changes after MPTP- and tDCS- treatment

This study provides evidence that electric stimulation modulates responses of non-neuronal cells in the brain, and relevantly contributes to our scarce knowledge about the neurobiological effects of tDCS. Neurons in PD brain were well preserved after transcranial anodal stimulation compared to those in the MPTP group. The results obtained are of importance because they demonstrate that neuronal damage after PD can be reduced by transcranial anodal stimulation. The mechanism of the protective effect of repeated transcranial anodal stimulation may involve modulation of the activities of calcium channel and NMDA receptor, activations of which cause neuronal damage due to excessive glutamate release (Nitsche et al., 2003). Some other factors might involve this phenomenon and further animal studies must be performed to clarify this.

5.5. Limitations

From our results, transcranial direct current stimulation may have a neuroprotective effect on neuronal cells in the Parkinson's disease brain. However, we only observed histopathologic changes in neurons as whole, not in axons and myelins using immunohistochemistry techniques, the Luxol fast blue-periodic acid Schiff stains. The addition of other stains would reveal the protection of neuronal injury more accurately. tDCS was found to make a functional improvement and well-preserved neurons in our mice

PD model. These findings may be useful for studies about the therapeutic mechanism of tDCS.

CONCLUSION

The results shown herein demonstrate that it is possible to improve motor impairment caused by MPTP in PD using tDCS treatment. It is also possible to acquire and extract information from brain electrical activity using a non-invasive approach in conscious rats by monitoring the EEG activity with accuracy and precision.

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