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The alteration of dopamine receptors in L-DOPA (L-3,4-dihydroxyphenylalanine) induced dyskinesias.

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Preface

The experimental work described in this thesis was conducted at the Westville campus, University of KwaZulu-Natal, Durban, South Africa, from March 2019 to November 2019 under the guidance and supervision of Msibi Z.N.P. and Mabandla M.V. This work has not been submitted to any tertiary institution including the University of KwaZulu-Natal for the purposes of obtaining an academic qualification, whether by myself or any other party. Use of other people's work has been duly acknowledged as they occurred in the text.

Makwena Mokgokong

Signed: Date: 15 March 2021

As the candidate's supervisor and co-supervisor, we, agree to the submission of this thesis.

Mabandla M.V.

Signed _____ Date 26 April 2016

Msibi Z.N.P.

Signed _____ Date 22 April 2021

Declaration

I, Miss Makwena Mokgokong (213514384), declare that

1. The work described in this thesis titled, The alteration of dopamine receptors in L-DOPA (L-3,4-dihydroxyphenylalanine) induced dyskinesias, resulted from my own investigation and research under the guidance and supervision of Mabandla M.V. and Msibi Z.N.P.
2. All the work from other authors that were used when compiling this work was cited and given due credit.
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Mabandla M.V.

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Msibi Z.N.P.

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PD	Parkinson’s disease
BG	Basal Ganglia
DA	Dopamine
MFB	Medial forebrain bundle
L-DOPA	L-3,4-dihydroxyphenylalanine
LIDs	L-DOPA-induced dyskinesias
AIMs	abnormal involuntary movements
HPA	hypothalamus-pituitary-adrenal
PFC	Prefrontal cortex
TNF-α	tumour necrosis factor-alpha
AChE	acetylcholinesterase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
RT-PCR	Reverse transcription-polymerase chain reaction
6-OHDA	6-hydroxydopamine
VTA	ventral tegmental area
DAT	DA reuptake transporter
SPECT	single-photon emission computed tomography
GCs	Glucocorticoids
GRs	glucocorticoid receptors
BBB	blood-brain barrier
D1R	D1 receptor
D2R	D2 receptor
ACh	Acetylcholine
s.c	subcutaneous injection
SD	Sprague-Dawley
EBWT	elevated beam-walk test
NORT	novel object recognition test
MWMT	Morris water maze test
ROS	Reactive oxygen species
G	Grams
cm	Centimeter
mg	Miligram
kg	Kilogram
mm	Milimeter
µg	Microgram

µL	Microliter
mL	Mililiter
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent assay
RNA	Ribonucleic acid
cDNA	Complementary deoxyribonucleic acid
HRP	Horse raddish peroxidase
Xg	Times gravity
ChIs	Cholinergic interneurons
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

Abstract

L-3,4-dihydroxyphenylalanine (L-DOPA) can ease symptoms of Parkinson's disease (PD), but extended use of L-DOPA causes abnormal involuntary movements (AIMs) called L-DOPA induced dyskinesias (LIDs). The present study aims to investigate alterations in HPA axis stimulation, neuroinflammation, DA signalling, and cholinergic signalling using molecular markers in a rat model of LIDs. A unilateral 6-hydroxydopamine (6-OHDA) lesion in the medial forebrain bundle of male Sprague-Dawley rats was used to model Parkinsonism. The PD rat model was treated with L-DOPA to further model LIDs. L-DOPA treated groups included rodents treated for 14 days and rats that developed AIMs during 28 days of treatment. LIDs severity was rated using the AIMs score. Motor skills were assessed using the elevated beam walking test. Cognitive functions were assessed using the Morris water maze test and the novel object recognition test. The concentrations of tumour necrosis factor-alpha (TNF-α), corticosterone, acetylcholinesterase (AChE), and dopamine (DA), and the expressions of D1 receptor (D1R) and D2 receptor (D2R) were quantified. L-DOPA treatment for 14 days improved the 6-OHDA-induced hypokinesia, incoordination, spatial learning, and spatial memory but did not improve recognition memory impairment. Prolonged (28 days) L-DOPA treatment led to AIMs development and failed to improve 6-OHDA-induced spatial memory impairment. L-DOPA treatment significantly increased striatal TNF-α and striatal DA concentration, cerebellar TNF-α and DA concentration, prefrontal cortex (PFC) DA and AChE concentration, but significantly reduced striatal AChE concentration, the concentration of TNF-α and D1R expression in the PFC, plasma corticosterone, and hippocampal AChE concentration. When treatment was prolonged for 28 days, striatal D2R expression significantly increased, while cerebellar TNF-α and DA concentration significantly decreased. Increased striatal D2R signalling increases motor output since the direct basal ganglia (BG) pathway is activated in LIDs. The present study showed significantly increased cerebellar DA concentration in response to BG hypoactivity; however, as striatal D2R increased cerebellar DA decreased. The connectivity between the BG and cerebellum in PD increases off L-DOPA and lowers On L-DOPA. The cognitive decline in the 6-OHDA lesioned rodents and those treated with L-DOPA results from increased AChE concentration. High AChE concentration leads to increased ACh catabolism which impairs cognitive function.

Keywords: Parkinson's disease, L-3,4-dihydroxyphenylalanine, Dopamine, tumour necrosis factor-alpha, acetylcholinesterase, corticosterone

Chapter 1: Literature review

1. Introduction

Parkinson's disease (PD) is a neurodegenerative motor disorder, that results from a dysregulation in the basal ganglia (BG) (Boecker et al., 1999). This disease results from the death of dopamine (DA) neurons in the pars compacta of the substantia nigra, and this deficiency of DA signalling in the BG leads to motor symptoms characteristic of PD (Fearnley and Lees, 1991, Gibb and Lees, 1991). The motor symptoms used to diagnose PD include bradykinesia, akinesia, resting tremors, postural instability, and rigidity (Hughes et al., 1992, Jankovic and Tolosa, 2007, Postuma et al., 2015). PD affects 1-2 people in a population of 1000, affecting most people above the age of 60 years (Tysnes and Storstein, 2017). Approximately 10% of PD patients in the early, untreated disease state met the traditional cognitive impairment criteria (Weintraub and Claassen, 2017).

The cognitive deficits expressed by PD patients include impaired visuospatial learning, spatial recognition memory, attention deficiencies and others (Chaudhuri et al., 2006, Amboni et al., 2015, Pfeiffer, 2016). The cognitive deficits in PD may result from irregular dopaminergic, noradrenergic, and cholinergic inputs to the cortex (Bäckström et al., 2018). DA deficiency in PD is related to mild cognitive deficits in frontostriatal functions, memory and attention deficits prevalent in PD (Bäckström et al., 2018). The most effective pharmacotherapy for PD is L-3,4-dihydroxyphenylalanine (L-DOPA) combined with monoamine oxidase and catechol-O-methyltransferase inhibitors (Kang et al., 2016). L-DOPA is a DA precursor molecule that can cross the blood-brain barrier, and the inhibitors reduce the peripheral degradation of L-DOPA, therefore increasing DA signalling in the brain (Ruonala et al., 2018).

Long-term use of L-DOPA results in abnormal involuntary movements called levodopa-induced dyskinesias (LIDs) (Luquin et al., 1992). LIDs affect 40% of PD patients that have been treated with L-DOPA for six years, and this percentage surges to 60% after ten years of treatment (Ahlskog and Muenter, 2001, Tran et al., 2018). Studies to elucidate the pathophysiology of LIDs have shown the involvement of oxidative stress, dysregulated serotonin signalling, dysregulated DA signalling, dysregulated cholinergic signalling, dysfunction of the glutamatergic pathway, dysfunction of the GABAergic pathway and others (Murer et al., 1998, Shen et al., 2015, Ndlovu et al., 2016, Smith et al., 2016). These molecular dysregulations in brain areas, like those of the basal ganglia, can lead to abnormal involuntary movements in PD patients treated with L-DOPA (Ndlovu et al., 2016). However, the effect of L-DOPA on the cognitive decline prevailing in PD is yet to be established.

Molecular dysfunctions in LIDs were studied using animal models (Branchi et al., 2008, Tadaiesky et al., 2008). Numerous animal models of PD were modified to model dyskinesias that result from treating parkinsonism with L-DOPA over a specified period (Jackson et al., 2014, Iderberg et al., 2015, Ndlovu et al., 2016). Models that use neurotoxins like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) are used if a nigrostriatal lesion is required for the study (Calne et al., 1985, Mandel et al., 1997). The unilateral lesion into the medial forebrain bundle (MFB) has been shown to induce memory impairments showing that DA depletion may play a role in the cognitive decline evident in PD (Tadaiesky et al., 2008). The lesion in the MFB is thought to reduce DA signalling in the striatum, prefrontal cortex (PFC), and hippocampus, which are brain areas that play a crucial role in cognition (Du et al., 2018). The lesion in the nigrostriatal pathway affects the BG pathway, contributing to the initiation of the desired movement and inhibition of undesired movement (Ungerstedt, 1968, Middleton and Strick, 1994).

2. The Basal Ganglia pathway and symptoms of Parkinson's disease

The basal ganglia (BG) comprises subcortical nuclei that span the telencephalon, midbrain and diencephalon (Albin et al., 1989). The nuclei that make up the BG include the striatum, substantia nigra (the pars compacta and the pars reticulata), the Globus pallidus (the internal and the external segment), and the subthalamic nucleus (figure 1a) (Heimer, 1983, Rommelfanger and Wichmann, 2010). Dopamine (DA) is known for modulating the functions of the BG, and the disrupted DA signalling in the striatum and other areas is thought to play a significant role in the development of symptoms of Parkinson's disease (PD) (Sian et al., 1994, Ruskin et al., 1999, Szewczyk-Krolikowski et al., 2016). The BG dysregulation can be attributed to the death of dopaminergic neurons in the pars compacta of the substantia nigra (figure1b), which supplies the BG, specifically the striatum, with DA (Hajdu et al., 1973, Burns et al., 1983). Motor symptoms of PD become apparent when the concentration of DA in the striatum is low (Zarow et al., 2003). The striatum projects to the substantia nigra par reticulata and the outer segment of the Globus pallidus (figure 1a), and therefore plays a crucial role in both the direct and indirect basal ganglia pathways (Domesick et al., 1983, Staines and Fibiger, 1984). The classical PD model implies that the degeneration of the DA neurons (figure 1b) results in hyperactivity of the neurons in the striatum (Albin et al., 1989). This leads to uncurbed inhibition of the neurons of the outer segment of the Globus pallidus and, therefore, a reduced inhibitory tone of the subthalamic nucleus (Starr et al., 2005, Milosevic et al., 2017).

The resultant hyperactivity in the subthalamic nucleus contributes to the neuron's hyperactivity in the Globus pallidus' internal segment (DeLong and Wichmann, 2007). The hyperactivity of the neurons of the inner segment of the Globus pallidus leads to an inhibited thalamus, and therefore, reduced signals

to the motor cortex (DeLong and Wichmann, 2007). The above results in difficulty initiating and executing movement, which accounts for many clinical features used to diagnose PD (DeLong and Wichmann, 2007). Other brain areas that play a critical role in the movement are the cerebellum and other regions of the cerebral cortex (Middleton and Strick, 1994, Bostan et al., 2013). The cerebellum receives DA signals from the substantia nigra and the ventral tegmental area (VTA), and the dysfunction in the BG can lead to impaired signalling in the connected brain areas (Panagopoulos et al., 1991, Ikai et al., 1992, Melchitzky and Lewis, 2000). Cerebellar dysregulations and the BG mediate both motor and non-motor symptoms of PD (Wu and Hallett, 2013). This is supported by the anatomical connections between the BG and the cerebellum (figure 2) (Bostan et al., 2010). There are morphological alterations in the cerebellum and the primary motor cortex, which may result from the hyperactivity observed in both brain areas in PD patients (Sabatini et al., 2000, Haslinger et al., 2001, Borghammer et al., 2010). The hyperactivity within the brain areas is thought to be a compensatory mechanism by the defective BG (Sabatini et al., 2000, Wu and Hallett, 2005). The cerebellar-BG-motor cortex loop is correlated with motion while the neurons in the BG and cerebellum that circles (figure 2) with areas of the prefrontal cortex (PFC) is related to cognitive function (Middleton and Strick, 1994). Damage in the BG or the cerebellum will affect the motor function while the resultant dysregulation in the non-motor brain areas leads to cognitive deficits and other non-motor symptoms of PD (Middleton and Strick, 1994).

L-DOPA treatment has shown to improve clinical symptoms of PD on the Unified Parkinson's Disease Rating Scale motor score (Festini et al., 2015, Mueller et al., 2019). The above improvements were accompanied by a connectivity increase between the cerebellum and other brain areas that make up the motor system like the brainstem, thalamus, and globus pallidus (Festini et al., 2015, Mueller et al., 2019). The cerebellum connections to the other brain areas were recognised almost immediately after the first L-DOPA dose (Mueller et al., 2019). The improved motor performance was associated with increased cerebellar-motor system connectivity, which implies that cerebellar networks can be a crucial pharmaceutical target in PD treatments (Mueller et al., 2019). The increased connectivity shows that patients on dopaminergic medication recruit the cerebellum during periods of increasing variability (Gilat et al., 2017).

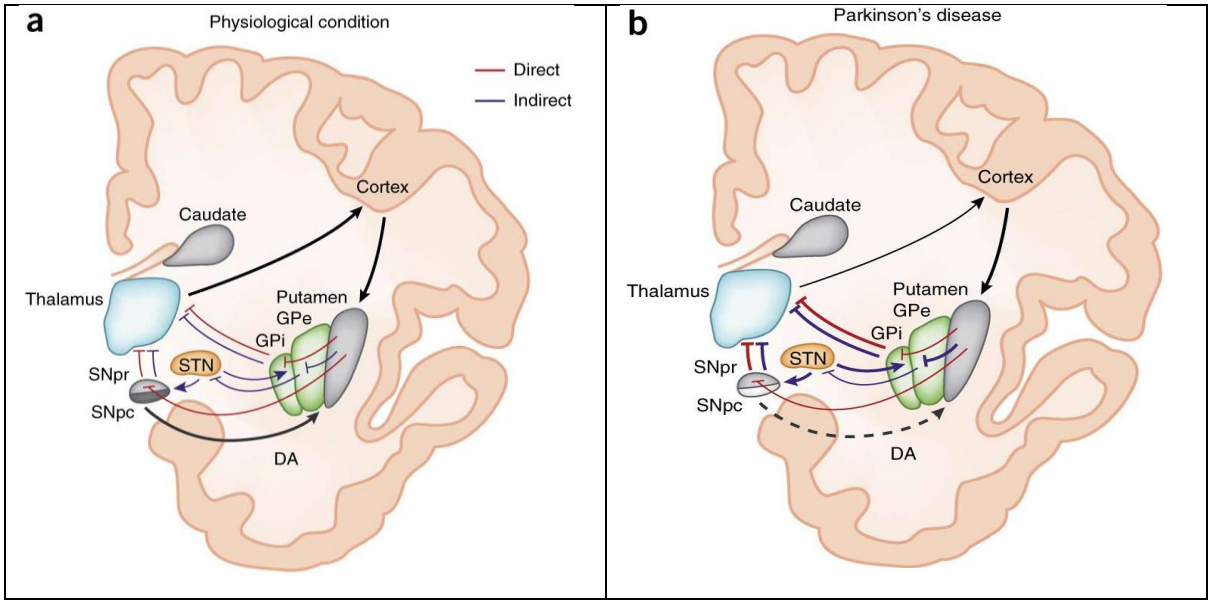

Figure 1: an illustration of DA neurons projecting from the substantia nigra pars compacta to the striatum, activating the dopamine D1 receptor on the medium spiny neurons in the direct BG pathway (red lines) and dopamine D2 receptor which inhibits the neurons of the indirect BG pathway (blue lines) (Calabresi et al., 2014).

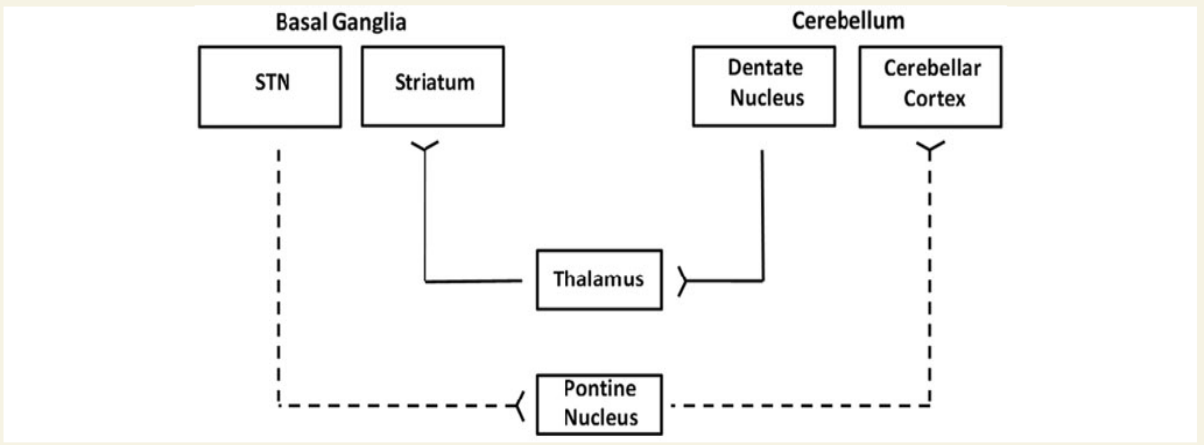

Figure 2: showing the structural connections between the basal ganglia and the cerebellum. The solid lines show the neuronal projections to the striatum from the dentate nucleus, and the dotted lines show the axonal projections to the cerebellar cortex from the subthalamic nucleus (Bostan et al., 2010, Wu and Hallett, 2013).

3. Diagnosis of PD

No one test can definitively diagnose PD; however, it can be diagnosed using a clinical criterion (Jankovic, 2008, Mahlknecht et al., 2015). The Movement Disorder Society has criteria for clinical

research and PD diagnosis (Postuma et al., 2015). Accurate diagnosis of PD is possible during life, and 75%-90% of diagnosed cases have been confirmed posthumously (Rizzo et al., 2016). Central to the criteria for the diagnosis of PD is cardinal motor symptoms, like bradykinesia, rigidity, resting tremors and postural instability (Hughes et al., 1992). Current criteria not only focuses on the motor symptoms of PD but also acknowledges non-motor symptoms (Postuma et al., 2015). Non-motor symptoms include impaired sense of smell, sleep dysfunction, dysautonomia, and cognitive deficits (Zis et al., 2015). Motor parkinsonism can be defined as bradykinesia and resting muscle rigidity or resting tremor (Postuma et al., 2015). The revised version of the Movement Disorder Society for the confirmation of parkinsonism involves four parts, namely, information of their daily non-motor experiences, daily motor experiences, an examination of motor skills, and motor complications (Goetz et al., 2008, Postuma et al., 2015, Berg et al., 2018). During the diagnosis, the doctor may require other laboratory examination from blood to imaging tests, even though these tests cannot confirm PD they can be used to rule out other diseases (Kim et al., 2002). A patient must show motor parkinsonism, and those symptoms must not be ascribable to any other confounding factors (Postuma and Berg, 2016).

Proving that PD is the causal factor for parkinsonism observed in a patient requires the following categories of diagnostic features to be fulfilled, absolute exclusion criteria, at least two supportive criteria, and no red flags (Postuma et al., 2015). The absolute exclusion criteria are used to debar PD in patients with other mental disorders like behavioural variant frontotemporal dementia (Postuma et al., 2015). The supportive criteria are met when the patient has a beneficial response to DA replacement therapy that is dose-dependent, and a predicted end of the dose wearing off, i.e., a marked on/off fluctuation (Postuma et al., 2015). The no red flag criteria are ideal, but the diagnosis of probable PD can be made even when the patient presents some red flags provided that for every red flag, there is a supportive criterion (Postuma et al., 2015). Examples of red flags include an absence of motor symptom progression over five years of observations and rapid gait impairment progression that requires the use of a wheelchair (Postuma et al., 2015). The accuracy of diagnosis is dependent on the age of the individual, the clinical expertise of the attending clinician, and the duration of the disease (Adler et al., 2014, Postuma et al., 2015).

In recent years, there has been an increase in the use of imaging techniques used in diagnosing PD (Galinowski et al., 2017). Modern technology used in magnetic resonance imaging can show the absence of the standard swallow tail sign in the substantia nigra of PD patients and has a 90% accuracy of diagnosing PD (Schmidt et al., 2017). The computed tomography scans cannot show a distinct difference between patients with PD and the control, but this technology can be used to rule out other diseases, making it a useful tool for the diagnosis of PD (Galinowski et al., 2017). Striatal DA activity can be determined using the DA reuptake transporter (DAT)–single-photon emission computed tomography (SPECT) (Noyce et al., 2018). This technique quantifies striatal DA activity by using the

transcranial sonography and striatal binding ratio, which can be used to identify the hyperechogenicity of the substantia nigra of PD patients (Noyce et al., 2018).

A range of cognitive impairments occur in PD, from mild deficits to dementia (Aarsland et al., 2010, Litvan et al., 2012, Wang et al., 2015). There are two criteria for diagnosing cognitive deficits in PD as defined by clinical presentation (Yang et al., 2016). Level 1 diagnosis is a brief assessment based on the Queen's Square Brain Bank criteria for PD's cognitive deficits (Gibb and Lees, 1991, Hughes et al., 1992). The mild cognitive decline occurs slowly and can be described by caregivers, patients, or a physician can observe it during an examination (Yang et al., 2016). The criteria exclude impairments that result from the manifestation of parkinsonism, other possible causes of cognitive decline and another factor that occur comorbid with PD that may influence the mental examination results (Emre et al., 2007). The mild cognitive deficits eventually progress to dementia in most cases and avoid to errors in the diagnosis that may arise more than one neurophysiological test is used (Emre et al., 2007). Mild cognitive deficits in PD can be diagnosed with a second criterion which requires two neuropsychological tests examining five cognitive domains described by the Movement Disorder Society (Yang et al., 2016). The five cognitive domains are neuropsychiatric functions, instrumental functions, memory, executive functions and global mental efficiency (Muslimović et al., 2005).

4. Treatment of PD

The cure for Parkinson's disease (PD) is currently non-existent, but there are numerous ways for PD patients to maintain a reasonable standard of living. There are medications, physical treatments, and surgery that help the patients cope with the disease (Weaver et al., 2009). Surgical intervention is only required when the symptoms are not adequately ameliorated with medical therapy (Williams et al., 2010, Diestro et al., 2018). The subthalamic nucleus can be electrically stimulated to attenuate the severity of the advanced PD symptoms (Limousin et al., 1998, Weaver et al., 2009). Examples of such cases include PD that presents with essential tremors that are still disabling, even though the patient receives adequate doses of medication (Picillo and Fasano, 2016). Patients may need deep brain stimulation or lesional surgery in some basal ganglia regions, which can improve severe symptoms of PD (Laitinen, 1966, Kumar et al., 1998, Ruonala et al., 2018).

PD patients require rehabilitation to help improve their standard of living reduced by the disease (Hely et al., 2008). A rehabilitation program for PD patients includes occupational therapy, speech therapy, and physiotherapy (Radder et al., 2017). The above helps improve and maintain mobility, flexibility, strength gait speed, speech and overall quality of life (Kluger et al., 2017). The palliative care approach provides patients and families affected by PD with some relief from suffering and information that will help manage the symptoms (Kluger et al., 2017).

The most effective pharmacotherapy given to help manage PD symptoms is dopamine (DA) replacement therapy, which increases DA signalling in the brain (Calne et al., 1969, Qi et al., 2016). DA cannot cross the blood-brain barrier because of the charge on the molecule, so a precursor of DA called L-3,4-dihydroxyphenylalanine (L-DOPA) is administered orally instead (Kang et al., 2016). Enzymes like monoamine oxidase degrade L-DOPA in the periphery, which poses a problem since this will reduce the amount of L-DOPA reaching the brain (Brannan et al., 1992, Doudet et al., 1997). Therefore, L-DOPA is administered with monoamine oxidase inhibitors and catechol-O-methyltransferase inhibitors (Brannan et al., 1992, Doudet et al., 1997). L-DOPA can be transported across the blood-brain barrier via transcellular passive diffusion (Kageyama et al., 2000). There are alternative medications, but none are as effective as L-DOPA. However, L-DOPA causes L-DOPA induced dyskinesias (LIDs) in PD patients (Mones et al., 1971).

5. Pathophysiology of L-DOPA induced dyskinesia

The Pathophysiology of L-3,4-dihydroxyphenylalanine (L-DOPA) induced dyskinesias (LIDs) is still not clearly understood. There are numerous neurotransmitter pathways whose dysregulations are implicated in the pathology of LIDs. The molecular pathways implicated include oxidative stress, inflammation, mitochondrial dysregulation, imbalanced dopaminergic signalling, impaired serotonergic pathway, dysfunction of cholinergic neurotransmission and other molecular pathways (Ding et al., 2011, Devos et al., 2013, Michel et al., 2015, de Farias et al., 2016). Oxidative stress due to reactive species, nitrogen or oxygen, and reduced activity of catalase enzymes increase the chances of dopamine (DA) neuron death in Parkinson's disease (PD) (de Farias et al., 2016). L-DOPA promotes excessive DA release in the striatum, increasing the oxidative stress and the release of pro-inflammatory cytokines (de Farias et al., 2016). DA replacement therapy, like L-DOPA, does not necessarily reduce the rate of degeneration of DA neurons. The loss of DA neurons in the substantia nigra causes impaired DA storage capacity and impaired physiological DA release (Ding et al., 2011, Tronci et al., 2017). It is not clear whether L-DOPA promotes DA and serotonin's production and secretion from serotonin neurons (Ng et al., 1970, Melamed et al., 2007, Miguelez et al., 2016). Reduction of striatal cholinergic tone and the ablation of striatal cholinergic interneurons ameliorates LIDs (Ding et al., 2011, Won et al., 2014). Further details need to be elucidated in each pathway to help paint a clear picture of the pathology of LIDs.

5.1. Inflammatory response

The neuropathology of PD includes non-apoptotic and apoptotic cell deaths, and this injury in the nigrostriatal pathway results in activated microglia (Devos et al., 2013). The microglia in the substantia nigra and the striatum of PD patients are activated (Kim et al., 2000). Once activated, the microglia respond via hypertrophy, proliferation, and release of inflammatory mediators (Devos et al., 2013).

Mediators like interleukin-1β, interleukin-6, and tumour necrosis factor-alpha (TNF-α), have been shown to play a role in metabolism, ion channel activity and growth factor expression (Mogi et al., 1994, Kim et al., 2000). TNF-α is a pro-inflammatory cytokine elevated in the striatum, prefrontal cortex (PFC), cerebellum and cerebrospinal fluid of PD patients (Mogi et al., 1994, Khadrawy et al., 2017). This cytokine is thought to work as a transducer of the immune response in brain injuries (Mogi et al., 1994). It also has stimulator and cytotoxic properties that prevent infection in the injured area and enhances the immune response to brain tissue damage (Mogi et al., 1994). Gliosis may be beneficial at times but deleterious at other times as it can lead to increased production of reactive oxygen species (ROS) (Kim et al., 2002). ROS generation is counterbalanced by enzymes eliminating ROS compounds by detoxifying them (Olufunsho and Ayodele, 2017). One of the features of PD is reduced antioxidant capacity, which leads to the oxidation of biomolecules that make up the DA neurons (Campolo et al., 2016). Unfortunately, the gliosis can lead to the death of DA neurons in a process called glial cell-mediated neurotoxicity (Liao and Chen, 2001).

The progressive death of DA neurons is a predominant risk factor for LIDs development in PD patients (Hong et al., 2014, Bastide et al., 2015). In-vitro studies show that high L-DOPA concentrations can initiate the degeneration of cultured DA neurons by inducing both apoptosis and necrosis; how this could relate to the brain in the PD condition is unclear (Basma et al., 1995, Ziv et al., 1997). A low L-DOPA concentration did not show any toxic effects on the cultured dopamine neurons (Mytilineou et al., 1993). However, it showed some protective effects against toxicity that could have been mediated by peroxide and the upregulation of antioxidant molecules (Han et al., 1996, Warren, 2015). In vivo studies and clinical trials have not demonstrated any indication of toxicity, although none of the animal models of LIDs is a precise replication of the PD condition (Perry et al., 1984, Lyras et al., 2002). The augmented inflammation in PD may lead to heightened effector responses, and one such effector is the hypothalamus-pituitary-adrenal (HPA) axis (Ericsson et al., 1994).

5.2. Hypothalamus-pituitary-adrenal axis

Potent inflammatory cytokines like TNF-α can induce the release glucocorticoids (GCs) by stimulating the HPA axis (Sapolsky and Pulsinelli, 1985, Dunn, 2000). The GCs released from the adrenal glands by inflammatory mediators from injured cells or presence of pathogens (Dunn, 2000, Galon et al., 2002). GCs is thought to have pro-inflammatory and anti-inflammatory properties (Cruz-Topete and Cidlowski, 2015). The anti-inflammatory properties are due to the GCs' ability to repress the pro-inflammatory genes through signal transduction using glucocorticoid receptors (GR) on the surface of the immune cells (Cruz-Topete and Cidlowski, 2015). Studies of genome-wide microarrays of human mononuclear cells show that GCs can upregulate some innate immune-related genes (Galon et al., 2002, Chinenov and Rogatsky, 2007). The GCs bind to GRs and regulate inflammation by altering the transcription of target genes and inhibiting the transcriptional factors like NF-kB, AP-1 or interferon

regulatory factors (Dunn, 2000, Cruz-Topete and Cidlowski, 2015). In PD patients, the HPA axis is hyper-stimulated due to heightened pro-inflammatory cytokines concentration (Herrero et al., 2015). However, this imbalance in the HPA axis secretions leads to a high cortisol level and eventual dysregulated GRs function in the immune cells (Phillips et al., 2017). In the PD model, microglial GRs are activated, which has a significant effect on reducing microglia cell activation and diminishing DA degeneration (Pålhagen et al., 2010). GCs play a role in the brain's regulations as it affects the blood-brain barrier's permeability (BBB) (Kortekaas et al., 2005, Desai et al., 2007). In PD, an impaired BBB could lead to cytotoxic molecules' infiltration resulting in increased DA neuron death through chronic inflammation (Kortekaas et al., 2005). High secretion of GCs can cause brain tissue injury especially in the hippocampus, PFC, and amygdala because of oxidative stress, mitochondrial dysfunction and apoptosis (Dinkel et al., 2002, Sato et al., 2010). High oxygen, high iron content, fatty acid peroxidation, and low antioxidant capacity in the brain makes it susceptible to oxidative stress (Madrigal et al., 2006). The high GCs may increase the risk to develop cognitive or anxiety symptoms, common to brain diseases like dementia and depression in which plasticity changes occur (Lucassen et al., 2015).

5.3. Dopamine signalling

DA plays a crucial role in sleep, learning, attention, pain processing, and working memory (Ayano, 2016). DA exerts its function via DA receptors which are classified into Gs/Golf-coupled D1-like receptors (D1 receptor (D1R) and D5 receptor) and Gi/o-coupled D2-like receptors (D2 receptor (D2R), D3 receptor, and D4 receptor) (Ryoo et al., 1998, Yano et al., 2018). DA reuptake transporter (DAT) is responsible for terminating DA signalling by pumping the DA molecules back into the cytosol (Howe and Dombeck, 2016, Weintraub and Claassen, 2017). In the indirect basal ganglia (BG) pathway, D2Rs are inhibitory as they are thought to play a critical role in inhibiting undesired movements (Surmeier et al., 2010). The stimulation of D2R modulates neuronal activity and inhibits DA and acetylcholine (ACh) release (Tozzi et al., 2011, Threlfell et al., 2012). This complex interaction further affects the striato-thalamicortical circuits which affects DA signalling in the cerebellum (Kharkwal et al., 2016, Avila-Luna et al., 2018). DA axons that project to the cerebellum are from the substantia nigra and ventral tegmental area (Ikai et al., 1992, Flace et al., 2018). Studies have shown the presence of DA regulatory machinery and tyrosine hydroxylase, i.e., a rate-limiting enzyme in the synthesis of catecholamine in the cerebellum (Diaz et al., 1995, Melchitzky and Lewis, 2000, Flace et al., 2018, Qian et al., 2018). DA axons that project to the cerebellum may play a key role in motor control and posture regulation (Schweighofer et al., 2004, Dirkx et al., 2017). DA also regulates essential cognitive functions through DA receptors expressed in the prefrontal cortex (PFC), striatum and hippocampus (Cools et al., 2002, Da Cunha et al., 2002). DA neurotransmission in the PFC plays an essential role in mediating executive functions such as the working memory (Arnsten et al., 2015). DA exerts these

effects by acting on D1Rs because blockade or stimulation of these receptors in the PFC can impair performance on delayed response tasks (Floresco and Magyar, 2006). Little is known about how DA mediates the executive functions regulated by the PFC (Meltzer et al., 2019).

Reduced DA signalling, such as in PD, leads to increased D2R expression, i.e., an increased inhibitory drive of the indirect pathway and therefore inducing motor impairments (Gerfen et al., 1990, Petzinger et al., 2013). Basal ganglia (BG) impairment affects signals that directly or indirectly connect to the BG (Floresco and Magyar, 2006, Arnsten et al., 2015). Furthermore, there is an anatomical association of cortico-striatal circuitry with the PFC, resulting in executive deficits in PD (Robbins and Cools, 2014, Sook et al., 2017). The reduced DA signalling in the BG leads to reduced DA signalling in the PFC, but little is known about how cerebellar DA neurotransmission is affected (Parenti et al., 1986, Floresco and Magyar, 2006, Petzinger et al., 2013, Arnsten et al., 2015). L-DOPA treatment ameliorates PD symptoms by altering cerebellar, limbic, and striatal processing L-DOPA has been shown to improve learning and executive cognitive deficits in patients and spatial memory in rodents and monkeys (Murphy et al., 1972, Fernández-Ruiz et al., 1999, Cools et al., 2002). However, logical memory deficits have been found in treated PD patients compared to untreated patients; moreover, a pre-clinical study showed no improvement in learning and memory in rodents (Hietanen and Teräväinen, 1988, Gevaerd et al., 2001, Zhao et al., 2016).

Increased striatal D2R signalling in LIDs causes increased motor output, i.e., more severe dyskinesia since the direct nigrostriatal pathway is the principal signalling cascade activated in LIDs (Murer and Moratalla, 2011, Suárez et al., 2014). Dopaminergic terminals convert L-DOPA into DA which is released in the synaptic cleft in a controlled way due to the expression of a regulatory mechanism composed of the dopaminergic D2R and the DAT proteins (Bertolino et al., 2009). Thus, D2R and DAT can regulate the firing rate of dopaminergic neurons and DA reuptake, respectively, leading to a controlled DA release (Bertolino et al., 2009). LIDs associated with long-term L-DOPA use occur due to maladaptive striatal plasticity (Bezard et al., 2013, Aristieta et al., 2016). The development of LIDs is dependent on the degree of degeneration of dopaminergic neurons, the type of treatment and the schedule of administration of the drug (Michel et al., 2015). The first potentiating factor for LIDs is the progressive degeneration in the nigrostriatal pathway, which leads to reduced capacity for storing DA in the presynaptic vesicles and a reduced physiological DA secretion (Bastide et al., 2015, Tronci et al., 2017). Additionally, genetically modified mice lacking D1 receptors do not develop LIDs, and a combination of D2R agonists and L-DOPA in L-DOPA naïve rodents resulted in zero to mild cases of LIDs (Blanchet et al., 1993, Pearce et al., 1998, Darmopil et al., 2009). Furthermore, deletion of D3 receptors decreases LIDs via targeting D1R-mediated signalling (Darmopil et al., 2009, Solís et al., 2015). The development of LIDs involves the interaction between DA and the cholinergic system (Conti

et al., 2016). DA receptors play a crucial role in the complex interactions between the DA and cholinergic systems because the neurons in both systems express D2R (Kharkwal et al., 2016).

5.4. Cholinergic Signalling

DA and ACh have a feed-forward mechanism since DA can stimulate (via D5R) or inhibits the release (via D2R) of ACh depending on which receptors are stimulated, while ACh stimulates the release of DA via nicotinic and muscarinic receptors present on DA neurons (Kharkwal et al., 2016). The striatum contains the highest level of ACh and cholinergic markers in the brain, including choline acetyltransferase and acetylcholinesterase (AChE) (Kawaguchi et al., 1995, Zhou et al., 2002). ACh is an excitatory neurotransmitter in the brain and AChE terminates the neurotransmission by hydrolysing ACh to acetate and choline (Maiti et al., 2017). The cholinergic system in the PFC and the hippocampus plays a role in learning and memory, and dysfunction in cholinergic signalling may result in cognitive deficits (Toyoda, 2018, Gargouri et al., 2019). The loss of nigral DA neuron innervation to the striatum in PD leads to a hypercholinergic tone in the BG (Fino et al., 2007, Salin et al., 2009, Tubert et al., 2016). The increased ACh distorts the function of the network in the brain, which induce structural alterations that contribute to PD symptoms (Pisani et al., 2007).

Interestingly, pharmacological reduction of striatal cholinergic tone and ablation of striatal cholinergic interneurons (ChIs) have been shown to decrease the severity of LIDs (Ding et al., 2011, Won et al., 2014). A recent optogenetic study showed that stimulation of cholinergic interneurons modulates LIDs severity (Bordia et al., 2016). The changes in striatal cholinergic signalling and DA depletion contribute to PD pathology and the appearance of LIDs (Perez et al., 2018). Muscarinic antagonists were among the first treatments for PD, although their use is limited by low selectivity and side effects (Duvoisin, 1967). Striatal DA depletion provokes a reduction in the efficacy of M4 auto-receptors on ChIs, resulting in the self-disinhibition of acetylcholine release that contributes to the hypercholinergic tone observed with DA depletion (Ding et al., 2006). Dysregulated striatal cholinergic transmission via M1 and M4 muscarinic receptors has directly been shown to contribute to the motor impairments observed with DA depletion in PD (Ztaou et al., 2016). There is an association between LIDs and the aberrant striatal output caused by an impaired nigrostriatal pathway and chronic L-DOPA administration (Cenci, 2014, Bastide et al., 2015). Enhanced cholinergic tone and its facilitation of long-term potentiation, along with DA deficiency, are thought to be contributing factors to the development of LIDs (Fino et al., 2007, Salin et al., 2009, Cenci and Konradi, 2010, Tubert et al., 2016).

6. Animal models of LIDs

Studies in rodent and primate models of L-3,4-dihydroxyphenylalanine (L-DOPA) induced dyskinesias (LIDs) have been widely used to understand the pathophysiology related to LIDs and facilitate pre-clinical testing of novel therapeutics targets (Perez et al., 2018). Animal models represent a pre-clinical tool since factors such as disease severity, drug treatment, and comorbidities are easily controlled (Nestler and Hyman, 2010). Animal models have been useful in investigating pathogenic pathways and therapeutic targets of Parkinson's disease (PD) and LIDs (Francardo and Cenci, 2014). These models generally show PD cardinal signs and biochemical and behavioural features (Bové et al., 2005). Most animal models of PD show non-motor symptoms such as abnormal olfactory function, gastrointestinal problems and sleep disorders (Issy et al., 2015). Animal models of PD are mostly based on dopamine (DA) neuron death in the substantia nigra and cellular dysregulation due to toxins like 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone and paraquat (Bové et al., 2005, Bezard et al., 2013, Bez et al., 2016). The MPTP-lesioned non-human primate model is an excellent symptomatic PD model and was the first model used to reproduce LIDs experimentally (Johnston et al., 2010). MPTP recapitulates the motor features of human dyskinesia, that is chorea and dystonia and is considered a reliable animal model to define novel therapies (Johnston et al., 2010).. The nigrostriatal system's partial lesion is more likely to mimic these earlier stages of PD if a lower dose of the toxin was used (Sebastianutto et al., 2016). The unilateral 6-OHDA-lesioned rat model can mimic the status of advanced PD and has been widely used for many years to assess drugs' symptomatic effects (Picconi et al., 2003, Boix et al., 2015, Michel et al., 2015). The unilateral 6-OHDA model presents a side-biased motor impairment, and it allows control of the extent of DA neuron degenerations (Beal, 2001). Progression of DA neuron degeneration is most likely the predominant risk factor for the appearance of LIDs, but only in subjects with an extensive DA neuron degeneration, resulting in more than 80% reduction in striatal DA levels (Hong et al., 2014, Bastide et al., 2015). Ulusoy and colleagues (2010) showed that rats whose striatal DA levels had been reduced by 70%, without any damage to the striatal dopaminergic innervation (obtained by employing AAV vector overexpressing a siRNA for the tyrosine hydroxylase) could develop dyskinesias induced by apomorphine. However, they were refractory to dyskinesias generated by L-DOPA (Ulusoy et al., 2010). Showing that LIDs cannot be induced as long as a sufficient number of DA terminals are present to mediate the regulated release of L-DOPA-derived DA (Carta and Björklund, 2018).

7. Basis of the study

The dopamine (DA) neuron death in Parkinson’s disease (PD) leads to the alterations in the molecular markers within the basal ganglia pathways, which affects all the other brain areas that interact directly or indirectly with basal ganglia (BG). The brain areas affected lead to more symptoms other than motor symptoms, including impaired attention, memory, executive and visuospatial functions. It has been

shown that L-3,4-dihydroxyphenylalanine (L-DOPA) ameliorates resting tremors, bradykinesia and muscle rigidity in PD patients (Ruonala et al., 2018). L-DOPA can remediate some cognitive deficits. It can also induce non-motor fluctuations with cognitive dysfunction, e.g. there is no apparent improvement in episodic memory and visuospatial learning and memory (Dubois and Pillon, 1996, Robbins and Cools, 2014). Altered DA signalling and cholinergic signalling in the prefrontal cortex (PFC) and hippocampus impacts episodic memory acquisition (Morici et al., 2015). Studying the effect of L-DOPA on the Hypothalamus-pituitary-adrenal axis and expression of pro-inflammatory markers like tumour necrosis factor α will show if L-DOPA exacerbates glial cell linked neurotoxicity in the BG, PFC, and the cerebellum. If this is so, the exaggerated neurotoxicity in PD patients may play a significant role in the development of LIDs and cognitive decline (Pisanu et al., 2018, Boi et al., 2019). L-DOPA medication results in LIDs and these behavioural fluctuations may not only be limited to motor fluctuations. More work focusing on the social aspects, cognitive impairment, and mood dysfunction in PD need to be done to understand other effects of LIDs. The present study aimed to investigate the gradual effect of L-DOPA treatment on the inflammation, HPA axis, cholinergic pathway and dopaminergic pathway using molecular markers for each pathway in the cerebellum, PFC and striatum of rats that were unilaterally lesioned with 6-OHDA in the medial forebrain bundle.

Objectives

- To assess basal motor function and dyskinesias.
- To assess cognitive deficit.
- To determine the level of corticosterone (plasma)
- To assess the level of TNF-α (striatum and PFC).
- To assess the level of DA (PFC and striatum) and the expression of D1 (PFC) and D2 (striatum).
- To assess the level of AChE in the PFC, hippocampus, and striatum.

8. General methodology

The present study received ethical approval (AREC/077/018M) from the University of KwaZulu-Natal's Animal ethics committee. Seventy-two adult male Sprague-Dawley (SD) rats with weights ranging from 220-250 g were obtained from the Biomedical Resource Unit at the University of KwaZulu-Natal and were used in the present study. After the rats were acclimatised, they were trained for the elevated beam walking test (EBWT) for two days and assessed on the third day. A day later the SD rats were separated into two groups where one group was intracranially injected with saline (4 µL) and the second group was lesioned with 6-hydroxydopamine (6-OHDA) (8 µg/4 µl dissolved in 0.2% ascorbic acid) in the right side of the medial forebrain bundle. The SD rats included in the first phase of the present study went through handling or received a subcutaneous injection of saline or L-3,4-

dihydroxyphenylalanine (L-DOPA) (50 mg/kg) and benserazide (10 mg/kg) for 14 days. During the first day of treatment, 1 hour after handling and administering the L-DOPA or saline, the SD rats' balance and coordination were assessed using the EBWT (figure 3). The SD rats' object recognition memory, visuospatial learning and spatial memory were assessed using the novel object recognition test (NORT) and the Morris water maze test (MWMT) (figure 3).

The second phase of the present study includes SD rats that developed dyskinesias after subcutaneous injections of L-DOPA and benserazide for the treatment duration of 28 days. The dyskinesias experienced by the group were recorded between 9 am to 10 am and scored using the abnormal involuntary movements (AIMs) score. Once the AIMs have receded, the animals are given an hour to rest. Once the SD rats were rested, the SD rats' balance and coordination were assessed using the EBWT. The SD rats' objection recognition memory, visuospatial learning and spatial memory were assessed using the NORT and the MWMT (figure 3).

SD rats were sacrificed, and the blood, cerebella, striata, hippocampus, and the prefrontal cortex (PFC) collected for neurochemical analysis. The collected tissues were analysed to determine striatal dopamine (DA) concentration, cerebellar DA concentration, striatal tumour necrosis factor α (TNF-α) concentration, striatal acetylcholinesterase (AChE) concentration, cerebellar TNF-α concentration, PFC DA concentration, PFC AChE, PFC TNF-α concentration, hippocampal AChE concentration, and plasma corticosterone concentration using Enzyme-linked Immunosorbent assay (ELISA) kit. Furthermore, D1 and D2 receptor expressions were determined using reverse transcription-polymerase chain reaction to analyse the PFC and striatum, respectively. The study design and timeline are illustrated in figure 3 below.

9. Experimental Design

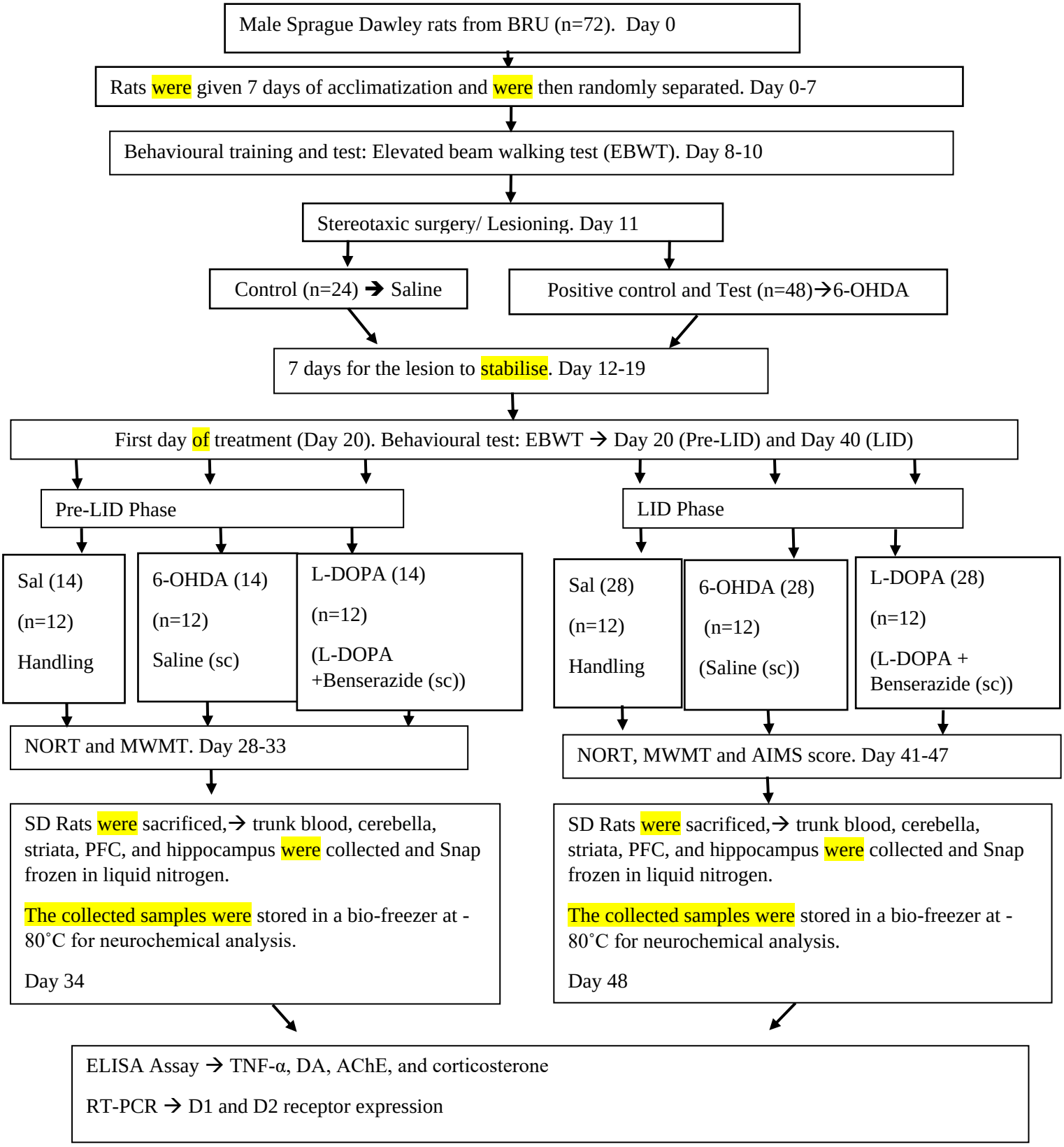

Figure 3: illustration of the study design and timeline for the induction of parkinsonism and treatment with L-DOPA.

10. References

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Chapter 2

Prologue

Manuscript 1

Dopamine (DA) signalling deficiency within the basal ganglia has been shown to result in motor symptoms that are features of Parkinson's disease (PD). The golden standard for treating PD symptoms is the DA replacement therapy called L-3,4-dihydroxyphenylalanine (L-DOPA), which increases DA signalling in the basal ganglia. However, prolonged use of L-DOPA leads to abnormal involuntary movements called L-DOPA induced dyskinesias (LIDs). L-DOPA remains the most effective PD treatment, hence gathering information to ascertain dysregulations that lead to the development of LIDs is essential. Studies have used the 6-OHDA rat model of PD to imitate dyskinesias' development by treating 6-OHDA lesioned rodents with L-DOPA. In addition to the basal ganglia, the cerebellum is also associated with motor function. The present study investigates the alteration of striatal AChE and cerebellar DA as abnormal involuntary movements develop in a rat model of LIDs.

The present manuscript was prepared according to the author guidelines for the Journal brain research.

The alteration of the D2 receptor as abnormal involuntary movements develop due to L-DOPA therapy in a 6-OHDA rat model of PD.

The alteration of dopamine signalling in the striatum and the cerebellum as abnormal involuntary movements develop due to L-DOPA therapy in a 6-OHDA rat model of PD.

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Abstract

Forty to sixty per cent of Parkinson's disease (PD) patients on chronic L-3,4-dihydroxyphenylalanine (L-DOPA) treatment develop abnormal involuntary movements (AIMs) called L-DOPA induced dyskinesias (LIDs). The present study investigates the alteration in striatal acetylcholinesterase (AChE) and cerebellar dopamine (DA) concentration as abnormal involuntary movements develop in a rat model of LIDs. 6-hydroxydopamine (6-OHDA) was used to unilaterally lesion the medial forebrain bundle of Sprague-Dawley rats to induce parkinsonism which was treated with L-DOPA. The motor skills were assessed using the elevated beam walking test and the abnormal involuntary movement score. Tumour necrosis factor-alpha (TNF-α), AChE, and DA concentrations, and the expression of dopaminergic D2 receptor (D2R) were measured. L-DOPA treatment alleviated 6-OHDA-induced hypokinesia and motor incoordination, while prolonged L-DOPA treatment led to AIMs. L-DOPA treatment increased striatal TNF-α and DA concentration as well as D2R expression but reduced striatal AChE concentration. Reduced AChE concentration impairs the regulation of ACh signalling indirectly affecting DA signalling. The progressive increase in striatal D2R and DA between 14 and 28 days of treatment may play a vital role in the appearance of AIMs. Cerebellar TNF-α and DA concentrations were elevated following 14 days of L-DOPA treatment, but both markers were decreased following 28 days of treatment. Increased cerebellar DA concentration in the L-DOPA treated group resulted in improved motor skills. However, reduced cerebellar DA occurred with AIMs and hypokinesia, demonstrating the cerebellum's involvement in PD and LIDs pathology.

Key words: Parkinson‘s disease, dopamine, tumour necrosis factor-alpha, acetylcholinesterase, and 6-hydroxydopamine.

1. Introduction

Parkinson's disease (PD) is a disease whose phenotype includes motor symptoms like bradykinesia and akinesia which are a result of impaired basal ganglia (BG) signalling that prevails in the disease state (Boecker et al., 1999, Postuma and Berg, 2016). The BG is a subcortical nuclei group that plays a role in initiating desired and inhibition of undesired movements (Ungerstedt, 1968, Middleton and Strick, 1994). PD is characterised by dopamine (DA) neurons' death in the pars compacta of the substantia nigra (Issy et al., 2015). The death of DA neurons results in diminished DA neurotransmission in the striatum, which results in motor symptoms such as hypokinesia, that are characteristic of PD (Zhai et al., 2018).

Effective medication used to treat motor symptoms of PD is the DA replacement therapy that includes a combination of L-3,4-dihydroxyphenylalanine (L-DOPA) and inhibitors of monoamine oxidase and catechol-O-methyltransferase (Cotzias et al., 1969, Männistö et al., 1990, Kang et al., 2016). The inhibitors are administered to lessen the peripheral degradation of L-DOPA, therefore enhancing the concentration of L-DOPA that reaches the central nervous system, where it can be converted to DA- thus, increasing DA neurotransmission in PD patients' striatum (Törnwall and Männistö, 1993, Stacy and Galbreath, 2008, Ruonala et al., 2018). One of the significant side-effects of long-term use of L-DOPA is the development of abnormal involuntary movements called L-DOPA induced dyskinesias (LIDs) (Cotzias et al., 1969, Ko et al., 2014, Conti et al., 2016). Forty per cent of PD patients develop LIDs after using L-DOPA for six years, this percentage increases to sixty per cent after ten years of treatment (Ahlskog and Muenter, 2001, Tran et al., 2018). Factors that predispose PD patients to develop LIDs include, age of PD onset, body weight, the dose of L-DOPA prescribed and others (Daneault et al., 2013).

The pathophysiology of LIDs is still under investigation. However, some of the findings implicated impaired striatal DA signalling, increased conversion of L-DOPA to dopamine by striatal serotonin neurons, impaired cholinergic fibre signalling, and neuroinflammation (Murer et al., 1998, Shen et al., 2015, Ndlovu et al., 2016, Smith et al., 2016). Neuroinflammation is central to the pathology of PD as it is thought to not only be a result of the disease itself but also to propagate the death of DA neurons via pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF-α) (Mogi et al., 1994, Jin et al., 2008, Tufekci et al., 2012). The release of pro-inflammatory cytokines like TNF-α is beneficial in the early stages of the disease as it enhances the immune response to the tissue damage occurring in PD (Boka et al., 1994, Tufekci et al., 2012). However, as the disease progresses, this augmented inflammatory response leads to increased synthesis of reactive oxygen species that eventually cause the death of DA neurons (Hunot et al., 1999, Felger et al., 2015, Peter et al., 2018).

DA neurons can convert L-DOPA into dopamine which is released in the synaptic cleft in a controlled manner due to the expression of a delicate regulatory mechanism composed of the dopaminergic D2 receptor (D2R) and the DA active transporter proteins (Bertolino et al., 2009, Kintz et al., 2017). The decreased DA signalling in PD has been shown to lead to increased D2R sensitivity as a compensatory mechanism (Petzinger et al., 2013, Kintz et al., 2017). The increased endogenous DA production due to L-DOPA therapy and increased D2R sensitivity in the basal ganglia causes increased motor complications, i.e., LIDs (Parenti et al., 1986, Surmeier et al., 2010, Murer and Moratalla, 2011, Petzinger et al., 2013).

Striatal cholinergic interneurons express D2R, which, when stimulated, inhibits ACh secretion and reduced DA signalling, such as in PD, leading to increased ACh signalling (Soreq, 2015, Souza et al., 2015). The changes in striatal cholinergic signalling and DA depletion contribute to PD pathology and the appearance of LIDs (Perez et al., 2018). The ablation of cholinergic neurons, i.e., decreased striatal ACh concentration, has been shown to decrease the severity of LIDs (Ding et al., 2011, Won et al., 2014). ACh signalling is regulated by a group of enzymes called Acetylcholinesterase (AChE) that break down ACh to acetate and choline, a critical process that regulates ACh signalling (Zugno et al., 2015). Studying the role that AChE plays in the pathology of PD and LIDs is essential for the development of more pharmaceutical interventions that can reduce the severity of LIDs.

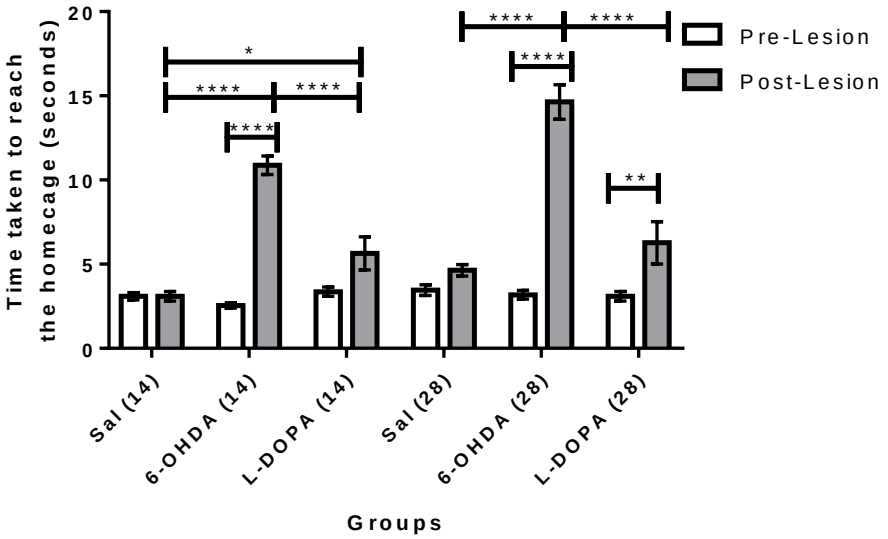
The study of molecular changes in PD that lead to the development of LIDs has been done in humans and animal models. One such animal model that can mimic the death of DA neurons in the medial forebrain bundle and the resulting parkinsonism is the 6-hydroxydopamine (6-OHDA) rodent model of PD (Paxinos and Watson, 1986). The neurotoxin is injected into the medial forebrain bundle, affecting the DA neurons in the substantia nigra (Ngema and Mabandla, 2017). The reduced DA signalling affects the basal ganglia, especially the striatum leading to impaired motor skills (Zarow et al., 2003). The ventral tegmental area and substantia nigra supply DA to the cerebellum but not much is known about cerebellar DA content in PD patients (Ikai et al., 1992, Flace et al., 2018). The administration of L-DOPA can affect DA signalling in the cerebellum. The present study investigates the alteration of striatal AChE and Cerebellar DA as abnormal involuntary movements develop in a rat model of LIDs.

2. Results

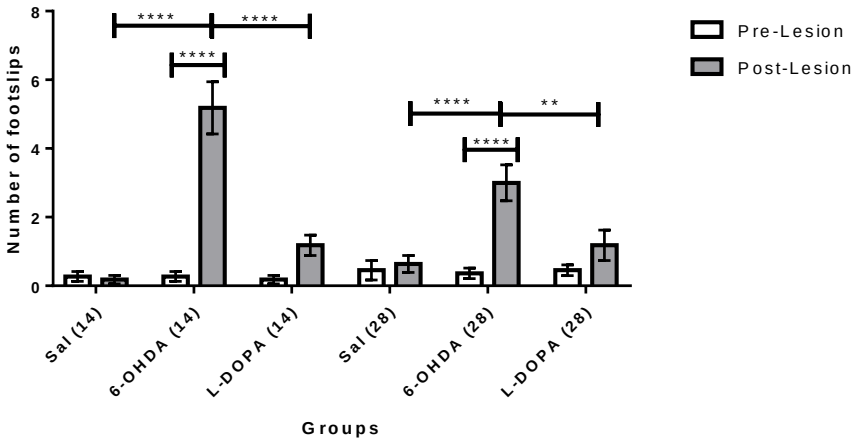
2.1 The Elevated Beam walking test

Motor coordination and balance were assessed before (pre-lesion) and seven days after stereotaxic surgery (post-lesion) in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 1. The two-way analysis of variance followed by Sidak's multiple

comparisons test was used to analyse the data in figure 1. The 6-OHDA lesion significantly increased the time taken to cross the beam (pre-lesion vs post-lesion; 6-OHDA (14); 6-OHDA (28); F 5, 60 = 28,53, p<0.0001, figure 1a) and the number of foot slips observed when crossing the beam (pre-lesion vs post-lesion; 6-OHDA (14); 6-OHDA (28); F5, 60= 16.04, p<0.0001, figure 1b). Treatment with L-DOPA significantly reduced the time to cross the beam (6-OHDA (14) vs L-DOPA (14); 6-OHDA (28) vs L-DOPA (28); F 5, 60 = 22,64, p<0.0001, figure 1a) and significantly reduced the number of foot slips (6-OHDA (14) vs L-DOPA(14); F5, 60= 14,4, p<0.0001, figure 1b) and L-DOPA (28) (6-OHDA (28) vs L-DOPA (28); F5, 60 = 14,4, p=0,0001, figure 1b). AIMs development in the L-DOPA (28) group increased the time the SD rats took to reach the home cage (pre-lesion vs post-lesion, L-DOPA (28); F5, 60= 28.53, p< 0,0022, figure 1a).



(a)



(b)

Figure 1: Graph showing (a) the time it took the SD rats to reach the home cage and (b) the number of foot slips observed as SD rats crossed the beam in the Elevated beam walking test (n=11per group).

2.2 The abnormal involuntary movement score

Abnormal involuntary movements were rated in L-DOPA (28), figure 2. The two-way analysis of variance followed by Sidak's multiple comparisons test was used to analyse the data in figure 2. There was no significant difference in the severity of AIMs in the L-DOPA (28) group in the eight days of observation (L-DOPA (28); F9, 63 = 3,393, *p*=0,6162, figure 2).

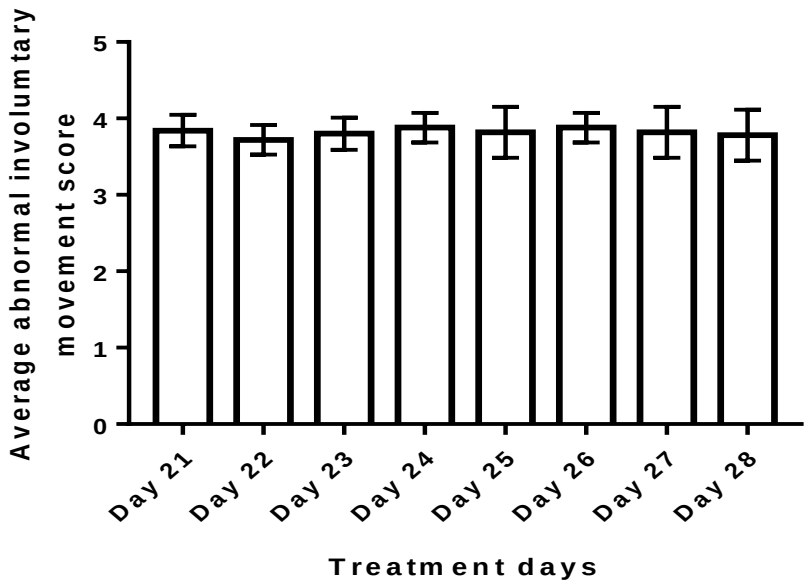


Figure 2: Graph showing the average severity of the abnormal involuntary movements in the L-DOPA (28) group for eight days of treatment (n=10).

2.3 The striatal tumour necrosis factor-alpha concentration

Striatal TNF-α concentration was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 3. One-way analysis of variance plus Tukey's multiple comparisons test was used to examine the data in figure 3. TNF-α concentration was significantly increased in 6-OHDA-lesioned rats at day 14 of saline injections (Sal (14) vs 6-OHDA (14); F5, 12 = 27.2, *p*= 0,0078, figure 3). This effect was also observed at day 28 of saline injections (Sal (28) vs 6-OHDA (28); F5, 12 = 27.2, *p*< 0,0088 figure 3). TNF-α concentration was significantly heightened after 14 days (Sal (14) vs L-DOPA (14); F5, 12 = 27.2, *p*= 0,0154, figure 3) and 28 days (Sal (28) vs L-DOPA (28); F5, 12 = 27.2, *p*= 0,0081, figure 3) of L-DOPA treatment.

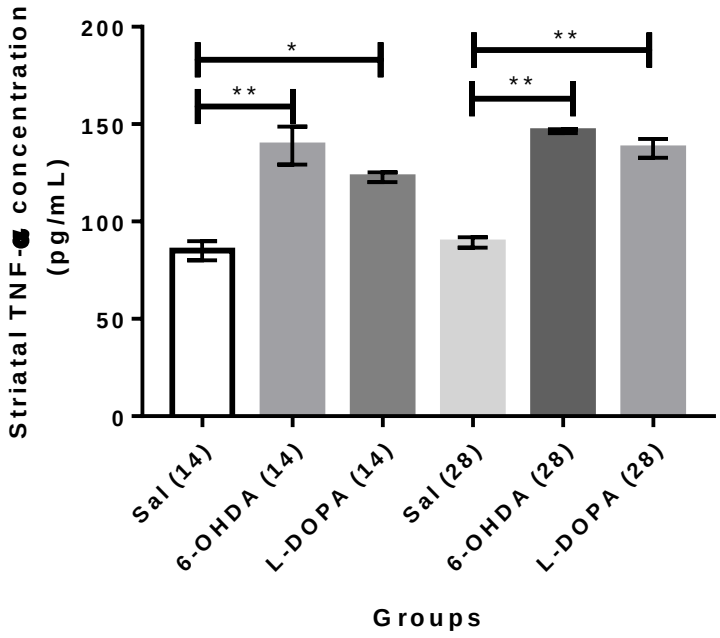


Figure 3: Graph showing striatal TNF-α concentration in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

2.4 The concentration of cerebellar Tumour necrosis factor-alpha (TNF-α)

Cerebellar TNF-α concentration was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 4. One-way analysis of variance plus Tukey's multiple comparisons test was used to examine the data in figure 4. TNF-α concentration was significantly increased in 6-OHDA-lesioned rats at day 14 (Sal (14) vs 6-OHDA (14); F5, 12 = 29.47, p= 0.0067, figure 5) and day 28 (Sal (28) vs 6-OHDA (28); F5, 12 = 29.47, p< 0.0001, figure 5). Treatment with L-DOPA for 14 days significantly increased TNF-α concentration (Sal (14) vs L-DOPA (14); F5, 12 = 29.47, p= 0.0024, figure 5), while 28 days of treatment significantly decreased it (6-OHDA (28) vs L-DOPA (28); F5, 12 = 29.47, p< 0,0001, figure 4). TNF-α concentration significantly increased with time in the 6-OHDA lesioned groups only (6-OHDA (14) vs 6-OHDA (28); F5, 12 = 29.47, p= 0,0051, figure 4).

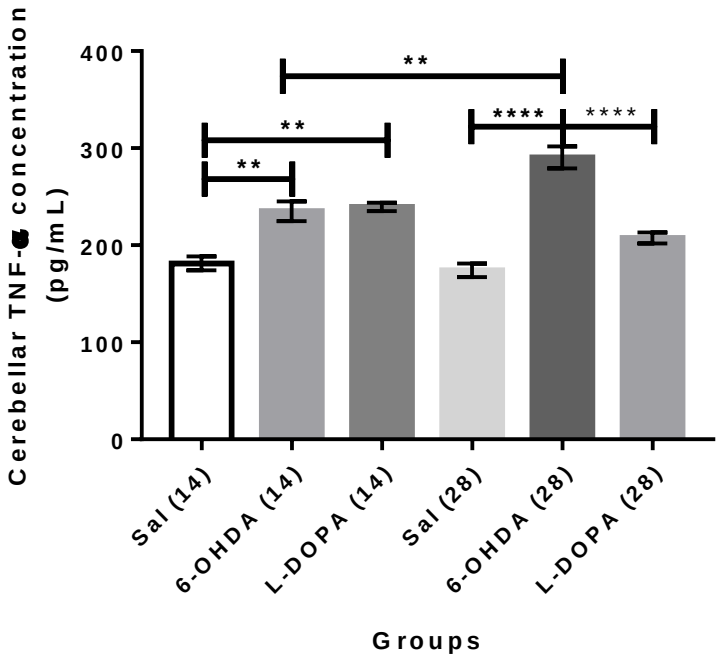


Figure 4: Graph showing cerebellar TNF-α concentration in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

2.5 The concentration of dopamine in the striatum

Striatal DA concentration was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 5. One-way analysis of variance plus Tukey's multiple comparisons test was used to examine the data in figure 3. DA concentration was significantly decreased in 6-OHDA-lesioned rats 14 days (Sal (14) vs 6-OHDA (14); F2, 10 = 3.288, p<0,0001, figure 5) and (Sal (14) vs L-DOPA (14); F2, 10 = 3.288, p= 0.0003, figure 5) and 28 days (Sal (28) vs 6-OHDA (28); F2, 10 = 3.288, p<0.0001, figure 5) and (Sal (28) vs L-DOPA (28); F2, 10 = 3.288, p= 0.0019, figure 5) post treatment. However, there was a treatment effect in the 6-OHDA lesioned groups (6-OHDA (14) vs L-DOPA (14); F2, 10 = 3.288, p= 0.0147, figure 5) and (6-OHDA (28) vs L-DOPA (28); F2, 10 = 3.288, p= 0.0011, figure 5). Furthermore, there was a time effect in the L-DOPA treated groups (L-DOPA (14) vs L-DOPA (28); F2, 10 = 3.288, p=0.0014, figure 5).

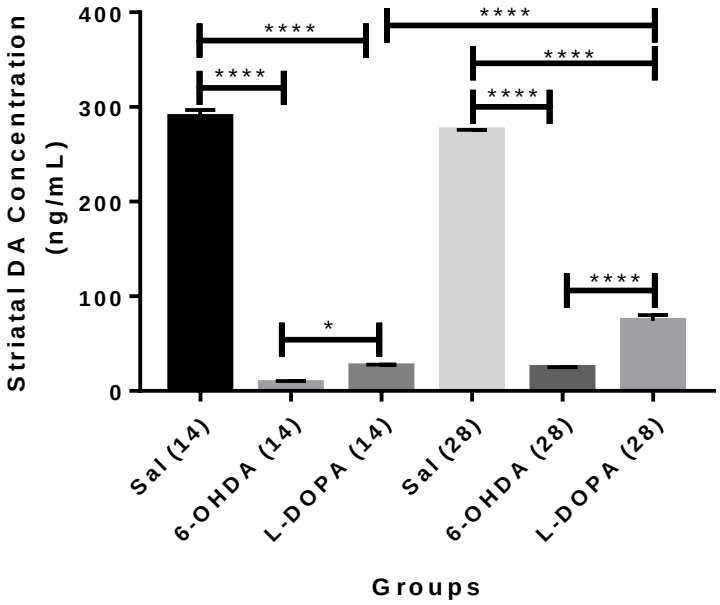


Figure 5: Graph showing striatal DA concentration in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

2.6 The concentration of DA in the cerebellum

Cerebellar DA concentration was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 6. One-way analysis of variance plus Tukey's multiple comparisons test was used to examine the data in figure 6. DA concentration was significantly increased in 6-OHDA-lesioned rats 14 days of treatment (Sal (14) vs 6-OHDA (14); F5, 12 = 34.1, p= 0,0035, figure 6) and (Sal (14) vs L-DOPA (14); F5, 12 = 34.1, p= 0,0006, figure 6). Cerebellar DA was significantly elevated in the 6-OHDA lesioned rats 28 days post-lesion (Sal (28) vs 6-OHDA (28); F5, 12 = 34.1, p= 0,0002, figure 6). However, the 6-OHDA lesioned rats that were treated with L-DOPA for 28 days had a significantly lower cerebellar DA concentration (6-OHDA vs L-DOPA (28); F5, 12 = 34.1, p< 0,0001, figure 6). The rats treated with L-DOPA for 28 days had a significantly decreased cerebellar DA concentration as compared to the group treated for 14 days (L-DOPA (14) vs L-DOPA (28), F5, 12 = 34.1, p< 0,0001, figure 6).

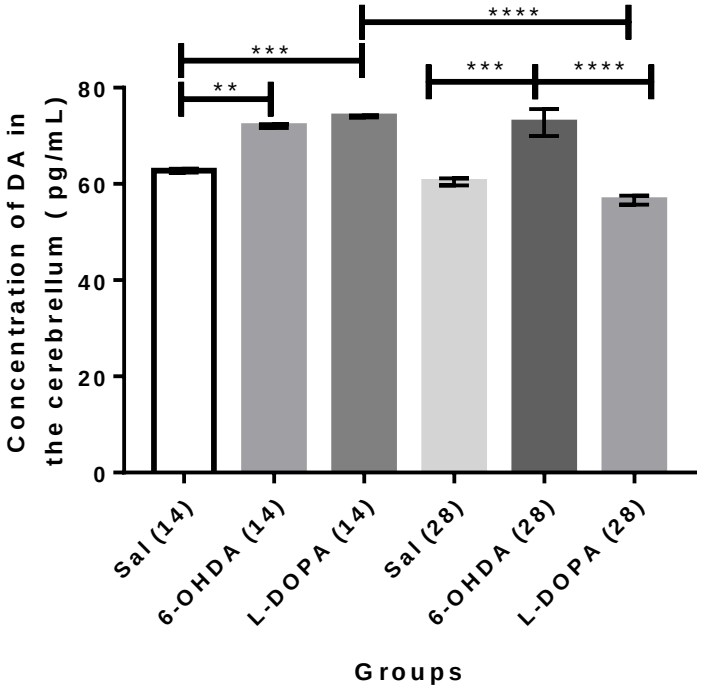


Figure 6: Graph showing cerebellar DA concentration in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

2.7 The expression of Dopamine 2 receptor in the striatum

Striatal D2R expression was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 7. One-way analysis of variance plus Tukey's multiple comparisons test was used to examine the data in figure 7. There was a significant upregulation of D2R gene expression as time progressed in the 6-OHDA lesioned groups (6-OHDA (14) vs 6-OHDA (28), F5, 12 = 25.86, p= 0,0004, figure 7) and (L-DOPA (14) vs L-DOPA (28); F5, 12 = 25.86, p= 0,0011, figure 7). There was a lesion effect 35 days post lesion (Sal (28) vs 6-OHDA (28); F5, 12 = 25.86, p<0.0001, figure 7) and (Sal (28) vs L-DOPA (28); F5, 12 = 25.86, p= 0.0083, figure 7).

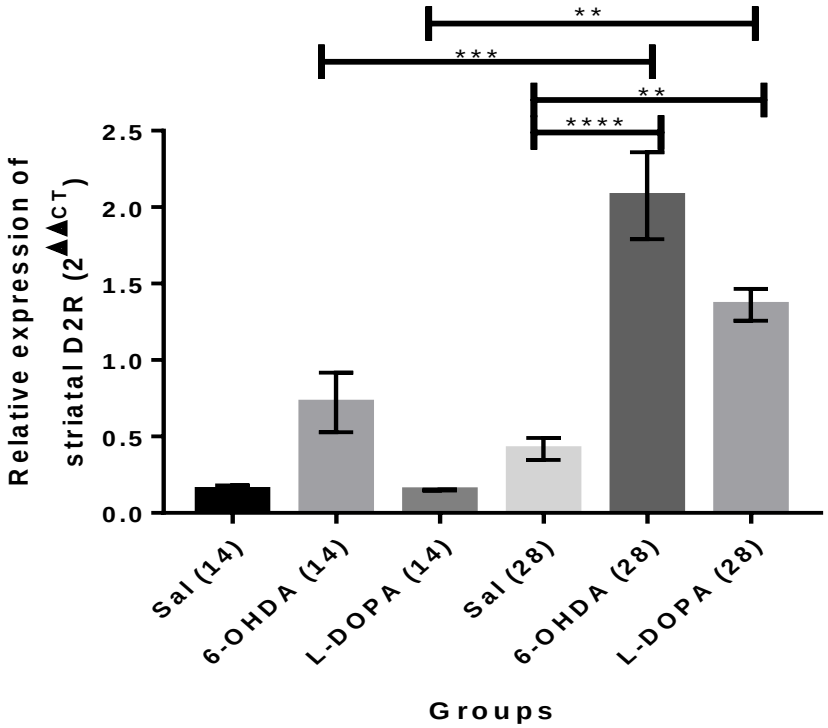


Figure 7: Graph showing the change in D2R expression relative to GAPDH expression in the striata of the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

2.8 The concentration of acetylcholinesterase in the striatum

Striatal AChE concentration was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 8. One-way analysis of variance plus Tukey's multiple comparisons test was used to examine the data in figure 8. AChE concentration was significantly increased in 6-OHDA lesioned rats (Sal (14) vs 6-OHDA (14); F5, 12 =114.1, $p <$ 0,0001, figure 8) and (Sal (28) vs 6-OHDA (28); F5, 12 = 114.1, $p <$ 0,0001, figure 8). AChE concentration was significantly reduced in 6-OHDA lesioned rats that were treated with L-DOPA(6-OHDA (14) vs L-DOPA (14); F5, 12 = 114.1, $p<$ 0,0001, figure 8) and (6-OHDA (28) vs L-DOPA (28); F5, 12 = 114.1, $p<$ 0,0001 figure 8).

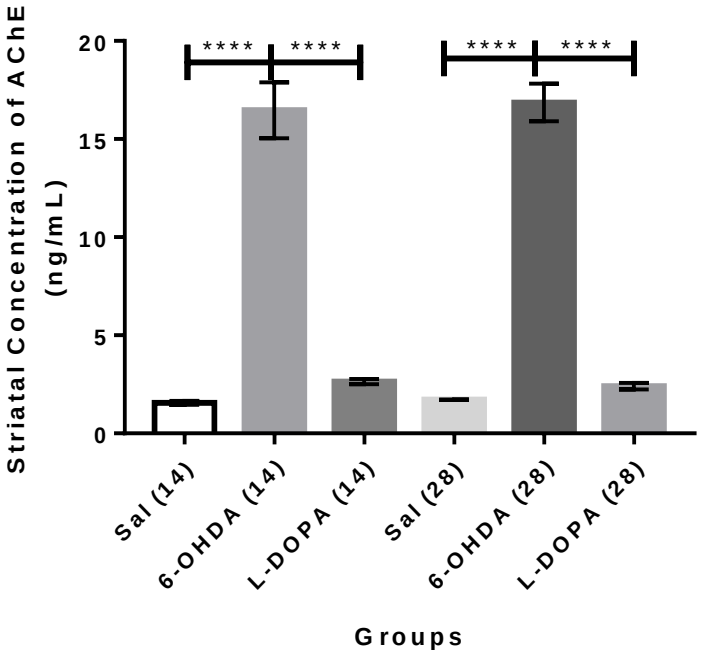


Figure 8: Graph showing striatal AChE concentration in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

3. Discussion

L-DOPA is still the most effective pharmacological intervention for motor complications related to Parkinson's disease (PD) (Kang et al., 2016). However, chronic treatment of PD with L-3,4-dihydroxyphenylalanine (L-DOPA) has been known to progressively lead to abnormal involuntary movements (AIMs) (Bezard et al., 2013, Aristieta et al., 2016). The neurotoxin 6-hydroxydopamine (6-OHDA) causes the degeneration of dopamine (DA) neurons in the nigrostriatal pathway, which leads to motor impairment in a rat model of PD (Schober, 2004). The rodent 6-OHDA model has been modified by treating the rodents with a combination of L-DOPA and benserazide for 28 days post-6-OHDA surgery to model the development of L-DOPA-induced dyskinesias (LIDs) (Picconi et al., 2003, Lundblad et al., 2005, Ndlovu et al., 2016). Previous studies have shown that the administration of L-DOPA led to the development of AIMs after 21 days of treatment (Ndlovu et al., 2016). The present study investigated motor complications related to prolonged exposure to L-DOPA in a unilaterally lesioned 6-OHDA rat model of PD. The present study further explored cerebellar (DA and tumour necrosis factor-alpha (TNF-α)) and striatal (TNF-α, Acetylcholinesterase (AChE), DA and dopaminergic D2 receptor (D2R)) molecular marker alterations in the progression of L-DOPA treatment, before and after AIMs developed.

In the present study, the elevated beam walking test (EBWT) was used to assess two motor skills, viz. hypokinesia and fine coordination (Carter et al., 2001, Luong et al., 2011). The 6-OHDA lesion caused hypokinesia, incoordination and motor imbalance which was observed on the 8th and 27th-day post-surgery. These results agree with reports showing rats intracranially injected with 6-OHDA in the medial forebrain bundle showed motor impairments (Pierucci et al., 2009, Chen et al., 2018). The motor impairments were ameliorated by L-DOPA as assessed on the first day of treatment and the 20th day of treatment using the EBWT. These findings agree with literature that showed that the motor impairment that resulted from a unilateral 6-OHDA lesion is ameliorated by L-DOPA treatment (Strome et al., 2007, Ordoñez-Librado et al., 2010). However, prolonged exposure to L-DOPA resulted in AIMs with a consistent score for the eight days of observation (from the 21st day to the 28th day of treatment). These findings align with Ndlovu *et al.*, (2016), who found that rats treated with L-DOPA after a unilateral 6-OHDA lesion in the medial forebrain bundle resulted in AIMs 21 days post-L-DOPA administration. The development of AIMs has been attributed to impaired DA signalling in the striatum (Ahlskog and Muenter, 2001).

PD is associated with chronic inflammation, and the injection of 6-OHDA in the medial forebrain bundle has shown to result in an inflammatory response (Farbood et al., 2015). One of the main effectors of inflammation is the pro-inflammatory cytokine TNF-α, and the increased expression of this biomarker in PD shows that it may be a mediator of neural damage via oxidative stress (Boka et al., 1994, Nagatsu et al., 2000). Under normal conditions, TNF-α is not toxic to DA cells but persistently high expression of TNF-α can lead to notable death of DA neurons (Boka et al., 1994, Leal et al., 2013). The present study showed that a 6-OHDA lesion increased striatal TNF-α concentration, suggesting that the neurotoxin causes hyper-inflammation in the striatum, a state that remained in the striatum throughout treatment with L-DOPA (Mogi et al., 1994, Mogi et al., 1999). The 6-OHDA lesion increased cerebellar TNF-α concentration with a progressive increase observed from week two to week four in the untreated 6-OHDA groups. Increased cerebellar and striatal TNF-α concentration show a heightened inflammatory state, leading to the death of DA neurons via glial cell-mediated neurotoxicity (Hwang et al., 2008, Chung et al., 2016). These findings show that PD has pathophysiological features that allow the death of DA neurons to occur in other brain areas besides the basal ganglia (BG) and prevails in the disease state (Khadrawy et al., 2017).

DA signalling is tightly regulated by DA receptors and DA reuptake transporter (Howe and Dombeck, 2016). DA neurons convert endogenous L-DOPA to DA which is released into the synaptic cleft, and the DA transporters facilitate reuptake of DA into the presynaptic neuron (Männistö et al., 1990, Howe and Dombeck, 2016). In PD and animal models of the disease, there is a death of DA neurons resulting in reduced DA signalling in the striatum (Lai et al., 2019). In our study, the intracranial injection of 6-OHDA into the medial forebrain bundle led to DA concentration reduction in the striatum. These results

align with Lai *et al*., 2019, who showed that the intracranial injection of 6-OHDA in the medial forebrain bundle reduced striatal DA content. The administration of L-DOPA increased DA signalling in the striatum and along with the hypersecretion of DA in the cerebellum ameliorated the motor impairment that prevails due to the 6-OHDA lesion (Bertolino et al., 2009). However, comparing the striatal DA concentration after 14 days of L-DOPA treatment and after 28 days of L-DOPA administration showed a progressive increase in striatal DA concentration. The severely depressed striatal DA signalling due to the loss of DA neurons causes the recruitment of striatal serotonin neurons that uptake exogenous L-DOPA and produce DA (Ng et al., 1970, Maeda et al., 2005, Bastide et al., 2015). The issue with the above mechanism is that it does not have the same regulatory mechanism for DA release as the DA neurons (Tanaka et al., 1999). DA released from serotonergic neurons will act in coordination with orally administered L-DOPA to cause pulsatile stimulation of DA receptors, which cause changes in downstream signalling pathways in striatal neurons and contributes to the development of LIDs (Tanaka et al., 1999, Björklund et al., 2007, Carta and Björklund, 2018). The unregulated release of striatal DA by serotonin neurons leads to uncontrolled movements, i.e., AIMs observed in the present study.

Cerebellar DA sources are axonal projections from the ventral tegmental area and the substantia nigra (Ikai et al., 1992, Flace et al., 2018). There was increased cerebellar DA concentration in groups lesioned with 6-OHDA except in the group treated with L-DOPA for 28 days. Humans and animal studies showed hyperactivity in the cerebellum and motor cortex in response to the reduced signalling in the substantia nigra. Furthermore, there is increased connectivity between the cerebellum and other brain areas in PD patients off L-DOPA treatment (Festini et al., 2015). The increased activity and connectivity is thought to compensate for the depressed neurotransmission in the BG in PD. In the present study, the hyperactivity observed in the cerebellum is increased DA signalling in rodents lesioned with 6-OHDA. The above may be due to increased connectivity to the VTA in response to depressed DA signalling in the BG. PD patients on L-DOPA treatment had reduced connectivity between the cerebellum and other brain areas (Festini et al., 2015). Initially, the treatment with L-DOPA for 14 days showed a high cerebellar DA concentration, but there was a significant reduction of cerebellar DA concentration after 28 days of treatment.

The second factor that plays a role in the regulation of DA transmission is DA receptors. DA receptors help facilitate the action of DA and are often the target site for DA therapy drugs (Ryoo et al., 1998). In the present study, the unilateral lesion using 6-OHDA and the resultant DA depression resulted in heightened D2R expression in the ipsilateral striatum. The upregulated D2R expression becomes more prominent as time progressed from 14 days to 28 days of treatment. These findings align with literature which shows that in PD the reduced DA signalling causes an increased D2R expression in the striatum as a compensatory mechanism, i.e., rendering the post-synaptic neuron more sensitive to DA (Gerfen

et al., 1990, Nikolaus et al., 2003, Smith et al., 2016). Initially, the treatment with L-DOPA for 14 days showed a tendency to reduce the D2R expression, but after 28 days of treatment, the expression of D2R increased significantly. These findings align with Narang and Wamsley (1995) who found a significant increase in D2R expression from the second week to the sixteenth-week post-lesion. This increased expression and increased DA production from exogenous L-DOPA administration may increase motor output, i.e., AIMs (Murer and Moratalla, 2011).

D2Rs are expressed on acetylcholine (ACh) neurons, and once stimulated, they inhibit the activation and release of ACh (Kharkwal et al., 2016). The reduced activity of the dopaminergic system in the striatum has been shown to occur in parallel with the cholinergic system's hyperactivity (Dagaev et al., 2004, Fino et al., 2007, Salin et al., 2009, Tubert et al., 2016). In normal physiological conditions, ACh signalling is tightly regulated by cholinergic receptors and AChE (Kawaguchi et al., 1995, Zhou et al., 2002). AChE is an enzyme that hydrolyse extracellular ACh and is sometimes used as a cholinergic biomarker (Zaretsky et al., 2016). In the present study, the 6-OHDA lesion alone showed increased striatal AChE concentration. The reduced DA signalling in the 6-OHDA lesioned groups has been shown to lead to an increased ACh as a compensatory mechanism since ACh stimulates DA secretion from DA neurons (Ding et al., 2011). The heightened ACh signalling increases AChE concentration, allowing for pulsatile release of ACh and diminishing the chances of receptor desensitization on the DA neuron (Zhou et al., 2001). Increased DA signalling, such as in the groups treated with L-DOPA, reduces ACh's secretion and eventually decreased the concentration of the enzyme AChE. ACh and DA have a feedforward mechanism since DA neurons also have nicotinic and muscarinic receptors that, when activated lead to the DA release (Kharkwal et al., 2016). The reduced striatal AChE in groups treated with L-DOPA increases ACh signalling which may play a vital role in the development of LIDs since the pharmaceutical reduction of cholinergic signalling has been shown to reduce the severity of LIDs (Ding et al., 2011, Won et al., 2014).

4. Conclusion

The present study showed that L-DOPA attenuates the hypokinesia and impaired motor coordination caused by the injection of 6-OHDA into the medial forebrain bundle. The hypokinesia returned after the development of AIMs because of increased DA signalling and reduced AChE concentration in the striatum. The present study showed that the hyperactivity and increased connectivity previously reported in PD patients' cerebellum off L-DOPA treatment would translate to increased cerebellar DA concentration and hyper-inflammation (Festini et al., 2015, Mirdamadi, 2016). The prolonged treatment with L-DOPA reduced cerebellar DA concentration and hyperinflammation. This reduction may be because of decreased connectivity between the cerebellum and other brain areas (Festini et al., 2015). There was a progressive increase in DA signalling in the striatum during prolonged L-DOPA treatment

which causes a progressive decline in DA signalling in the cerebellum. Furthermore, the present study showed that prolonged treatment reduces hyper-inflammation in the cerebellum, which reduces the chance of cerebellar neuronal degeneration. These findings suggest that DA plays a crucial role in the pathology of PD and LIDs. The findings may be beneficial to reduce the disease's progression and reduce the chances of developing dyskinesias. This increases the longevity and quality of life for PD patients. Future studies may focus on the progressive alteration of the apoptotic marker in the cerebellum, thalamus, and motor cortex to illuminate the heightened inflammatory response's effect on the neurons in the cerebellum.

5. Materials and methods

5.1 Animal Care and Housing

The University of KwaZulu-Natal's Animal Research Ethics Committee (AREC/077/018M) granted the present study ethical approval. Upon ethical approval, seventy-two adult male Sprague-Dawley rats with weights ranging from 220-250 g were obtained from the Biomedical Resource Unit of the University of KwaZulu-Natal. The rats were housed in clear cages and given standard rat feed and water freely. The holding area where the rats were kept for the study's duration was under standard laboratory conditions and a 12-hour light/dark cycle with lights automatically switching on at 06:00. The rats were given an acclimatisation period of 7 days before any behavioural tests.

5.2 Elevated Beam walking test

In the present study, the elevated beam walking test was used to assess two motor skills, i.e., fine coordination and balance (Carter et al., 2001, Luong et al., 2011). The elevated beam walking test (EBWT) was performed twice, before the 6-OHDA lesion and after the lesion has stabilised (figure 9). This task includes a training phase that happens over two days, followed by a 1-day assessment (figure 9). The training phase included the rats being trained to cross an 80 cm long, 4 cm wide beam on the first day, followed by a 60 cm long, 3 cm wide wooden beam on the second day to reach the goal box. On the day of the assessment, the rats crossed a 60 cm long, 2 cm wide wooden beam to reach the goal box. During the training and assessment, the wooden beam was 30 cm above a soft surface on a wooden frame. The rats that fell off were returned to the starting position, and the timer restarted. The parameters measured during the assessment was the time taken for the rat to cross the narrow beam and the number of foot slips that the animals displayed as they crossed the beam.

5.3 Stereotaxic surgery

The rats were placed under chemical anaesthesia prior to surgery. The chemicals used were a combination of ketamine and xylazine (125 mg/kg and 10 mg/kg, respectively) which was administered via intraperitoneal injection (Veilleux-Lemieux et al., 2013). The ketamine keeps the rats in a state of unconsciousness, and xylazine helps increase the heart rate to prevent hypotension during surgery

(Bennett and Bullimore, 1973, Veilleux-Lemieux et al., 2013). Once the rat was unconscious, it was placed in the stereotaxic frame (David Kopf Instruments, Tujunga, USA). The scalp was marked at the following coordinates from the lambda 4.7 mm anterior and -1.6 mm from the midline (Paxinos and Watson, 1986). A small hole was drilled at the marked area and a Hamilton syringe loaded with 4 µl of the 6-OHDA (8 µg/4 µL dissolved in 0.2% ascorbic acid; Sigma, Sigma-Aldrich, United States) or saline solution was lowered 8.4 mm below the skull (Paxinos and Watson, 1986, Kang et al., 2016, Ndlovu et al., 2016). The 6-OHDA solution or 0.9% saline was unilaterally infused at a rate of 1 µL per minute. In total the needle was left inside the brain for 6 minutes allowing for the diffusion of the 6-OHDA or 0.9% saline solution into the surrounding tissue after which the needle was slowly retracted (Mabandla et al., 2015).

5.4 Animal grouping

The SD rats were divided into two phases which define the overall study design, the pre-LID and the LID phase. The groups in the pre-LID phase comprised of the rats that were treated for 14 days. The first day of treatment was 7 days post-surgery. Saline-lesioned rats were handled for 14 days and served as the negative control (Sal (14)) group, while the 6-OHDA-lesioned rats were further divided into two groups. The 6-OHDA-lesioned rats that received a subcutaneous injection (s.c) of saline for 14 days served as the positive control (6-OHDA (14)). The other 6-OHDA-lesioned group received a s.c with a combination of L-DOPA (50 mg/kg) and benserazide (10 mg/kg) for 14 days, which served as the test group (L-DOPA (14)). The LID phase groups comprised of SD rats that received treatment for 28 days. Saline-lesioned rats were handled for 28 days and served as a negative control (Sal (28)). The 6-OHDA-lesioned rats that received a s.c of saline for 28 days served as the positive control (6-OHDA (28)) and the remaining 6-OHDA-lesioned received s.c with a combination of L-DOPA (50 mg/kg) and benserazide (10 mg/kg) for 28 days, which served as the test group (L-DOPA (28)) (figure 9).

5.5 L-DOPA treatment

Seven days after the surgery, L-DOPA (50 mg/kg; Sigma, Sigma-Aldrich, United States), combined with the peripheral DOPA–decarboxylase inhibitor, benserazide (10 mg/kg; Sigma, Sigma-Aldrich, United States), was dissolved in 0.9% saline (Huang et al., 2011). Benserazide was administered to reduce the metabolism of L-DOPA in the periphery. The drugs were administered twice daily (9 am and 4 pm) for 14 days (pre-LID phase) or 28 days (LID phase).

5.6 Abnormal involuntary movement scoring

Abnormal involuntary movements (AIMs) were scored daily between 9 am, and 10 am from the 21st day of L-DOPA injection (Ndlovu et al., 2016). On the day of observation, the rats were individually placed in transparent empty plastic cages 10 min before drug administration. Fifteen minutes after the drug administration, each rat was observed for 60 minutes for AIMs scoring. The AIMs scoring was

done for the last eight days of the 28-day treatment. The AIMs being observed and scored were A) axial dystonia (contralateral twisted posturing of the neck and upper body), (B) abnormal orolingual movements (stereotyped jaw movements and contralateral tongue protrusion) and (C) abnormal forelimb movements (repetitive rhythmic jerks or dystonic posturing of the contralateral forelimb and grabbing movements of the contralateral paw) (Ostock et al., 2011). The severity of the three AIMs were rated from 0 to 4, where 0 denotes the absence of AIMs, 1 represents occasional occurrence, 2 represents frequent occurrence, 3 represents uninterrupted occurrence, and 4 means that the dyskinetic behaviour was continuous and not interrupted by sensory distraction (Ndlovu et al., 2016). Total AIMs were determined from each rat's total scores, which were averaged to determine the group total, and reported (Ndlovu et al., 2016).

5.7 Euthanasia

The rodents were brought to the autopsy suite an hour before decapitation. The rats were decapitated using a guillotine at 12 pm, and trunk blood was collected in EDTA coated blood vails; after centrifugation for ~5 minutes, the plasma collected and stored at -80°C for further analysis. The cerebella and the striata from the rodent's brain were collected in Eppendorf tubes. The tissue was then snap-frozen in liquid nitrogen and stored at -80°C for further analyses (figure 9).

5.8 Neurochemical analysis

5.8.1 Tumour necrosis factor-alpha ELISA assay

Frozen striatal/cerebella tissue samples were thawed at room temperature. A competitive ELISA kit from Elabscience science (Biocom Africa (Pty) Ltd, South Africa) was used to determine the concentration of TNF-α, as per the manufacturer's guidelines. The tissue samples were minced and washed using iced-cold PBS to remove excess blood. The tissue samples were homogenised in PBS buffer (1 g of tissue: 9 mL of PBS) and further centrifuged at 5000 ×g for 5 minutes. The supernatant was collected, and the standards and other reagents were prepared separately. The standard solution (100 µL) was added to each well in the first two columns of the 96 well plate. The samples (100 µL) were added to the remaining wells. The sealed plate was incubated at 37°C for 90 minutes. The liquid in the plate was decanted, and Biotinylated Detection Ab working solution (100 µL) was added to the wells. The sealed plate was incubated at 37°C for 60 minutes. The solution was decanted, and the plate washed using the wash buffer. The HRP Conjugate working solution was added to each well, the plate sealed and incubated at 37°C for 30 minutes. The plate was then decanted and washed using the wash buffer and plate washer. Substrate Reagent was added into each well; the plate was sealed and incubated for 15 minutes at 37°C. Stop solution (50 µL) was added to each well, and the optical density immediately determined using the SPECTROstar^{NANO} micro-plate reader (BMG LABTECH) set to 450 nm.

5.8.2 Dopamine ELISA assay

Frozen striatal/cerebella tissue samples were left on the benchtop, allowing it to thaw at room temperature. A competitive ELISA kit from Elabscience science (Biocom Africa (Pty) Ltd, South Africa) was used to determine the concentration of dopamine (DA). This assay was executed according to the manufacturer's guidelines. The tissue samples were minced and washed using iced-cold PBS to remove excess blood. The tissue samples were homogenised in PBS buffer (1 g of tissue: 9 mL of PBS) and further centrifuged at 5000 ×g for 5 minutes. The supernatants were collected; standards and other reagents were prepared separately. The standard working solutions (50 µL) were added to the first two strips of the antibody-coated 96 well plate, and the sample homogenates (50 µL) were added to the remaining wells. Immediately after, 50 µL of Biotinylated Detection solution was added to each well. The plate was sealed and incubated for 45 minutes at 37℃. The solution was decanted, and the plate washed using the wash buffer. HRP Conjugate working solution (100 µL) was added to each well. The plate was sealed and incubated for 30 minutes at 37℃. The plate was then decanted and washed using the wash buffer and plate washer. Substrate Reagent was added into each well. The sealed plate was incubated for 15 minutes at 37℃. Stop Solution (50 µL) was added to each well. Stop solution (50 µL) was added to each well, and the optical density immediately determined using the SPECTROstar^{NANO} micro-plate reader (BMG LABTECH) set to 450 nm.

5.8.3 Reverse transcription-polymerase chain reaction

5.8.3.1 RNA extraction

The extraction process was performed in three steps: homogenisation of tissue, clearing, and RNA purification. The RNA extraction was done using the Zymo Research's Quick-RNA Miniprep kit (Inqaba Biotechnical Industries (Pty) Ltd, South Africa), following the manufacturer's guidelines. Frozen striata tissue samples were homogenised in RNA lysis buffer. The homogenates were centrifuged for 1 minute at 10000 xg, and the supernatants were further filtered, and the flow-through was saved. Absolute ethanol (95-100%) was added to the sample (1 mL of lysis buffer: 1 mL of ethanol). The mixture was filtered, and the flow-through was discarded. RNA Prep Buffer (400 µL) was added to the Spin-Away filters in collection tubes, and the tube was centrifuged for half a minute. The filtrate was kept, and the flow-through was discarded. The RNA wash buffer was added to the filter tube and centrifuged for 2 minutes at 10000 xg. The filter tubes were placed in RNase free tubes and 100 µL DNase/RNase-Free water was added into the filter tubes and centrifuged for 30 seconds.

5.8.3.2 cDNA synthesis

Synthesis of cDNA was done using the BioLabs Inc. ProtoScript II First-strand cDNA synthesis kit (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). This procedure was done following the manufacturer's guidelines. The cDNA synthesis reaction components were as follows: extracted RNA

(1 µg), 2 µL of d(T)23 VN, 10 µL of ProtoScript II Reaction Mix, 2 µL of ProtoScript II Enzyme Mix (10X) and nucleases-free water was added to make a final volume of 20 µL in a 0.2 mL tube. The reaction components were briefly mixed, and the tubes were incubated for 60 minutes at 42℃, and the enzyme was inactivated at 80℃ for 5 minutes.

5.8.3.3 Gene expression

The primers used in this experiment were prepared by Inqaba Biotechnical Industries (Pty) Ltd, South Africa. The reference gene used is glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The forward and revise primer sequences that were used to amplify the expression of GAPDH and D2R are in table 1. The cDNA (5 µL) sample was used as a template for PCR amplification using Luna Universal qPCR Master Mix (Inqaba Biotechnical Industries (Pty) Ltd, South Africa) as a fluorophore. The real-time PCR detection system used was the LightCycler® 96 Instrument (Roche Life Science, South Africa). The reaction had a denaturation and polymerase activation procedures for 30 seconds at 95℃ and amplification process comprising of denaturation for 15 seconds at 95℃ and annealing for 60 seconds at 60℃ for 40 cycles. The system read the plate at 60℃, and the LightCycler® 96 software (Roche Life Science, South Africa) was used to analyse the data collected. The ΔΔCq method was used to compare the expression of the reference gene to the expression of D2R.

Table 1: The primers sequences for D2R, and GAPDH that were used in the present study

Primer	Forward primer	Reverse primer
D2R	5'-CAA-CAA-TAC-AGG-CAA-ACC-AGA-ATG- AG- 3'	5'-ACC-AGC-AGA-GTG-ACG-ATG-AAG-G-3'
GAPDH	5'-GCG-AGA-TCC-CGT-CAA-GAT-CA-3'	5'-CCA-CAG-TCT-TCT-GAG-TGG-CAG-3'

5.8.4 *Acetylcholinesterase ELISA assay*

Frozen striata tissue samples were thawed at room temperature. A competitive ELISA kit from Elabscience science (Biocom Africa (Pty) Ltd, South Africa) was used to determine the concentration of AChE, following the manufacturer's guidelines. The tissue was minced and washed using iced-cold PBS to remove excess blood. The tissue samples were homogenised in PBS buffer (1 g of tissue: 9 mL of PBS) and further centrifuged at 5000 ×g for 5 minutes. The supernatant was collected, and the standards and other reagents were prepared separately. The standard solution (100 µL) was added to each well in the first two columns of the 96 well plate. The samples (100 µL) were added to the remaining wells. The sealed plate was incubated at 37℃ for 90 minutes. The liquid in the plate was decanted, and Biotinylated Detection Ab working solution (100 µL) was added to the wells. The sealed plate was incubated at 37℃ for 60 minutes. The solution was decanted, and the plate washed using the

wash buffer. The HRP Conjugate working solution was added to each well, the plate sealed and incubated at 37°C for 30 minutes. The plate was decanted and the plate washed using the wash buffer. The substrate solution (90 µL) was added to each well; the plate was sealed and incubated at 37°C for 15 minutes. Stop solution (50 µL) was added to each well, and the optical density immediately determined using the SPECTROstar^{NANO} micro-plate reader (BMG LABTECH) set to 450 nm.

5.9 Statistical analysis

The statistical software GraphPad Prism version 7 (GraphPad Software Inc, United States) was used to analyse the data. Shapiro-Wilk normality test was used to ascertain the distribution of the data. The two-way analysis of variance followed by the Sidak's multiple comparisons test was used to analyse the behavioural tests' data. One-way analysis of variance plus Tukey's multiple comparisons test was used to examine the neurochemical results. All the data were expressed as standard error of the mean, and a p-value of less than 0.05 was considered significant.

Table 2: Showing the symbols that will be used to describe the p-values obtained in the multiple comparison test

Symbol	p-value
*	$0.01 \geq p < 0.05$
**	$0.001 \geq p < 0.01$
***	$0.0001 \geq p < 0.001$
****	$p < 0.0001$

5.10 Experimental design

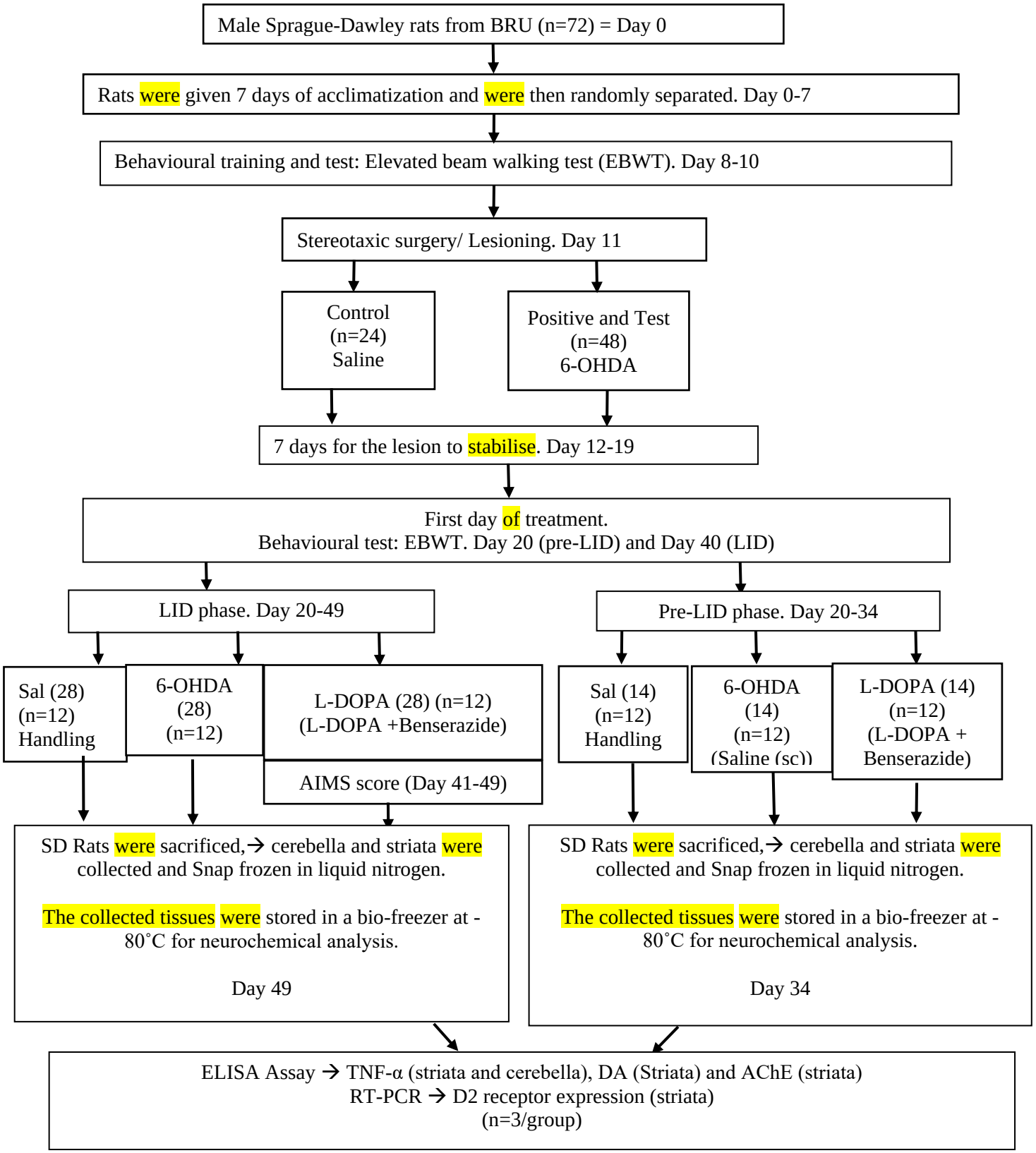


Figure 9: Illustrates the study design and timeline for the induction of parkinsonism in SD rats and the treatment using L-DOPA.

6 Conflicts of Interest

The author declares that there is no conflict of interest regarding this current work.

7 Acknowledgement

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Chapter 3:

Prologue

Manuscript 2

Previous studies showed that reduced DA signalling plays a significant role in the development of both motor and cognitive deficits in PD. The unilateral 6-hydroxydopamine (6-OHDA) lesion of the medial forebrain bundle has been shown to reduce DA signalling in the striatum, hippocampus, amygdala and the PFC. The reduced DA signalling and other molecular dysfunction in these brain areas have been associated with cognitive decline. The treatment with L-DOPA improves DA signalling in the brain and has been shown to alleviate some cognitive deficit. Still, other studies have also reported L-DOPA's detrimental effects on cognition. The present study investigates how chronic L-DOPA treatment affects spatial learning, spatial memory, and recognition memory.

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The present manuscript was prepared according to the College of Health guidelines.

The alteration of prefrontal cortex D1 receptor expression as abnormal involuntary movement develops in a rat model of L-DOPA induced dyskinesias.

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Abstract

L-3,4-dihydroxyphenylalanine (L-DOPA) increases dopamine (DA) signalling and has been shown to ease some of the cognitive difficulties caused by Parkinson's disease (PD). The present study investigates how chronic L-DOPA treatment affects spatial learning, spatial memory, and recognition memory. The Morris water maze test and the novel object recognition test were used to assess cognitive function. The concentration of tumour necrosis factor-alpha (TNF-α), corticosterone, acetylcholinesterase (AChE), and DA were quantified along with dopaminergic D1 receptor (D1R) expression. The results showed that a unilateral 6-OHDA lesion resulted in impaired spatial learning, spatial memory, and object recognition memory. Treatment with L-DOPA did not improve object recognition memory. However, L-DOPA treatment for 14 days improved spatial learning and memory, but spatial memory was impaired when the treatment was prolonged for 28 days. There was a significantly increased concentration of TNF-α in the prefrontal cortex (PFC), plasma corticosterone, hippocampal AChE, and upregulated D1R expression in 6-OHDA lesioned rats. The treatment with L-DOPA significantly reduced the concentration of TNF-α in the prefrontal cortex, plasma corticosterone, hippocampal AChE, and D1R expression in the PFC but increased PFC DA and AChE concentration. There were cognitive deficits induced by the 6-OHDA lesion, but L-DOPA was only able to improve spatial learning and memory. However, prolonged treatment failed to improve 6-OHDA induced spatial memory impairment. This resulted from heightened AChE concentration in both the hippocampus and PFC that persisted since L-DOPA was able to ameliorate imbalances in the HPA axis, neuroinflammation in the PFC, and maintained DA signalling.

Keywords: Parkinson 's disease, dopamine, tumour necrosis factor-alpha, acetylcholinesterase, and 6-hydroxydopamine.

1. Introduction

Parkinson's disease (PD) is a disorder that is characterised by motor symptoms that result from the loss of dopaminergic neurons specifically in the nigrostriatal pathway (German et al., 1989, Gibb and Lees, 1991). PD's hallmark features are motor symptoms, but PD also prevails with non-motor symptoms, including cognitive deficits (Baggio et al., 2014, Zis et al., 2015, Yang et al., 2016). Studies have shown that PD patients present with cognitive deficits even in the early stages of the disease (Dubois and Pillon, 1996, Baggio et al., 2015). The incidences of cognitive deficits in PD increases from 10% to 80% from early to advanced stages of the disease (Williams-Gray et al., 2007, Hely et al., 2008). Cognitive deficits associated with PD include impaired visuospatial learning, spatial recognition memory, attention deficiencies and others (Muslimović et al., 2005, Paolo et al., 2011, Amboni et al., 2015). The development of cognitive deficits in PD patients depend on several factors, including the age of disease onset (Muslimović et al., 2005, Babiloni et al., 2019).

Studies have reported cognitive decline while others showed no deviation in cognitive behaviours (Miyoshi et al., 2002, Braga et al., 2005, Branchi et al., 2008). The unilateral lesion into the medial forebrain bundle (MFB) has been shown to induce memory impairment showing that dopamine (DA) depletion may play a role in the cognitive decline evident in PD (Tadaiesky et al., 2008, Du et al., 2018). The lesion in the MFB is thought to reduce DA signalling in the prefrontal cortex and hippocampus, two areas that play a crucial role in cognition (Du et al., 2018). DA signalling is dependent on DA receptors, especially DA D1 receptor (D1R), which is thought to modulate aspects of cognition (Song et al., 2016, Meltzer et al., 2019). This receptor is highly expressed in the PFC, striatum, and hippocampus of PD patients in response to low DA signalling (Ledonne and Mercuri, 2017).

The excitatory neurotransmitter acetylcholine (ACh) can also stimulate DA release via nicotinic and muscarinic receptor present on DA neurons (Kharkwal et al., 2016). However, the presence of DA D2 receptor on ACh neurons, which stimulated the release of ACh, shows that both neurotransmitters have a feedback mechanism (Kharkwal et al., 2016). ACh may play a vital role in the cognitive deficits in PD since ACh alterations in the hippocampus, and the PFC have been associated with cognitive decline (Tzavara et al., 2003, Croxson et al., 2011). ACh's function is also regulated by acetylcholinesterase (AChE), an enzyme that hydrolysis ACh to acetate and choline (Bourne et al., 2003, Maiti et al., 2017). Reduced DA signalling such as in PD, distorts cholinergic signalling, which would impair cognitive function (Ngoupaye et al., 2018). L-DOPA treatment may improve ACh signalling and, therefore, improve cognitive skills in PD patients (Toyoda, 2018). Another factor that may lead to cognitive deficits in PD is a hyperactive hypothalamic-pituitary-adrenal axis which results in an increased secretion of cortisol in humans and corticosterone in rodents (Hartmann et al., 1997, Ngema and Mabandla, 2017). Glucocorticoids like corticosterone in high concentration have a neurotoxic effect which can play a crucial role in exacerbating the rate of DA neuron degeneration in PD (Kibel and

Drenjančević-Perić, 2008). The elevated glucocorticoid levels promote the release of pro-inflammatory cytokines (Shini et al., 2010). In addition to high glucocorticoids, the death of DA neurons stimulates an immune response, propagated by pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF-α) (Mogi et al., 1994, Jin et al., 2008, Tufekci et al., 2012). The increased secretion of these cytokines is associated with higher oxidative stress, leading to the death of DA neurons (Boka et al., 1994, Tufekci et al., 2012).

Standard treatment for PD patients remains the DA precursor, L-3,4-dihydroxyphenylalanine (L-DOPA) which increases DA signalling and is, therefore, able to ameliorate the motor symptoms characteristic of PD (Gotham et al., 1988, Kulisevsky et al., 1996). Continual use of L-DOPA usually results in abnormal motor fluctuations called L-DOPA induced dyskinesias (LIDs) (Ndlovu et al., 2016). In some studies with L-DOPA as a treatment for PD, there was a significant improvement in the cognitive deficits associated with PD (Celesia and Wanamaker, 1972, Gul and Yousaf, 2018). However, other findings showed that L-DOPA therapy does not improve episodic, spatial learning and spatial memory in PD (Hietanen and Teräväinen, 1988, Gevaerd et al., 2001). The above is possibly due to dopamine influx to cortical areas related to cognitive function (Swainson et al., 2003, Cools, 2006, Ko et al., 2016). L-DOPA therapy's effect on cognitive function is still not apparent because although it can remediate some cognitive deficits, it can also induce non-motor fluctuations with cognitive dysfunction and neuropsychiatric symptoms (Calabresi et al., 2015). The present study investigates how the development of abnormal involuntary movements (AIMs) affects spatial learning, spatial memory, and episodic memory during L-DOPA treatment. Understanding the alteration of D1 and AChE in the different PD stages could provide the knowledge required to develop preventative or more effective treatments as PD progresses.

2. Materials and methods

2.1 Animal Care and Housing

All the experimental procedures in the present study were approved by the University of KwaZulu-Natal's Animal Research Ethics Committee (AREC/077/018M). Male Sprague Dawley rats weighing 220 -250 g were obtained from Biomedical Resource Unit of the University of KwaZulu-Natal. The 72 rats were housed in clear polycarbonate cages in the holding area during the study. The holding area was kept under standard laboratory conditions: 22 +/-1^{o}C room temperature, 50 - 70% humidity, and a 12h light/dark cycle with lights on at 06h00. The rats were given seven days to acclimatise to the holding area's conditions with standard rat feed and water given liberally.

2.2 Stereotaxic surgery

The combination of Ketamine (125 mg/kg) and xylazine (10 mg/kg) was used as anaesthesia (Veilleux-Lemieux et al., 2013). Ketamine keeps the rat unconscious, and xylazine increased the heart rate to prevent low blood pressure during surgery (Bennett and Bullimore, 1973, Veilleux-Lemieux et al., 2013). The Hamilton syringe was loaded with 4 µL of the 6-hydroxydopamine (6-OHDA) solution (8 µg/4 µL dissolved in 0.2% ascorbic acid; Sigma, Sigma-Aldrich, United States) or saline and mounted on the stereotaxic frame (David Kopf Instruments, Tujunga, USA). The anaesthetic was administered via intraperitoneal injection before surgery. The unconscious rats were placed into the stereotaxic frame, and a small hole was drilled at the following coordinates from the lambda 4.7 mm anterior and -1.6 mm from the midline (Paxinos and Watson, 1986). The Hamilton syringe was then lowered 8.4 mm below the skull, and the 6-OHDA solution or 0.9% saline was released at a rate of 1 µL per minute. The syringe was left in place for 6 minutes to allow the 6-OHDA to diffuse into the surrounding tissue after which the needle was slowly retracted (Mabandla et al., 2015). The rats were given 7 days to recover from the surgery.

2.3 Animal grouping

The rats were divided into 2 timelines, the pre-LID and the LID timeline. In the first timeline, the rats were sacrificed after 14 days of handling, subcutaneous saline injections, or L-DOPA treatment. The negative control (Sal (14)) consists of rats that were intracranially injected with 0.9% saline and handled for 14 days. The positive control group (6-OHDA (14)) consists of rats lesioned with 6-OHDA solution and were subcutaneously injected with 0.9% saline for 14 days. The test group (L-DOPA (14) comprised of rodents that were lesioned with 6-OHDA and later treated with L-DOPA. The LID timeline consisted of rats sacrificed after 28 days of handling, subcutaneous saline injections, or L-DOPA treatment. The negative control (Sal (28)) consists of rats that were intracranially injected with 0.9% saline and handled for 28 days. The positive control group (6-OHDA (28)) consists of rats lesioned with 6-OHDA solution and were subcutaneously injected with 0.9% saline for 28 days. The test group (L-DOPA (28) comprised of rats that were lesioned with 6-OHDA solution and treated with L-DOPA for 28 days.

2.4 L-DOPA treatment and administration schedule

A combination of L-DOPA (50 mg/kg s.c; Sigma, Sigma-Aldrich, United States) combined with the peripheral DOPA–decarboxylase inhibitor, benserazide (10 mg/kg s.c; Sigma, Sigma-Aldrich, United States), was dissolved in 0.9% saline (Huang et al., 2011). The drug was administered seven days after surgery. This combination was administered two times a day (9 am and 4 pm) for 14 days or 28 days.

2.5 Behavioural tests

2.5.1 Novel object recognition test

The novel object recognition test (NORT) was conducted to assess a rodent's recognition memory, determined by the rodent's exploratory behaviour. The test is based on the rodent's tendency to study a novel object more than an object it has encountered. This test comprises of 3 stages: habituation, familiarisation, and the final test (Batool et al., 2016). The rats were given 3 days (figure 1) to get habituated to being in the square wooden box (100×100×50 cm). On the 4th day (figure 1), the two identical objects were placed in the wooden box so that the rats could study the objects for 5 minutes. The test stage commenced an hour after the familiarisation stage. In the final stage, one of the familiar objects was replaced by the novel object and the animals were given 5 minutes to explore the objects. The exploratory behaviour was recorded, i.e., the time the animals spent exploring the familiar object (F) and the time the rats spent exploring novel objects (N) (Ngoupaye et al., 2018). Intact recognition memory is evidenced by subjects spending more time investigating the novel object. A discrimination index (DI) defined as (N - F)/(N + F) was calculated for each animal (Ngoupaye et al., 2017).

2.5.2 Morris water maze test

Morris water maze test (MWMT) was used to assess spatial learning and memory. The MWMT apparatus is an open circular 1 m diameter pool with a black interior that is half-filled with water with a small transparent escape platform hidden inside. Two lines perpendicular to each other, divide the maze into four quadrants and visual cues will be placed inside each of the quadrants. The MWMT comprises two stages: the learning stage, which lasted for 3 days and the probe stage in 1 day (figure 1). During the learning stage of the MWMT, the rats were trained to find a submerged platform that was placed in one of the quadrants (target quadrant) (D'Hooge and De Deyn, 2001). The rats were trained to find the hidden platform using distal visual cues on the maze wall. The time taken by the rats to find the hidden platform was recorded. The time it took the rats to find the platform was used as an indication of spatial learning. The probe stage is a post-test for learning that measures an animal's ability to remember the target quadrant. The time the rodent spent in the target quadrant is deemed the measure of the rats' ability to remember or spatial memory (Cassim et al., 2015).

2.6 Euthanasia

Twelve rats per group were sacrificed on the 14th and 28th day of treatment or handling. The rats were decapitated using a guillotine and trunk blood, and brain tissue was collected. The blood was collected in EDTA coated tubes and centrifuged for 5 minutes at 5000 ×g. The isolated plasma was transferred to Eppendorf tubes and stored at -80°C for further analysis. The brain was dissected, and the prefrontal cortex (PFC) and hippocampus were collected and stored at -80°C for further analysis.

2.7 Neurochemical analysis

2.7.1 Tumour necrosis factor-alpha ELISA kit

A sandwich ELISA kit from Elabscience science (Biocom Africa (Pty) Ltd, South Africa) was used to determine the concentration of Tumour necrosis factor-alpha (TNF-α) in PFC samples. This process was done according to the manufacturer's guideline. Frozen rat PFC tissue was taken out of the bio-freezer and placed on ice, allowing it to defrost before use. The defrosted tissues were washed in PBS buffer to remove excess blood. Homogenisation of the tissue samples occurred in PBS buffer (1 g of tissue: 9 mL of PBS) after which the homogenate was centrifuged at 5000 ×g for 5 minutes. The standard and other reagents from the ELISA kit were prepared as per the manufacturer's guidelines. The supernatant was collected from the centrifuged homogenate and placed in Eppendorf tubes. A sealed 96 well plate was provided in the kit, and a 100 µL of the standard solutions were added to the wells in the first two columns with the blanks in the bottom two wells. A 100 µL of the supernatant samples were added to the remaining wells, and the plate was sealed and incubated at 37℃ for 90 minutes. The solution in the plate was decanted, then a 100 µL Biotinylated Detection Ab working solution was added to each well. The plate was incubated at 37℃ for 60 minutes. The wells' solution was decanted, and the plate washed three times using the provided wash buffer. Avidin-Horseradish Peroxidase conjugate working solution (100 µL) was added to each well; the plate was sealed and incubated at 37℃ for 30 minutes. The solution in the plate was decanted and washed five times. The substrate solution (90 µL) was added to each well, and the plate was sealed and incubated at 37℃ for 15 minutes. The stop solution (50 µL) was added to each well, and the optical density of the solution in each well was determined using the SPECTROstar^{NANO} micro-plate reader (BMG LABTECH) set to 450 nm.

2.7.2 Corticosterone ELISA kit

A competitive ELISA kit from Elabscience science (Biocom Africa (Pty) Ltd, South Africa) was used to determine plasma corticosterone concentration. This process was executed according to the manufacturers' guideline. The collected plasma was centrifuged 15 minutes at 1000 ×g at 4℃. The plasma, standards, and other reagents were prepared according to the manufacturers' guidelines. A sealed 96 well plate was provided in the kit, and 50 µL of the standard solutions were added to the wells in the first two columns with the blanks in the bottom two wells. The plasma samples' supernatants were added to the remaining wells, then Biotinylated Detection Ab working solution (50 µL) was added to all the wells. The plate was sealed and incubated for 45 minutes at 37℃. The solution was decanted from the plate; then it was washed three times using the ELISA kit's wash buffer. Avidin-Horseradish Peroxidase conjugate working solution (100 µL) was added to each well; the plate was sealed and incubated at 37℃ for 30 minutes. The solution was decanted, and the plate was washed five times. The substrate solution (90 µL) was added to each well; the plate was sealed and incubated at 37℃ for 15 minutes. The stop solution (50 µL) was added to each well, and the optical density of the solution in

each well was determined using the SPECTROstar^{NANO} micro-plate reader (BMG LABTECH) set to 450 nm.

2.7.3 A*cetylcholinesterase ELISA kit*

A sandwich ELISA kit from Elabscience science (Biocom Africa (Pty) Ltd, South Africa) was used to determine the concentration of acetylcholinesterase (AChE) in the PFC or hippocampal tissue samples. This process was done according to the manufacturer's guideline. Frozen rat PFC or hippocampal tissue samples were taken out of the bio-freezer and placed on ice, allowing it to defrost before use. The defrosted tissues were washed in PBS buffer to remove excess blood. Homogenisation of the tissue samples occurred in PBS buffer (1 g of tissue: 9 mL of PBS) after which the homogenate was centrifuged at 5000 ×g for 5 minutes. The standard and other reagents from the ELISA kit were prepared as per the manufacturer's guidelines. The supernatant was collected from the centrifuged homogenate and placed in Eppendorf tubes. A sealed 96 well plate was provided in the kit, and a 100 µL of the standard solution was added to the wells in the first two columns with the blank in the bottom two wells. A 100 µL of the supernatant samples were added to the remaining wells, and the plate was sealed and incubated at 37℃ for 90 minutes. The solution in the plate was decanted, then a 100 µL biotinylated Detection Ab working solution was added to each well. The plate was incubated at 37℃ for 60 minutes. The wells' solution was decanted, and the plate washed three times using the provided wash buffer. The Avidin-Horseradish Peroxidase conjugate working solution was added to each well; the plate was sealed and incubated at 37℃ for 30 minutes. The solution in the plate was decanted and washed five times. The substrate solution (90 µL) was added to each well; the plate was sealed and incubated at 37℃ for 15 minutes. The stop solution (50 µL) was added to each well, and the optical density of the solution in each well was determined using the SPECTROstar^{NANO} micro-plate reader (BMG LABTECH) set to 450 nm.

2.7.4 *Dopamine ELISA kit*

A competitive ELISA kit from Elabscience science (Biocom Africa (Pty) Ltd, South Africa) was used to determine the concentration of dopamine (DA) in the PFC tissue samples. This process was done according to the manufacturer's guidelines. Frozen rat PFC tissue samples were taken out of the bio-freezer and placed on ice, allowing it to defrost before use. The defrosted tissues were washed in PBS buffer to remove excess blood. Homogenisation of the tissue samples occurred in PBS buffer (1 g of tissue: 9 mL of PBS) after which the homogenate was centrifuged at 5000 ×g for 5 minutes. A sealed 96 well plate was provided in the kit, and 50 µL of the standard solutions were added to the wells in the first two columns with the blanks in the bottom two wells. The samples' supernatants were added to the remaining wells, then Biotinylated Detection Ab working solution (50 µL)was added to all the wells on the plate. The plate was sealed and incubated for 45 minutes at 37℃. The solution was decanted

from the plate. Then it was washed three times using the wash buffer provided in the ELISA kit. Avidin-Horseradish Peroxidase conjugate working solution (100 µL) was added to each well; the plate was sealed and incubated at 37℃ for 30 minutes. The solution was decanted, and the plate was washed five times. The substrate solution (90 µL) was added to each well; the plate was sealed and incubated at 37℃ for 15 minutes. The stop solution (50 µL) was added to each well, and the optical density of the solution in each well was determined using the SPECTROstar^{NANO} micro-plate reader (BMG LABTECH) set to 450 nm.

2.7.5 Reverse transcription-polymerase chain reaction

This process is divided into three main steps ribose nucleic acid (RNA) extraction, cDNA synthesis, and polymerase chain reaction (PCR). The RNA extraction was done using the Zymo Research's Quick-RNA Miniprep kit (Inqaba Biotechnical Industries (Pty) Ltd, South Africa) from crushed PFC tissue samples. The extracted RNA (1 µg) was used as a template to make complementary dihydroxy ribose nucleic acid (cDNA) using the BioLabs Inc. ProtoScript II First-strand cDNA synthesis kit (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). Dopamine type 1 receptor (D1R) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes were amplified using the Luna Universal qPCR Master Mix (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). The forward and revise primer sequences that were used for the amplification of GAPDH and D1R are in table 1, below. The primers used in this experiment were prepared by Inqaba Biotechnical Industries (Pty) Ltd, South Africa. The real-time PCR system used was the LightCycler® 96 Instrument (Roche Life Science, South Africa). The reaction had a denaturation and polymerase activation procedures for 30 seconds at 95℃ and amplification process comprising of denaturation for 15 seconds at 95℃ and annealing for 60 seconds at 60℃ for 40 cycles. The system read the plate at 60℃, and the LightCycler® 96 software (Roche Life Science, South Africa) was used to analyse the data collected. The ΔΔCq method was used to compare the expression of the reference gene to the expression of D1R.

Table 1: The primers sequence for D1R and GAPDH that were used in the present study (de Souza et al., 2018)

Primer	Forward primer	Reverse primer
D1R	5’-CTG-GAG-GAC-ACC-GAG-GAT-GAC-3’	5’-GTC-GAT-GAG-GGA-CGA-TGA-AAT -GG -3’
GAPDH	5’-GCG-AGA-TCC-CGT-CAA-GAT-CA-3’	5’-CCA-CAG-TCT-TCT-GAG-TGG-CAG-3’

2.8 Statistical Analysis

The collected data were analysed using GraphPad Prism version 7 software (GraphPad Software Inc, United States). The Shapiro-Wilk normality test was used to ascertain data distribution. One-way

analysis of variance was used to analyse the data followed by Tukey's multiple comparisons test to determine the differences between individual groups. A $p< 0.05$ is considered statistically significant. The data were expressed as mean± SEM.

Table 2: Shows the symbols that will be displayed in the graphs to describe the p-values obtained in the multiple comparison test

Symbol	p-value
*	$0.01 \geq p < 0.05$
**	$0.001 \geq p < 0.01$
***	$0.0001 \geq p < 0.001$
****	$p < 0.0001$

2.9 Experimental design

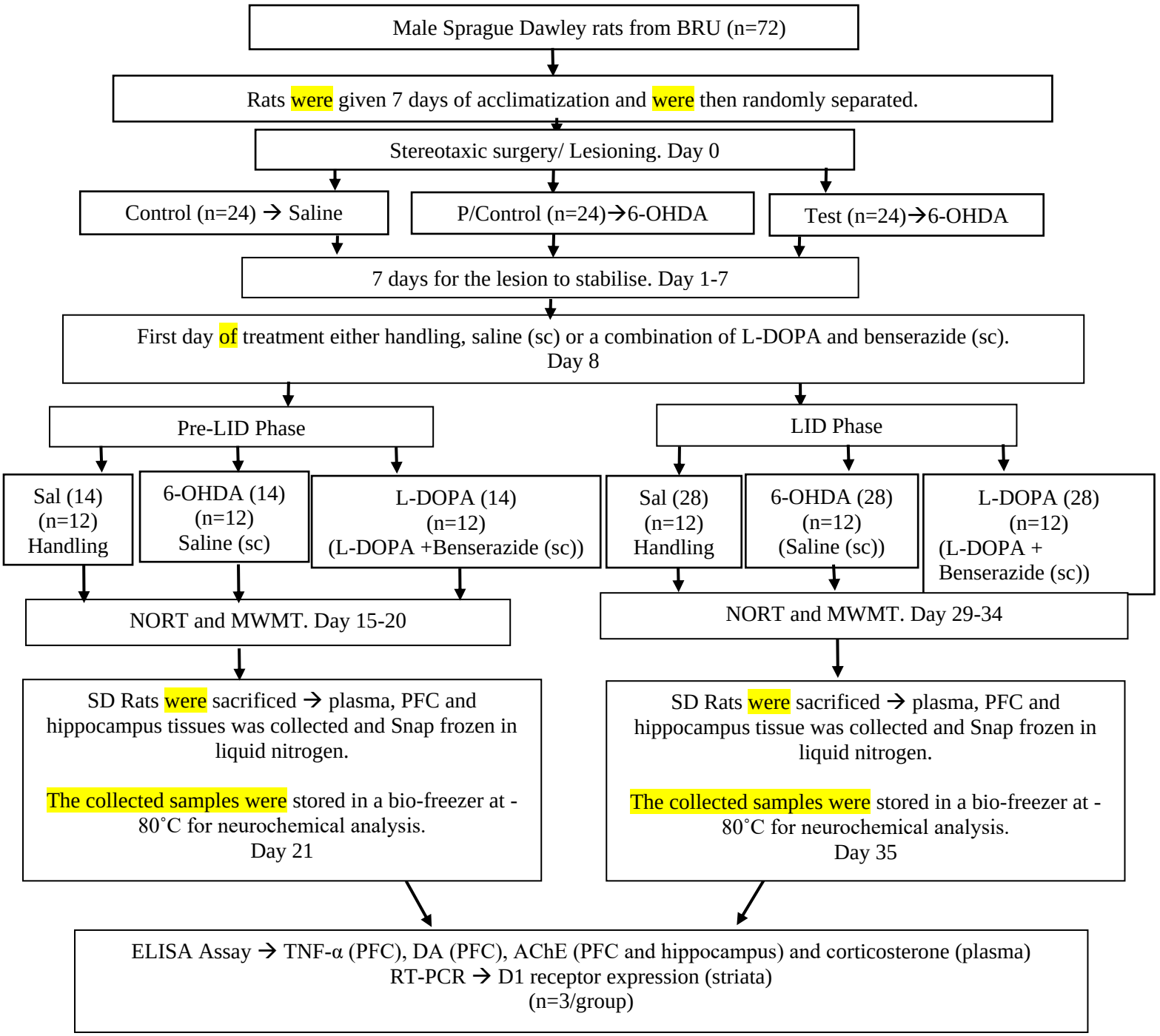

Figure 1: The study design and the timeline for the treatment of L-DOPA.

3. Results

3.1 Novel object recognition test

The recognition memory of the SD rats was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 2. The discrimination index was significantly reduced in the groups lesioned with 6-OHDA (Sal (14) vs 6-OHDA (14); F5, 60 = 15.38, p = 0,0012, figure 2), (Sal (14) vs L-DOPA (14); F5, 60 = 15.38, p = 0,0001, figure 2), (Sal (28) vs 6-OHDA (28); F5, 60 = 15.38, p = 0,0001, figure 2), and (Sal (28) vs L-DOPA (28); F5, 60 = 15.38, p = 0,0011, figure 2). There was a L-DOPA effect present when comparing the untreated and treated 6-OHDA lesioned groups after 14 days of treatment (6-OHDA (14) vs L-DOPA (14); F5, 12 = 7.128, p= 0.0033, figure 2).

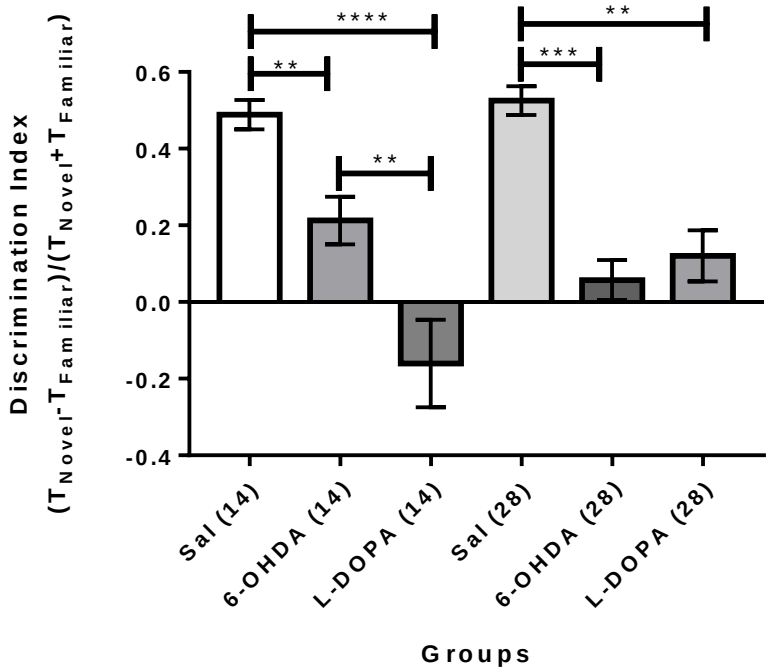

Figure 2: Graph showing the discriminatory index as assessed using the NORT (n=11 per group).

3.2 Morris water maze test

3.2.1 Spatial learning

Spatial learning was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 3. There was a 6-OHDA effect in finding the hidden platform (Sal (14) vs 6-OHDA (14); F5, 60 = 75.42, p<0.0001, figure 3) and (Sal (28) vs 6-OHDA (28); F5, 60

= 75.42, p<0.0001, figure 3). L-DOPA treatment attenuated the 6-OHDA effect (6-OHDA (14) vs L-DOPA (14); F5, 60 = 75.42, p<0.0001, figure 3) and (6-OHDA (28) vs L-DOPA (28); F5, 60 = 75.42, p<0.0001, figure 3).

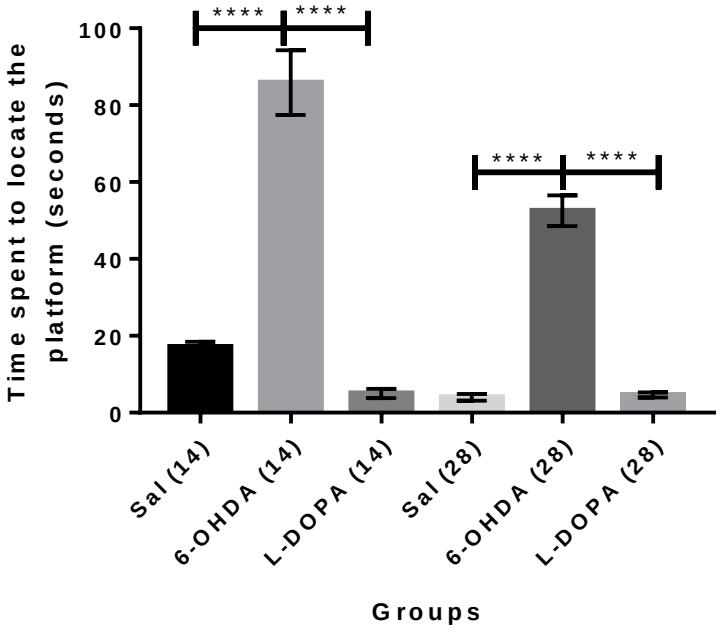

Figure 3: Shows the time taken for the rats to find the submerged platform during the third day of training in the MWMT (n=11 per group).

3.2.2 Spatial memory

Spatial memory of the SD rats was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 4. A 6-OHDA effect was observed in the time spent in the target quadrant (Sal (14) vs 6-OHDA (14); F5, 60 = 48.84, P<0.0001, figure 4) and (Sal (28) vs 6-OHDA (28); F5, 60 = 48.84, P<0.0001, figure 4). This effect was ameliorated in the group treated with L-DOPA for 14 days (6-OHDA (14) vs L-DOPA (14); F5, 60 = 48.84, P<0.0001, figure 4). However, L-DOPA treatment did not lessen the effects of 6-OHDA following 28 days of treatment. (Sal (28) vs L-DOPA (28); F5, 60 = 48.84, p<0.0001, figure 4). Prolonged L-DOPA treatment had a detrimental effect on spatial memory (L-DOPA (14) vs L-DOPA (28); F5, 60 = 48.84, p<0.0001, figure 4).

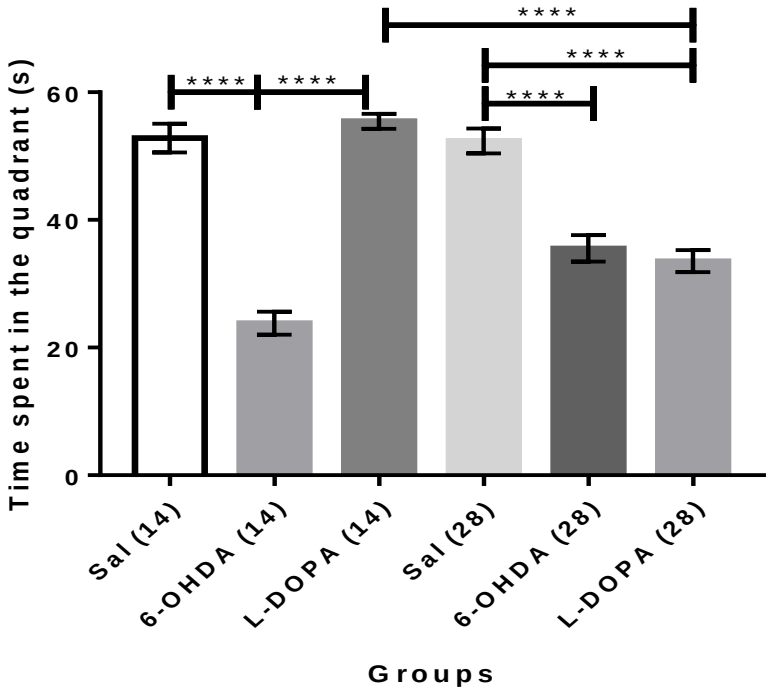

Figure 4: Shows the amount of time spent by the animals in the target quadrant during the test day of the MWMT (n=11 per group).

3.4 Prefrontal cortex tumour necrosis factor-alpha concentration

TNF-α concentration was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 5. There was a 6-OHDA effect on TNF-α concentration (Sal (14) vs 6-OHDA (14); F5, 12 = 8.074, *p*=0,0472, figure 5) and (Sal (28) vs 6-OHDA (28); F5, 12 = 8.074, *p*=0,0274, figure 5). The 6-OHDA effect was attenuated following 28 days of L-DOPA treatment (6-OHDA (28) vs L-DOPA (28); F5, 12 = 8.074, p= 0.0302, figure 5).

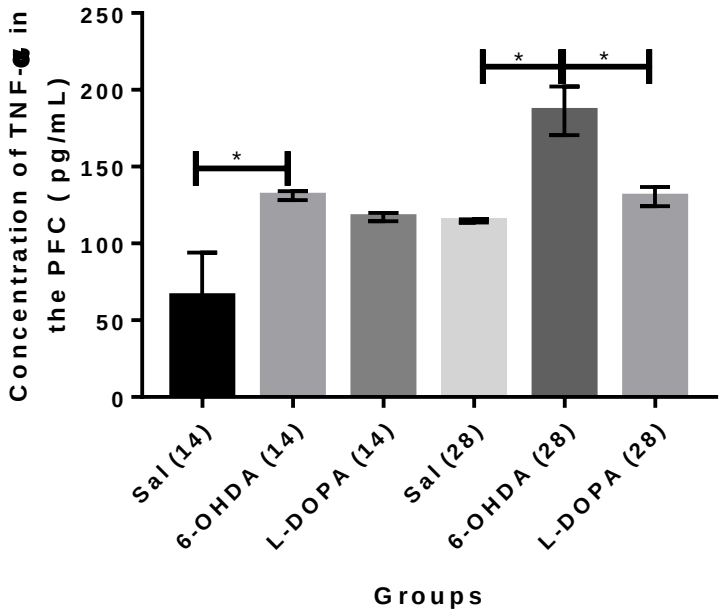

Figure 5: Graph showing PFC TNF-α concentration in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

3.5 Plasma corticosterone concentration

Plasma corticosterone concentration was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 6. The 6-OHDA lesion significantly increased plasma corticosterone concentration (Sal (14) vs 6-OHDA (14); $F_{5, 12} = 45.5$, p= 0,0005, figure 6) and (Sal (28) vs 6-OHDA (28); $F_{5, 12} = 45.5$, $P<0.0001$, figure 6), which was significantly reduced in L-DOPA treated animals (6-OHDA (14) vs L-DOPA (14); $F_{5, 12} = 45.5$, p= 0,0458, figure 6) and (6-OHDA (28) vs L-DOPA (28); $F_{5, 12} = 45.5$, $p < 0.0001$, figure 6). Plasma corticosterone concentration progressively increased in the 6-OHDA lesioned groups (6-OHDA (14) vs 6-OHDA (28); $F_{5, 12} = 45.5$, $p = 0.0002$, figure 6).

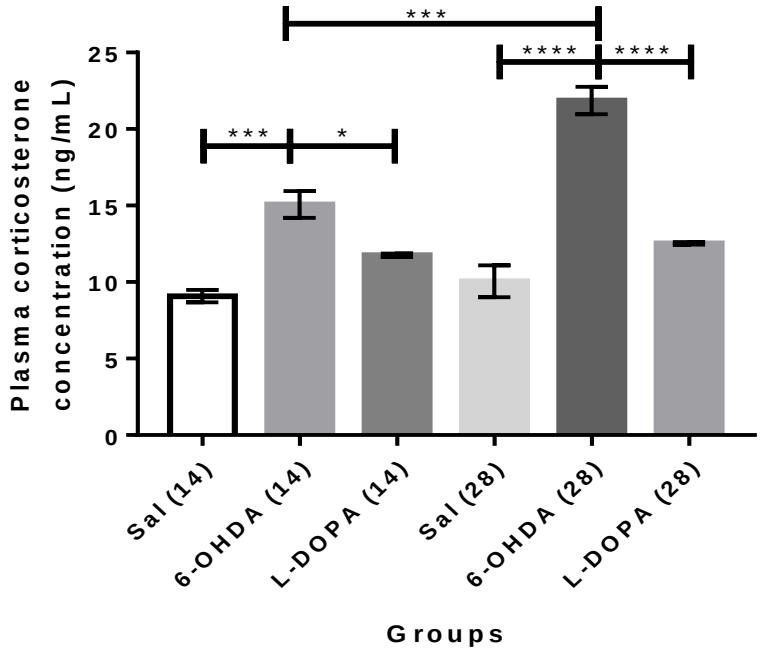

Figure 6: Graph showing plasma corticosterone concentration in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

3.6 Prefrontal cortex acetylcholinesterase concentration

AChE concentration was assessed in the ipsilateral PFC of the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 7. There was an L-DOPA effect present on AChE concentration (Sal (14) vs L-DOPA (14); $F_{5, 12} = 7.128$, $p = 0.0079$, figure 7) and (Sal (28) vs L-DOPA (28); $F_{5, 12} = 7.128$, $p = 0.0296$, figure 7). The L-DOPA effect was also present when comparing the untreated and treated 6-OHDA lesioned groups after 14 days of treatment (6-OHDA (14) vs L-DOPA (14); $F_{5, 12} = 7.128$, $p = 0.0384$, figure 7).

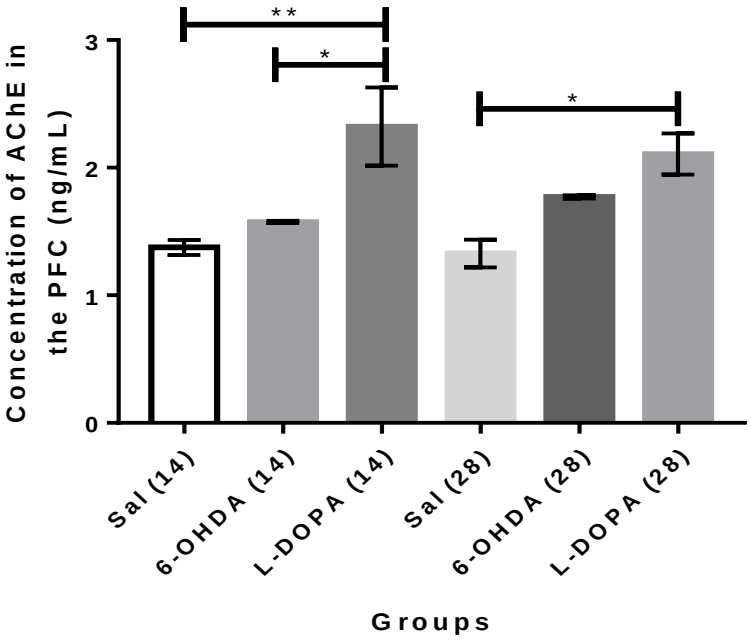

Figure 7: Graph showing PFC AChE concentration in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

3.7 Hippocampal acetylcholinesterase concentration

AChE concentration was assessed in the ipsilateral hippocampus of the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 8. There was a 6-OHDA effect on AChE concentration (Sal (14) vs 6-OHDA (14); F5, 12 = 42.99, p= 0,0229, figure 8) and (Sal (28) vs 6-OHDA (28); F5, 12 = 42.99, p= 0,0298, figure 8). This effect persisted after L-DOPA treatment for 14 days (Sal (14) vs L-DOPA (14); F5, 12 = 42.99, p= 0,0039, figure 8) and 28 days (Sal (28) vs L-DOPA (28); F5, 12 = 42.99, p< 0,0192, figure 8). However, there was a L-DOPA effect present when comparing the untreated and treated 6-OHDA lesioned groups after 14 days of treatment (6-OHDA (14) vs L-DOPA (14); F5, 12 = 42.99, p= 0.0496, figure 8).

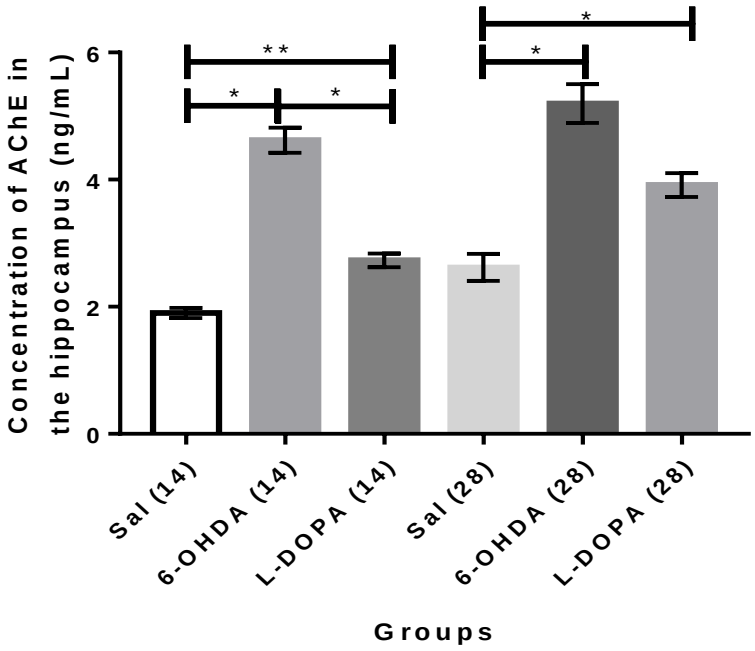

Figure 8: Graph showing hippocampal AChE concentration in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

3.8 Prefrontal cortex dopamine concentration

DA concentration was assessed in the ipsilateral PFC of the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 9. There was a 6-OHDA effect on DA concentration (Sal (14) vs 6-OHDA (14); F5, 12 = 163.2, p<0,0001, figure 9) and (Sal (28) vs 6-OHDA; F5, 12 = 163.2, P<0.0001, figure 9). This effect persisted after treatment with L-DOPA (Sal (14) vs L-DOPA (14); F5, 12 = 163.2, p<0,0001, figure 9) and (Sal (28) vs L-DOPA (28); F5, 12 = 163.2, P<0.0001, figure 9). However, L-DOPA was able to attenuate the effect of 6-OHDA on DA concentration (6-OHDA (14) vs L-DOPA (14); F5, 12 = 163.2, p= 0,0230, figure 9) and (6-OHDA (28) vs L-DOPA (28); F5, 12 = 163.2, p= 0,0225, figure 9).

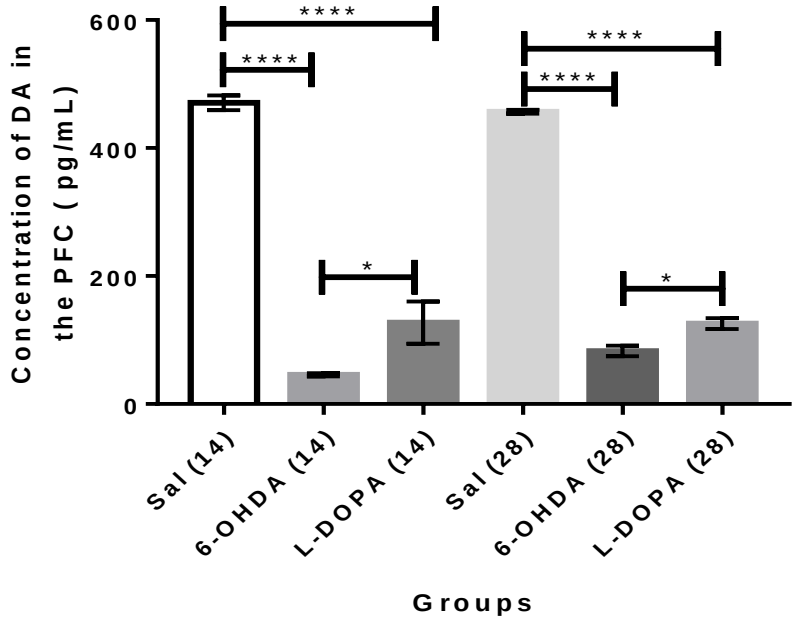

Figure 9: Graph showing PFC DA concentration in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

3.9 Prefrontal cortex D1R expression

The expression of D1R was assessed in the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28), figure 10. There was a 6-OHDA effect on D1R expression (Sal (14) vs 6-OHDA (14); $F_{5, 12} = 41$, $p<0.0001$, figure 10) and
(Sal (28) vs 6-OHDA (28); $F_{5, 12} = 41$, $p<0.0001$, figure 10). This effect was attenuated by 14 days (6-OHDA (28) vs L-DOPA (14); $F_{5, 12} = 41$, $p<0.0001$, figure 10) and 28 days (6-OHDA (28) vs L-DOPA (28); $F_{5, 12} = 41$, $p<0.0001$, figure 10) of L-DOPA treatment.

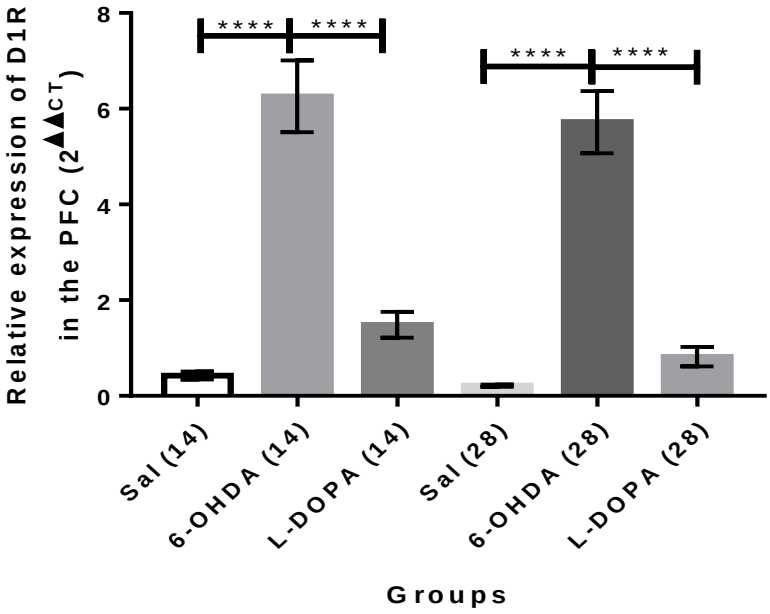

Figure 10: Graph showing the change in D1R expression relative to GAPDH expression in the PFC of the following groups: Sal (14), 6-OHDA (14), L-DOPA (14), Sal (28), 6-OHDA (28), and L-DOPA (28) (n=3 per group).

4. Discussion

The present study investigated L-3,4-dihydroxyphenylalanine (L-DOPA) treatment's effects on the learning and memory of rodents with a unilateral 6-hydroxydopamine (6-OHDA) lesion in the medial forebrain bundle (MFB). The present study also examined how the development of abnormal involuntary movements (AIMs) affect the hypothalamic-pituitary-adrenal axis (HPA axis), inflammation, cholinergic signalling, and dopamine (DA) signalling in unilateral 6-OHDA lesioned rats. The present study found that 6-OHDA lesion resulted in impaired spatial learning, spatial memory, and object recognition memory. The treatment with L-DOPA did not improve object recognition memory. However, L-DOPA treatment for 14 days improved spatial learning and memory, but spatial memory was impaired when the treatment was prolonged for 28 days. These behavioural changes observed result from increased pro-inflammatory cytokine secretion in the prefrontal cortex (PFC), increased plasma corticosterone concentration, reduced DA signalling in the PFC, increased cholinergic signalling and increased D1 receptor (D1R) expression in the 6-OHDA lesioned rats. The treatment with L-DOPA reduced the concentration of tumour necrosis factor-alpha (TNF)-α in the PFC, plasma corticosterone concentration, and D1R expression; however, increased PFC DA and acetylcholinesterase (AChE) concentration. Cholinergic signalling has been associated with learning and memory function.

The novel object recognition test (NORT) is a test that uses a rodent's spontaneous preference to explore novel objects relative to familiar objects to assess rodent's objection recognition memory (Darcet et al., 2014, Grayson et al., 2015, Nezhadi et al., 2016). Injecting 6-OHDA into the MFB induces impairment in learning and memory, suggesting that DA signalling is significant in cognitive function (Liu et al., 2016, Du et al., 2018). The rats lesioned with 6-OHDA showed a reduced discrimination index, which indicates impaired object recognition memory. The above findings are similar to Bonito-Oliva et al. (2014)'s findings, which showed that dopamine degeneration in 6-OHDA lesioned mice resulted in impaired recognition memory. The above is because the interaction between the PFC and nigrostriatal DA projections is essential for recognition memory (Chao et al., 2013). L-DOPA exposure worsened and did not progressively improve recognition memory because of impaired DA signalling in the PFC. These findings are in line with finding from treating MitoPark mice (mice genetically engineered to have DA degeneration in the midbrain) with L-DOPA, resulted in recognition memory deficits assessed using the NORT (Li et al., 2013). Human studies showed that high L-DOPA doses worsened some cognitive behaviours due to PFC DA fluctuations (Gotham et al., 1988, Cooper et al., 1992, Kulisevsky et al., 1996).

The present study further used the Morris water maze test (MWMT) to assess spatial learning and memory. The rats lesioned with 6-OHDA took longer to find the platform on the last day of the MWMT learning phase, and they spent less time in the target quadrant on test day. The above is evidence of

spatial learning and memory deficits in the animals lesioned with 6-OHDA. These results are in line with findings that showed that reduced DA signalling in hemiparkinsonian rodent model leads to impaired spatial learning and memory (Nezhadi et al., 2016). Furthermore, the present study indicated that L-DOPA improved spatial learning, although the occurrence of AIMs worsens spatial memory. The findings align with reports in human and animal studies showing increased DA signalling due to L-DOPA therapy improves spatial learning and memory (Ambrée et al., 2009, Wang et al., 2017). Studies have also shown that spatial learning and memory were impaired in aged rats treated with L-DOPA (Shohamy et al., 2006, Hernández et al., 2014). Heightened inflammatory cytokines like TNF-α have been associated with cognitive deficits that prevail in Parkinson's disease (PD), dementia, and Alzheimer's disease (Menza et al., 2010).

Chronic inflammation is a characteristic of PD, and it has been demonstrated in animal models and human studies using prominent pro-inflammatory molecules like TNF-α (Menza et al., 2010). In the present study, the PFC of rodents lesioned with 6-OHDA had a high concentration of TNF-α, which is an indicator of hyper-inflammation. Studies have shown that high concentrations of pro-inflammatory cytokines are released by activated glial cells in response to the injection of the neurotoxin 6-OHDA (Lindqvist et al., 2013). The consequences of increased pro-inflammatory cytokines have been associated with the progressive death of DA neurons through oxidative stress and increased glutamate activity (Liao and Chen, 2001). The combination of heightened glutamate and oxidative stress exaggerates neuronal death (Liao and Chen, 2001). The pro-inflammatory environment was not restricted to the nigrostriatal pathway, yet it spread to other areas, including the PFC. These findings are supported by literature showing that oxidative stress induced by 6-OHDA injection in the striatum can be propagated to the PFC and the hippocampus (de Araújo et al., 2013). The heightened inflammatory response in the PFC was attenuated by L-DOPA treatment, which showed a reduced TNF-α PFC concentration. This is in line with findings that showed that treating rodents lesioned with 6-OHDA in the striatum with L-DOPA reduced the pro-inflammatory response (de Araújo et al., 2013).

One other effector for inflammation is the HPA axis's stimulation to release glucocorticoids in response to cell injury, stress or pathogens (Herrero et al., 2015, McHugh Power, 2016). The upregulated inflammatory response in the rodents lesioned with 6-OHDA lead to a hyperactive HPA axis which is evident by heightened plasma corticosterone concentration whose toxic effects have been shown to result in spatial learning, spatial memory, and episodic memory impairment (Miyoshi et al., 2002, Barnum et al., 2008, Wang et al., 2010). These results are in line with findings that showed that 6-OHDA lesion in a rodent model results in a higher concentration of corticosterone in plasma (Hartmann et al., 1997, Ngema and Mabandla, 2017). L-DOPA was able to reduce the plasma corticosterone level caused by 6-OHDA lesion. Findings showed that increased DA signalling can result in DA mediated inhibition of adrenocorticotropic hormone release from the hypothalamus, which would result in

reduced corticosterone hormone secretion (Ulrich, 1973, Müller et al., 2007). The above contributed to improved spatial learning and memory caused by treating the 6-OHDA lesioned rodents with L-DOPA; however, episodic memory was still impaired, showing that there is another pathway involved in cognition affected.

Parts of the limbic system involved in cognitive function include the hippocampus and the PFC (Toyoda, 2018). DA and acetylcholine (ACh) signalling have an inverse relationship and a reduced DA signalling that occurs in the prefrontal cortex of PD patients and rodent models would result in heightened ACh signalling (Maiti et al., 2017). ACh is a neurotransmitter that plays a critical role in learning and memory formation (Ngoupaye et al., 2018). They are two parameters that regulate ACh signalling: ACh receptors and AChE (Kawaguchi et al., 1995, Zhou et al., 2002). In this current study, rodents treated L-DOPA had augmented AChE concentration in the hippocampus and PFC which is necessary to reduce ACh neurotransmission by breaking down ACh to acetate and choline (Zaretsky et al., 2016). Increased AChE is associated with impaired object recognition memory impairment because of increased ACh breakdown (Ngoupaye et al., 2018). L-DOPA treatment reduced the heightened AChE concentrations caused by the 6-OHDA injection into the MFB. However, this treatment was not enough to return the AChE concentration to normal, and as the treatment continued to the LID phase, there is a progressive increase of AChE content in the PFC. This heightened AChE concentration in the PFC may be the reason for episodic memory deficits in the L-DOPA (14) and L-DOPA (28).

The dentate nucleus of the hippocampus also received DA from the substantia nigra (via the mesolimbic pathway) and ventral tegmental area (VTA) (Nezhadi et al., 2016). Impairing DA neurotransmission in the substantia nigra will affect DA signalling, affecting ACh signalling in the hippocampus (Branchi et al., 2008). In the present study, rodents lesioned with 6-OHDA had a significantly higher AChE concentration in the hippocampus an indicator of hyperactive cholinergic signalling resulting from reduced DA signalling due to the 6-OHDA lesion (Soreq, 2015). Increased AChE activity in the hippocampus is associated with impaired spatial learning, spatial memory, and episodic memory impairment because of increased ACh signalling termination (Ngoupaye et al., 2018). L-DOPA treatment increased AChE concentration in the hippocampus leading to decreased ACh signalling (Sriraksa et al., 2012). It seems L-DOPA alleviates some cognitive deficits but impairs those that depend on an intact basal ganglion due to abnormal DA signalling in the striatum.

DA regulates essential cognitive functions through DA receptors expressed in the prefrontal cortex (PFC), striatum and hippocampus (Cools et al., 2002, Da Cunha et al., 2002). The Cognitive deficits in PD are thought to arise from two mechanisms: reduced DA signalling in the PFC and an imbalance in the corticostriatal circuits (Monchi et al., 2004). The injection of 6-OHDA into the MFB reduced DA concentration in the ipsilateral PFC. This finding is aligned with findings showing that the 6-OHDA

lesion in the MFB reduced DA concentration (Adu and Mabandla, 2019, Wu et al., 2019). Treatment with L-DOPA increased DA signalling in the PFC, and this increased DA signalling played a role in ameliorating the spatial learning and memory impairments that persisted due to the 6-OHDA lesion (Chiu et al., 2015). DA signalling is dependent on DA receptors expression (Ryoo et al., 1998). The hypoactivation of D1Rs in the PFC can impair cognitive function in Alzheimer's disease, Schizophrenia, and PD (Floresco and Magyar, 2006, Kintz et al., 2017, Meltzer et al., 2019). In the present study, there was increased D1R expression in the PFC of rats lesioned with 6-OHDA because of the reduced DA concentration in the PFC. These findings align with reports showing that 6-OHDA lesion leads to reduced DA concentration in the PFC and in response to the DA depletion the PFC post-synaptic neurons increase the expression of dopamine receptors like D1R (Cools, 2006, Kravitz et al., 2012). The treatment with L-DOPA increases DA concentration in the PFC (Frank, 2005) , which causes a reduced dopamine receptor expression, as seen in the present study.

5. Conclusion

The findings of the present study showed that a 6-hydroxydopamine (6-OHDA) lesion in the medial forebrain bundle (MFB) impairs recognition memory and although L-3,4-dihydroxyphenylalanine (L-DOPA) improved spatial learning and memory, it was not able to enhance object recognition memory. L-DOPA can reduce imbalances in the hypothalamic-pituitary-adrenal axis, neuroinflammation in the prefrontal cortex (PFC), but not the heightened cholinergic signalling in both the hippocampus and PFC, and it maintained DA signalling in the PFC. The death of dopamine (DA) neurons in the MFB significantly reduced DA signalling and increased neuroinflammation in other brain areas that play crucial roles in cognition. Future studies may focus on other brain areas involved in learning, memory function, and the interactions those brain areas have with the basal ganglia.

6. Conflicts of Interest

The authors declare that there is no conflict of interest regarding this current work.

7. Acknowledgement

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Chapter 4: Synthesis

Parkinson's disease (PD) is characterised by abnormal motor symptoms like postural instability and akinesia. However, PD patients also display non-motor symptoms like cognitive decline. These symptoms result from the death of dopamine (DA) neurons in brain areas collectively called the basal ganglia (BG). The BG is responsible for initiating and halting movement. In PD, some dysfunctions begin in the BG and spread to other brain areas via direct or indirect communication with the BG. The brain areas affected include the cerebellum, prefrontal cortex (PFC), hippocampus, amygdala, and other brain areas. Literature has shown that L-3,4-dihydroxyphenylalanine (L-DOPA) can alleviate motor symptoms of PD and some cognitive deficits by increasing DA signalling in the brain. However, the long-term use of L-DOPA is known to result in abnormal involuntary movements called L-DOPA induced dyskinesias (LIDs). Therefore, it became necessary to explore the effects that the development of LIDs would have on motor incoordination and cognitive decline experienced by PD patients.

The aid used to explore LIDs development's effects was a 6-hydroxydopamine (6-OHDA) model, resulting in LIDs-like behaviour when the rats are treated with L-DOPA. The present study showed that L-DOPA could alleviate motor incoordination, spatial learning, and spatial memory, but failed to ease object recognition memory. LIDs-like behaviour development in rodents leads to hypokinesia and impaired spatial memory. Altered behaviour is usually the result of impaired signalling in the brain and the case of PD, many of abnormalities stem from the death of DA neurons in the BG. L-DOPA slightly increased DA signalling, but even with this exogenous intervention, the brain progressively increased DA receptor expression to compensate for the significantly lower DA signalling. This is thought to play a crucial role in the progressive development of LIDs. The low BG DA signalling caused a reduced PFC DA concentration, which leads to reduced DA receptor activation is associated with impaired spatial learning, impaired spatial memory, and impaired object recognition memory. L-DOPA therapy improved spatial learning and memory. However, this changes as LIDs-like behaviour occurs because the continued exposure to exogenous DA will eventually lead to a reduced DA receptor expression in the PFC, which has been associated with a severe decline in working memory.

Along with DA signalling deficiencies, increased termination of cholinergic signalling in the brain underlies the cognitive impairments in PD. The present study found that in the two limbic brain areas that play a significant role in learning and memory had a higher concentration of the enzyme acetylcholinesterase (AChE) in groups treated with L-DOPA. This enzyme terminates cholinergic signalling, and other studies have shown that a high concentration of AChE in the hippocampus and PFC causes impaired object recognition memory. Most studies have focused on impaired DA signalling in the BG. Still, the present study also looked at other molecular alterations in different brain areas that may perpetuate the damage initiated by DA neurons' death.

The uncontrolled death of DA neurons causes a pro-inflammatory response. While this initiative is beneficial, this response's continued stimulation due to continued tissue damage has neurotoxic consequences. One such consequence is oxidative stress when reactive oxygen molecules break down the cell membrane and eventually kill the cell. The present study found that hyper-inflammation persists in the BG of the untreated brain and spreads to other brain areas like the cerebellum and the PFC. The neurotoxic effects of hyper-inflammation could be why some PD patients present with cognitive deficits even in the early stages of the disease. L-DOPA reduced the inflammatory response in the indirectly affected brain areas although after LIDs-like behaviour developed, there was hyper-inflammation in the cerebellum. In addition to oxidative stress, another consequence of hyper-inflammation is the stimulation of the hypothalamic-pituitary-adrenal (HPA) axis. This may increase the neurotoxic environment since the hyperactivation of the HPA axis results in an increased secretion of glucocorticoids, i.e., corticosterone (rodents) and cortisol (humans). Heightened glucocorticoids concentration is neurotoxic and has been associated with impaired spatial learning, impaired spatial memory, and impaired episodic memory. L-DOPA was able to reduce the plasma corticosterone level and therefore ameliorating imbalance caused by the heightened inflammation.

Conclusion

The present study showed that the dopamine (DA) replacement therapy with L-3,4-dihydroxyphenylalanine (L-DOPA) attenuated the hypokinesia, impaired motor coordination, impaired spatial learning, and spatial memory caused by the injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle. The progressive increase in the expression of D2 receptor (D2R) and the persisting hyper-inflammation in the BG seem to play a vital role in the development of L-DOPA induced dyskinesias (Mela et al., 2012, Suarez et al., 2016, Pisanu et al., 2018). Neuroinflammation makes the DA neurons vulnerable and increases the chance of degeneration. The high concentration of DA resulting from L-DOPA helps with the motor symptoms; however, the eventually increased expression of D2R receptors seem to increase the likelihood of abnormal involuntary movement (AIMs) development. The death of DA neurons in the medial forebrain bundle significantly reduced DA signalling and increased neuroinflammation in other brain areas that play crucial roles in cognition. However, L-DOPA can reduce imbalances in the hypothalamic-pituitary-adrenal axis, neuroinflammation in the PFC, but cholinergic signalling in both brain areas was significantly impaired during AIMs. This resulted in impaired episodic and spatial memory deficits. Furthermore, these cognitive deficits may be caused by dopamine influx to cortical areas related to these particular cognitive functions (Swainson et al., 2003, Ko et al., 2016). Future studies may focus on the progressive alteration of apoptotic markers in the cerebellum, thalamus, and motor cortex to elucidate the heightened inflammatory response's effect on the neurons.

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Appendix 1: AREC Approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

13 March 2019

Ms Makwena Mokgokong (213514384)
School of Laboratory Medicine & Medical Science
Westville Campus

Dear Ms Mokgokong,

Protocol reference number: AREC/077/018M
Project title: The alteration of dopamine receptors in L-DOPA (L-3,4-dihydroxyphenylalanine) induced dyskinesias

Approval Notification – Research Application

With regards to your revised application received on 21 December 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 13 March 2020.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil Singh, PhD
Deputy Chair: Animal Research Ethics Committee

/ms

cc Supervisor: Professor MV Mabandla
Cc Academic Leader Research: Dr Brenda de Gama
Cc Registrar: Mr Simon Molooena
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Appendix 2: Corticosterone ELISA kit protocol

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Rat CORT(Corticosterone) ELISA Kit

Synonyms: CORT

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96T

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Appendix 3: Tumour necrosis factor-alpha ELISA kit protocol

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Rat TNF-α(Tumor Necrosis Factor Alpha) ELISA Kit

Synonyms: DIF, TNF-alpha, TNFA, TNFSF2

Catalog No : E-EL-R0019
96T

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Appendix 4: Dopamine ELISA kit protocol

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DA(Dopamine) ELISA Kit

Synonyms: 2-(3, 4-dihydroxyphenyl)ethylamine, 3, 4-dihydroxyphenethylamine, 3-hydroxytyramine, DA, Intropin, Revivan, Oxytyramine

Catalog No : E-EL-0046
96T

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Appendix 5: Acetylcholinesterase ELISA kit protocol

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Rat AChE(Acetylcholinesterase) ELISA Kit

Synonyms: ACEE, ARACHE, N-ACHE, YT, acetylhydrolase

Catalog No : E-EL-R0355
96T

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Appendix 6: RNA extraction kit protocol

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Appendix 7: cDNA synthesis kit protocol

First Strand cDNA Synthesis Protocols (E6560)

Thaw kit components on ice and mix by inverting several times.

Easy Protocol

1. Mix the following components and incubate at 42°C for 1 hour. If Random Primer Mix is used, an incubation step at 25°C for 5 minutes is recommended before the 42°C incubation.

COMPONENT	VOLUME
Template RNA	up to 1 µg
$d(T)_{23}$ VN	2 µl
ProtoScript II Reaction Mix (2X)	10 µl
ProtoScript II Enzyme Mix (10X)	2 µl
Nuclease-free H_2O	to a total volume of 20 µl

2. Inactivate the enzyme at 80°C for 5 minutes. For downstream PCR application, the volume of cDNA product should not exceed 1/10 of the PCR reaction volume.

Appendix 8: Master Mix Protocol

Luna® Universal qPCR Master Mix Protocol (M3003)

- Prepare DNA or cDNA of interest using desired DNA extraction and purification method.
- Make dilutions of DNA or cDNA to be used for the standard curve. These should be prepared fresh before each experiment and can be diluted in either water or TE.

Reaction Setup: For best results, we recommend running each DNA standard and sample in triplicate.

COMPONENT	20 µl REACTION	FINAL CONCENTRATION
Luna Universal qPCR Master Mix	10 µl	1X
Forward primer (10 µM)	0.5 µl	0.25 µM
Reverse primer (10 µM)	0.5 µl	0.25 µM
Template DNA	variable	< 100 ng
Nuclease-free Water	to 20 µl	

Appendix 9: Brain research author information pack

BRAIN RESEARCH

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 0006-8993

DESCRIPTION

Brain Research is dedicated to publishing the highest quality and greatest impact articles within the ever-evolving field of Neuroscience. We recognize how technology has changed the way scientific breakthroughs are communicated and Brain Research is committed to serving as a dynamic journal meeting the needs of the neuroscience research community. We now offer multiple formats for scientists to share their work with the community. These include Short Reports, Technology Developments, and Commentaries, in addition to standard research articles and reviews that the journal has published in its storied 50-year history.

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Article Types: *Research Article* - Standard submission, graphical abstract required ***Review*** - Subject to Editorial Board approval prior to consideration, graphical abstract required ***Short Report*** - High impact, 3 figure max, expedited review, one-round only, can be moved to Research Article if major revisions are requested and interest is strong, graphical abstract required ***Neurotech Report*** - A methods paper whose impact is based purely on a technical or methodological advance, does not need to be hypothesis-driven science, graphical abstract required ***Point-Counterpoint*** - A pair

of articles addressing a contemporary debate or controversy within a field - can be broad or very focused in nature, merged graphical abstract required

AUDIENCE

Neuroscientists, neurophysiologists, neuroendocrinologists, neurochemists, neuroanatomists, neuropharmacologists, neurologists.

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INTRODUCTION

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Article Types

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Reference style
Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references can be listed either first alphabetically, then chronologically, or vice versa.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999).... Or, as demonstrated (Jones, 1999; Allan, 2000)... Kramer et al. (2010) have recently shown ...'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.
Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59. https://doi.org/10.1016/j.Sc.2010.00372.
Reference to a journal publication with an article number:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2018. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.
Reference to a website:
Cancer Research UK, 1975. Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ (accessed 13 March 2003).
Reference to a dataset:
[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T., 2015. Mortality data for Japanese oak wilt disease and surrounding forest compositions. Mendeley Data, v1. https://doi.org/10.17632/xwj98nb39r.1.

Journal abbreviations source
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Appendix 10: College of Health Science

GUIDELINES FOR PRESENTATION OF MASTERS AND PHD DISSERTATIONS/THESES BY RESEARCH

1. Purpose

The purpose of this document is to provide guidance to students and supervisors on how to prepare a dissertation/thesis for Masters by Research and PhD degrees using the manuscript or publication format..

2. Introduction

These guidelines must be read together with the College of Health Sciences (CHS) Handbook as well as the Jacobs documents on examination policies and procedures for PhD degrees. The rules on thesis format are based on modification of point 1 of the definition of terms section in the Jacobs document. In this section a thesis is defined as *"the supervised research component of all PhD degrees, whether by supervised research only, or coursework and research, or by papers that are either published or in manuscript form (the supervised research component of the PhD degree by paper(s) comprises the introduction, literature review, account of the methodology, selection of manuscripts, and conclusion)."* A dissertation is defined as "*the supervised research component of all Masters degrees, whether by supervised research only, or coursework and research, or by papers that are either published or in manuscript form (the supervised research component of the Masters degree by paper(s) comprises the introduction, literature review, account of the methodology, selection of manuscripts, and conclusion).*"

2.1 PhD thesis

In the CHS Handbook the rules for a PhD thesis are not in one place; they are stated in DR8 a i & ii, DR9 c and CHS 16. DR8 a i & ii and direct that a thesis be presented in the standard format together with one published paper or an unpublished manuscript that has been submitted to an accredited journal, arising from the doctoral research. CHS16 (thesis by publications states that the thesis may comprise of at least three published papers or in press in accredited journals; such papers must have the student as the prime author. The same CHS16 provides for a thesis by manuscripts that may have at least 3 papers with the student as the prime author that have not yet been published but are in the form of manuscripts; at least two of such papers must constitute original research. In both cases (thesis by publications and manuscripts), there must be introductory and concluding integrative material sections.

The standard type thesis is being phased out in many African countries in favour of the other options that originate from the Scandinavian countries. While this format ensures that all details of the work done for the doctoral degree are captured and thoroughly interrogated, they often remain as grey literature which is mainly useful to other students, usually within the same university, although with digitization of theses, such work may become more accessible beyond the source university. Apart from the risk of losing good work because of it not being on the public domain, as students rarely publish such work after graduating, this approach denies the college additional productivity units (PUs) emanating from publications.

The thesis by publication encourages students to publish key aspects of their doctoral research as they will not graduate if the papers are not published or in press. This approach ensures that the work of the student enters the public domain before the thesis is examined, providing the examiner with some assurance of prior peer review. The thesis must constitute a full study of the magnitude expected of a PhD with the papers providing a sound thread or storyline. Furthermore, the college maximizes the students' work as PUs are awarded for the papers as well as for graduating. However, this approach may negatively affect throughput and frustrate students as

they cannot graduate unless all the papers are published or in press, in addition to the synthesis chapter demonstrating the story line of the thesis.

The option of a thesis by manuscripts ensures that students make efforts to start publishing. The risk of not passing because of failure to publish all papers (as in the thesis by publication) does not exist under this option. However, the PUs emanating from publications from the doctoral work are not guaranteed as the submitted papers may eventually be rejected. Thus there is a possibility of the doctoral work remaining on the university library shelves as is the case for the standard thesis format. The standard thesis does have the advantage that more details of the doctoral work are usually included.

In view of the above, the best option for the college is that of a thesis by publication. However, in the interim, the attractive option is that of thesis by manuscripts, as it provides the possibility of publication without putting the student at risk of delayed graduation when some of the manuscripts are not published/accepted, which also disadvantages the college in terms of PU earnings. The standard thesis option should ultimately be phased out for the stated reasons and students are not encouraged to present their theses in that format. Consequently this document does not describe the standard thesis.

2.2 MSc dissertation

The rules on presentation of MSc dissertations are presented in CR13 (course work), CHS 14 (course work) and MR9 (research) in the CHS Handbook. CR13 c and MR9 c direct that a dissertation "may comprise one or more papers of which the student is the prime author, published or in press in peer-reviewed journals approved by the relevant college academic affairs board or in manuscripts written in a paper format, accompanied by introductory and concluding integrative material." Such a dissertation should include a detailed description of the student's own distinct contribution to the papers. Both CHS14 and CR13 specify that reviews and other types of papers in addition to original research paper/s may be included, provided they are on the same topic.

3 Length of thesis and dissertation by word count

Table 1 provides a guide of the length of a thesis or dissertation by word count excluding preliminary pages and annexes.

Table 1: Thesis length by word count

Sections	PhD		Masters	
	Minimum	Maximum	Minimum	Maximum
Introduction	2700	2700	2000	2000
Chapters	10000	25000	6000	11000
Synthesis	2000	2000	1700	1700
Bridging	300	300	300	300
Total	15000	30000	10000	15000

4. *Intention to submit*

A written intention to submit a thesis or dissertation should be submitted to the appropriate postgraduate office with endorsement of the supervisor at least three months before the actual date of submission which should be before November if the student intends to graduate in the following year. The actual submission will under normal circumstances require approval of the supervisor.

5. Format for theses/dissertation

There is little variation in the actual format of the PhD thesis and Masters dissertation for the various types described above. The box below summarise the outline of a thesis/dissertation for the thesis by manuscripts and thesis by publications.

Box 1: Outline of thesis

Preliminary pages

i. Title page
ii. Preface and Declaration
iii. Dedication
iv. Acknowledgements
v. Table of contents
vi. List of figures, tables and acronyms (separately presented)
vii. Abstract

Main Text

1. Chapter 1: Introduction
   Introduction including literature review
   Research questions and/or objectives
   Brief overview of general methodology including study design
2. Chapter 2
   First manuscript/publication
3. Chapter 3
   Second manuscript/publication
4. Chapter n
   Final manuscript/publication
5. Chapter n+1: Synthesis
   Synthesis
   Conclusions
   Recommendations
6. References Appendices

NB. Between the manuscripts or publications there must be a 1 page (maximum) bridging text to demonstrate the link between them

6. Details for thesis/dissertation subheadings

This section summarizes what is expected under each subheading shown in Boxes 1 and indicates where there might be variations between a Masters Dissertation and PhD Thesis.