UNIVERSITY OF KWAZULU-NATAL
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The role of neuroinflammation, serotonin deficiency and gene expression in the pathology of L-dopa-induced dyskinesia with prolonged Levodopa treatment in a Parkinsonian rat model.

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Submitted as the dissertation component in partial fulfillment for the degree of

Master of Medical Science (Human Physiology)

School of Laboratory Medicine and Medical Sciences

University of KwaZulu-Natal

09 February 2023

PREFACE

The experimental studies carried out in this thesis were conducted at the University of KwaZulu-Natal, Westville Campus, Durban, South Africa. The live animal experiment was conducted from March 2019 to August 2019 while the current study used samples stored from this period in August 2021- October 2021 under the guidance and supervision of Dr Z.N.P. Msibi. This work has not been submitted to any tertiary institution including the University of KwaZulu-Natal to obtain an academic qualification, whether by myself or any other party. The use of other people's work has been duly acknowledged as it occurred in the text.

DECLARATION

I, Nombulelo Agata Mthembu (214505143) declare that:

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

Supervisor: Dr Z.N.P Msibi

Signed _____ Date_____

Co-supervisor: Prof. M.V Mabandla

Signed _____ Date_____

ACKNOWLEDGEMENTS

I. My supervisor – Dr. Zama Msibi for her patience, guidance, and her natural care. While available but she always encouraged me to become more independent and confident in my work, thank you so much. My co-supervisor prof. M.V Mabandla for financial support on some reagents I needed, thank you.

II. My family, mom (Elizabeth Mthembu), and her deputy, the big sister (Nokukhanya Mthembu) – for your consistent love, prayers, emotional, and financial support, and, you guys have been with me since the very first day, my cheerleaders, never left me to be alone, I am grateful that you are my family. Not forgetting the rest of the family, thank you for your love and support!

III. My friends – Andile, sisi, you know we were supposed to be friends, but we became sisters, your emotional support, prayers, and love, are appreciated, Mavela. Zenele, KaMaphanga, you have been with me in my most dark days when I was at my lowest, thank you. To the physiology postgraduates, thank you for leading the way and encouraging me.

IV. God – for everything! He is so reliable, and I am grateful that He is my Father, so grateful, my praise will forever be lifted to You.

V. To all my nieces and nephews, I dedicate this dissertation to you so you can grow knowing time spent advancing your knowledge is never wasted.

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LIST OF ABBREVIATIONS

5HT	Serotonin
6-OHDA	6-Hydroxydopamine
ΔΔCt	Delta delta Ct
µL	microliter
AIMs	Abnormal involuntary movement score
ANOVA	Analysis Of Variance
AREC	Animal Research Ethics Committee
BRU	Biomedical Resource Unit
cDNA	complementary deoxyribonucleic acid
DA	Dopamine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
ELISA	Enzyme-Linked Immunosorbent Assay
FosB	FBJ murine osteosarcoma viral oncogene homolog B
IL1-β	Interleukin 1 - beta
L-dopa	L-dopa
LID	L-dopa-induced dyskinesia
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MWMT	Morris Water Maze Test
NORT	Novel Object Recognition Test
Nptx2	Neuronal Pentraxin 2
PFC	Pre-frontal cortex
PD	Parkinson's disease
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TH	Tyrosine Hydroxylase
TNF-α	Tumor Necrosis Factor-alpha

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder, which globally affects 2% of the population above 65 years old. It is characterized by motor symptoms (bradykinesia, tremor, and rigidity) and non-motor symptoms (cognitive deficit, sleeping, and mood disorders). There is no cure for PD, however, levodopa (L-dopa) therapy is a gold-standard pharmacotherapy for managing motor symptoms. Despite its effective results, its long-term consumption causes L-dopa-induced dyskinesias (LID). The pathology of LID is not clear, however growing evidence has indicated that neuroinflammation and cognitive decline may be significant factors. This study aimed to investigate the role of dysregulated Nptx2, TH, and FosB genes in the L-dopa-induced cognitive impairment and to assess the role of serotonin deficiency and neuroinflammation in LID pathology in a Parkinsonian rat model. 72 Male Sprague-Dawley rats were divided into two equal phases (n=36 per phase), each phase had 3 groups with n=12 per group. Phase 1 had 3 groups of rats that were injected with L-dopa for 14 days (pre-LID phase) while phase 2 had rats that were injected with L-dopa for 28 days (LID phase). Animals went through behavioral assessments and were sacrificed by decapitation to obtain the hippocampus, prefrontal cortex, and striatum for neurochemical analysis (ELISA and PCR). Overall, results from this study showed that the continuation of L-dopa triggers a neuroinflammatory response, hence highly expressing proinflammatory cytokine TNF-α. We found that Nptx2, TH, and FosB genes are downregulated in the PFC with continued L-dopa therapy. This downregulation was correlated with LID-induced cognitive decline. Findings from this study suggest that expression of TNF-α and deficiency of serotonin may play a significant role in the pathology of LID and downregulation of Nptx2, FosB, and TH genes contribute to LID-induced cognitive decline.

Chapter 1: Introduction, Literature review, research question, objectives, and general methodology

1. Introduction

Parkinson's disease (PD) is the second common and progressive neurodegenerative disorder following Alzheimer's disease (Poewe et al., 2017). PD globally affects approximately 2% of the world's population aged 65 or older, with a general prevalence of 113 per 100,000 cases in the age category of 50–59 years, up to 2953 per 100,000 cases in the age group of 80 + years (Vermeiren and De Deyn, 2017). The pathology of PD is unknown; however, recent studies have shed light on possible factors that may be associated with the cause. Around 80% of PD cases are considered idiopathic because of their unknown aetiology, whereas the remaining 20% are presumed to be genetic. i.e is genes with common and rare variants (Bhat et al., 2018, Ibanez et al., 2017).

There is no known cure for PD; however, dopaminergic drugs are used to relieve motor symptoms, of which levodopa (L-dopa) is currently the gold standard treatment for PD. External L-dopa therapy restores synaptic dopamine (DA) levels in the striatum, which is significant for the correct execution of movements (Pagano et al., 2017). L-dopa does not entirely reverse the cellular loss of DA, but it formulates a new functional and neurochemical state (Bezard et al., 2003). Regardless of its success in ameliorating motor symptoms, long-term consumption of L-dopa results in complications known as L-dopa-induced dyskinesias (LID) which are abnormal involuntary movements (Lindvall, 2015). Even though L-dopa is the most commonly used treatment, most patients need deep brain stimulation to control LID's motor symptoms (Vijayakumar and Jankovic, 2016). LID occurs in more than 50% of patients with PD that have been treated with L-dopa for about six months, but this side effect can be experienced even for a few weeks or months after L-dopa therapy usage (Vijayakumar and Jankovic, 2016).

Although L-dopa is currently a gold standard, it is only effective for motor symptoms of PD, while the majority of non-motor symptoms are very unresponsive to L-dopa therapy (Pantcheva et al., 2015). Growing evidence suggests that treating LID with non-DA drugs such as serotonin, noradrenaline, and cholinergic neurotransmitters may minimize dyskinesias while not exacerbating PD symptoms (Honig et al., 2009). This has been done in very few clinical trials where patients were given a combination of L-dopa and other neurotransmitters and it reduced complications caused by high doses of L-dopa (Abdel-Salam, 2008). The combination, however, did not cure PD as patients have to take L-dopa for the rest of their lives, which has severe side effects in the long run (Abdel-Salam, 2008). The mechanisms associated with LID pathology are not well understood, making it difficult to find treatment as the current therapies remains symptomatic.

In previous years, researchers have theorized that DA produced in serotonin terminals may be one of the mechanisms involved in LID development; hence serotonin therapy may be a practical approach to treating LID (Carta et al., 2007).

One of the major pathophysiological factors of PD is neuroinflammation (Wang et al., 2015). Inflammation-driven oxidative stress is predicted to have a significant role in PD (Dias et al., 2013, Hunot and Hirsch, 2003). Tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine, is primarily produced by activated macrophages and cytotoxic effects (Fiers, 1991). This cytokine is highly toxic to dopaminergic neurons. Increased cytokine levels decrease levels of neutrophils, and they then promote apoptotic cell death and subsequent phagocytosis of DA neurons (Fox et al., 2010).

Some pro-inflammatory cytokines, such as TNF-α and interleukin 1 (IL-1), are the key mediators of the inflammatory response (Kumar et al., 2010). In post-mortem brain analysis of PD patients, levels of TNF-α have been noted to be elevated (Barcia et al., 2005). This is not only with PD patients but also with animals treated with dopaminergic neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) (Barcia et al., 2005, Nagatsu et al., 2000).

Growing evidence has indicated that TNF-α can be either protective or harmful; however, this is still a debatable issue (A Frankola et al., 2011). If TNF-α is expressed mainly at a site where neurons are lost, it implicates this pro-inflammatory cytokine as a mediator of neuronal injury; hence it may be an attainable target in PD therapy (Leal et al., 2013).

Given all the previous studies, it is clear that neuroinflammation is not only a consequence of neuronal degeneration, but it is also possible that it plays a role in the progression of neuronal degeneration by producing pro-inflammatory molecules (Kempuraj et al., 2016). It is also possible that the high expression of serotonin receptors in the hippocampus exacerbates LID.

2. Literature review

2.1 History of Parkinson's disease

Emerging evidence is showing that neurological disorders are globally the main cause of disability, with PD as one of the leading causes (Feigin et al., 2017). PD was first described by James Parkinson as an "essay on the shaking palsy" (Parkinson, 1817), later on during the 1880s, Jean-Martin Charcot termed this essay Parkinson's disease in honor of James which is now a universally accepted and used term for this essay (Goedert and Compston, 2018). From the year 1990 up to 2015, statistics have shown that people living with PD have doubled and according to Global Burden of Disease, it is expected that the number doubles again by the year 2040 (Feigin et al., 2017).

2.2 Symptoms of PD

Upon discovery, significant motor signs of PD were outlined and are still considered the hallmarks of PD; these include bradykinesia, rigidity, and tremor (Tysnes and Storstein, 2017). These motor manifestations are believed to be mainly the result of dopaminergic neurons' depletion in the substantia nigra, resulting in loss of dopamine (DA) in the striatum (Poewe et al., 2017). Non-motor symptoms

such as depression, hallucinations, cognitive impairments, sleeping disorders, and urinary disturbances have also been reported in PD (Schrag et al., 2015) and are mainly secondary to non-dopaminergic pathway degeneration, they mostly play a role in advanced PD in patients having impairments and disabilities (Magrinelli et al., 2016). Non-motor symptoms were reported by almost 100% of patients (Kim et al., 2013) who were also experiencing motor fluctuations (Mendonça et al., 2017). Post-mortem analysis has revealed that there are other neurotransmitters involved that enhance PD pathology signs, this includes serotonin, noradrenaline, and cholinergic neurotransmitters (Lang et al., 2007, Lippa et al., 2007).

2.3 Risk factors

The prevalence of PD usually depends on a geographical location, some countries have a high prevalence while other countries have a small percentage of its occurrence (Ben-Joseph et al., 2020). The most common risk factor associated with PD is age, it tends to affect the elderly community mostly than young people (Pringsheim et al., 2014). Gene mutation is another factor that has been predicted to have a major role in the development of oxidative stress in PD, it is thought to contribute about 25% risk of developing PD, this includes a mutation in *PRKN, SNCA, PINK1, DJ1,* and *LRRK2* genes (Dorszewska et al., 2021, Day and Mullin, 2021). Amongst these genes, *PRKN* has been reported to be the most common for diagnosis of PD and it may have a therapeutic effect (Dorszewska et al., 2021). Other risk factors include gender, with males being the most affected, and ethnicity where studies have shown that whites are more susceptible to PD than blacks (Caslake et al., 2013, Bailey et al., 2020). The risk factors are not only limited to the above-mentioned, other risk factors have been revealed by most studies such as exposure to pesticides, herbicides, well-water drinking, and metals such as Manganese solvents and polychlorinated biphenyls (Bhat et al., 2018).

2.4 L-dopa as a gold standard for PD

There is currently no cure for PD, however, there are ways in which the disease can be managed. The age difference is usually considered when it comes to the management of PD (Marras et al., 2020). PD patients with advanced age have a poor response to some pharmacological therapies such as anti-cholinergic and dopamine agonists (Marras et al., 2020). Other therapies include monoamine oxidase inhibitors, surgery such as deep brain stimulation or neuroblative surgery, and gene therapy (Axelsen and Woldbye, 2018). These therapies do not prevent neurodegeneration progression and they have been predicted to have weighty side effects(Axelsen and Woldbye, 2018).

Due to the shortcomings of other therapies, dopamine replacement by L-dopa has been considered a gold standard in PD management (Chen et al., 2020). Upon initiation of L-dopa therapy, PD symptoms get reduced to the extent that the patient may be completely free from the motor symptoms of PD (Walton-Hadlock, 2005). L-dopa is usually administered combined with carbidopa, which is a decarboxylase inhibitor that helps in minimizing the peripheral breakdown of L-dopa (Haddad et al.,

2018). Although it is a first line of treatment, L-dopa causes L-dopa-induced dyskinesias in the long run.

2.5 L-dopa-Induced Dyskinesias

L-dopa-Induced Dyskinesias (LID) are abnormal involuntary movement motor symptoms that develop in PD patients on chronic L-dopa therapy (Antonini et al., 2016). LID affects cognition, daily activities, and social life, which negatively impacts the patients' quality of life. There has not been any approved therapy for LIDs (Poewe and Antonini, 2015). A study done in 1974 revealed that more than 50% of PD patients on L-dopa developed LID within six months (Duvoisin, 1974). It was noted that the longevity of L-dopa treatment is directly proportional to the severity of LID (Quinn et al., 1982). Another study by (Rascol et al., 2000) revealed that LID developed in 80% of L-dopa patients in five years. Several studies have shown that LID depends on factors such as cortico-striatal neurotransmission, post-synaptic changes in proteins and gene expression, as well as plasticity (Bastide et al., 2015). Another possible cause of LID is that in PD, most L-dopa is consumed by serotoninergic terminals; these serotoninergic terminals are not molecular structured to control DA release, causing DA levels in synapses to fluctuate uncontrollably (Carta and Bezard, 2011).

LID can be categorized into 3 types: 1) Peak-dose dyskinesia – which is universal and is usually treated by minimizing individual doses of L-dopa and adding DA agonists (Sawada et al., 2010). Peak-dose happens when L-dopa levels in plasma are high, and choreiform movements identify them; however, patients may experience any other mobility disorder, including dystonia and ballism, affecting upper limbs, trunk, and orofacial muscles (Vijayakumar and Jankovic, 2016). It was first described by Cotzias and Van Woert in 1967 (Cotzias et al., 1967). 2) Diphasic dyskinesia – which is the least common, occurs at about 10-15 minutes post-L-dopa administration. It is noted when levels of L-dopa in serum are fluctuating; this can happen at the same time as two peaks of abnormal movements which one take place when the drug is being administered, and the other one occurs when the drug stops being effective (Nutt, 1990). Here, dystonic movements in lower limbs are noted even though dystonia and chorea can be a combination, but this is very rare (Nicoletti et al., 2016). 3) Wearing-off – which results from chronic L-dopa treatment and is noted when levels of L-dopa in serum are at the lowest. They occur as an abnormal spasm that usually affects the foot or leg and is hardly seen on the arm and trunk (Nutt, 1990). It appears in the early morning pre-L-dopa administration. One of the strategies for managing the off-period is the addition of long-acting formulators during the night or early morning, while DA agonists can be added during the off-time in the day (Vijayakumar and Jankovic, 2016).

Mechanisms involved in LID development are still obscure, although in previous years there has been growing evidence shedding light on their development. These implicated factors such as neuroinflammation, and changes in non-dopaminergic systems such as serotonin and noradrenaline.

This research focuses on aspects of neuroinflammation, serotonin, and the expression of several genes believed to be involved in either PD pathogenesis and progression or LID pathology.

2.6 Neuroinflammation on LID pathology

Neuroinflammation has been implicated as one of the significant pathophysiological factors involved in PD and LID development (Wang et al., 2015). This is caused by microglial cells, which play a vital role as the first line of defense in the immune system of the central nervous system (CNS); they get activated if there is damage in neurons or if a pathogen invades the CNS (Lull and Block, 2010).

When activated, microglia complete their function by making and releasing different soluble factors such as pro and anti-inflammatory cytokines, giving microglia a pro or anti-inflammatory phenotype (Wolf et al., 2017). Evidence from previous studies has found pro and anti-inflammatory cytokines in the brain and blood of PD patients (López González et al., 2016). The production of cytokines may play a protective role during the initial stages of PD by producing large amounts of anti-inflammatory cytokines, which become pro-inflammatory and neurotoxic as the neurodegeneration continues.

A persistent pro-inflammatory environment in the brain, especially in DArgic areas, may worsen microglial response, which overexposes neurons to more potentially inflammatory stimuli (Gu et al., 2015). In their paper, Gu and colleagues further revealed that the brain is highly prone to oxidative stress by nature. It is known that there is an imbalance of antioxidant systems and oxidative load in the brain in PD; these are thought to contribute to neuroinflammatory response and neurodegeneration.

The already existing oxidative environment in DA sites, combined with continuous inflammatory status, can promote oxidative toxicity by L-dopa and dramatized inflammatory response (Pisanu et al., 2018). Contrarily, in cultured DA neurons, there is also a protective effect or absence of toxic neurodegenerative effects by L-dopa (Mena et al., 1997). In 2017, Carta and colleagues reported that this effect might be dependent on dosage and experimental conditions (Joers et al., 2017).

It is known that LID is mainly caused by the gold standard treatment of PD (L-dopa), however, recent reports revealed that there are non-neuronal mechanisms that may play a role in LID development. Numerous pre-clinical studies in rodent PD models have reported a dramatic inflammatory response to chronic dyskinetic-L-dopa treatment in the neuro-degenerated striatum (Mulas et al., 2016, Bortolanza et al., 2015). Their findings revealed that pharmacokinetics and style of delivery of L-dopa are significantly contributing factors towards the development of neuroinflammatory responses to L-dopa.

Systems playing a role in neuroinflammation contribution to LID include microglia and microglia-secreted soluble factors such as cytokines as they are known to have a vital role in neuromodulation. Microglia can discern neuronal activity to regulate synaptic function by expressing different receptors for neurotransmitters and neuromodulators (Joers et al., 2017). Furthermore, neurons and microglia communicate by the microglial release of cytokines through specific membrane receptors found in both

neurons and microglia (Tremblay et al., 2010). This neuro-microglial communication proposes that once microglia are chemically activated, they may develop an inadequate response to the loss of DA and L-dopa in PD.

A study conducted by Barnum and colleagues in 2008 showed that animals treated with L-dopa had elevated levels of pro-inflammatory cytokines such as IL-1β, which was prevented by treatment with corticosterone (Barnum et al., 2008). They also observed changes in the levels of TNF-α, although this was not statistically proven because of variability between animals. When they administered IL-1β receptor antagonists in the striatum, they observed a decrease in AIMs; this implies that IL-1β does play a role in the severity of LID.

Previous research has revealed that continuous intrajejunal delivery of L-dopa minimizes LID severity and effectively treats motor symptoms of PD (Antonini et al., 2016). Rats lesioned with 6-OHDA had a continuous development of AIMs when subjected to pulsatile delivery of L-dopa, (Mulas et al., 2016). It was found that there were elevated immunoreactivity of TNF-α and microgliosis in DA degenerated striatum of dyskinetic animals, while in non-dyskinetic animals, neuroinflammation was not affected by L-dopa treatment (Mulas et al., 2016). When the style of L-dopa delivery was changed from pulsatile to continuous; this speedily stopped the expression of AIMs while microglia showed basal levels of TNF-α immunoreactivity. This implies that elevated neuroinflammatory response caused by L-dopa in the striatum relied on the dyskinetic result of L-dopa treatment (Mulas et al., 2016).

Indirectly, TNF-α can negatively impact serotonin availability. In 2008 a group of researchers reported that TNF-α and other inflammatory cytokines could directly stimulate the enzyme responsible for boosting the kynurenine pathway; it drives the conversion of tryptophan into kynurenine resulting in minimized serotonin synthesis (Dantzer et al., 2008). Furthermore, inflammatory cytokines including TNF-α and IL-1β proved to elevate expression of the activity of serotonin transporter in the midbrain of mouse resulting in reduced extracellular levels of the neurotransmitter (Zhu et al., 2006)

2.7 Serotonin in LID pathology

Serotonergic systems have been recently implicated in PD as previous animal studies revealed that serotonin neurons could take up L-dopa and release DA into the synapses (Maeda et al., 2005). The release of DA by serotonin neurons is not well controlled since these neurons are unable to get a response from DA transporters responsible for the regulation of synaptic neurotransmitter levels (Carta et al., 2007).

Serotonin is widely distributed in the CNS and plays a role in physiological functions, including sleep, arousal, and mood changes. The function of serotonin is shown through numerous and widely distributed serotonin receptors (Peroutka, 1995). In a normal brain, there is a dense serotonergic innervation from raphe nuclei to basal ganglia; this also projects to the frontal cortex and limbic system, including the hippocampus (Van der Kooy and Hattori, 1980). Serotonin deficiency in some parts of the

limbic system in post-mortem studies of PD patients has been previously reported, although not to the extent of DA loss (Shannak et al., 1994, Kish et al., 2008).

Although serotonin deficiency has been reported in PD, very few studies have shown the link between serotonin loss and LID. A 2007 study revealed that decreased serotonin levels in the striatum were implicated in the LID neuropathology (Carta et al., 2007). It has also been shown that L-dopa reduces striatal serotonin tissue concentration in PD rats; this means that PD severity is also dependent on striatal serotonin tissue levels (Gil et al., 2011). In 2003, another study demonstrated that LID was reduced by acute therapy with serotonin precursor 5-hydroxytryptophan in a rat model with AIMs (Tronci et al., 2017).

The function of the serotonergic system is to regulate different cognitive and physiological processes (Štrac et al., 2016). There are approximately 16 types of serotonin receptors, but they can be grouped into seven subtypes (5HT1 – 5HT7); this grouping is done based on primary physiological mechanisms (Kroeze and Roth, 1998). Studies also put to light that serotonin receptors and serotonin itself play a role in cognitive dysfunction such as memory decline and learning inability (Meneses and Hong, 1999, Sumiyoshi et al., 2007).

It was found that specific serotonin receptor drugs were able to reduce LID, and these drugs included 5-HT1A agonists, 5-HT2A antagonists, and 5-HT1B (Carta et al., 2007). Serotonin usually acts on 5-HT1A and 5-HT1B to control neuronal activity as well as serotonin release. When these receptors are activated, they create measures to dampen serotonergic neuronal activity; these measures play a role in dyskinetic rats by decreasing the release of DA derived from L-dopa on serotonergic terminals (Carta et al., 2007). This receptor (5-HT2A) is the most widely distributed in the brain (Zhang and Stackman Jr, 2015).

Adult female macaque monkeys were lesioned with neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to induce PD, an increase in 5-HT2A receptor levels was seen in *de novo* that were given L-dopa compared to healthy controls (Riahi et al., 2011). This suggests that dyskinetic monkeys have more elevated levels of 5-HT2A receptors compared to non-dyskinetic animals. It was concluded that this receptor increase seen in dyskinetic animals plays a vital role in the development of LID (Riahi et al., 2011)

2.8 Gene expression on L-dopa-induced cognitive decline

The prefrontal cortex (PFC) is located in the frontal lobe of the brain, and it receives impulses from other cortical areas (Kolb, 2006). Amongst other functions, the PFC works by directing cognitive behavior and planning (Kolb et al., 2012). The cognitive control in the PFC is suspected to be the result of a prolonged period taken to stop the production of extra neurons during puberty (Petanjek et al., 2011). Higher cognitive abilities are thought to be dependent on early life (adolescence) development where PFC during this time, makes primary neuronal circuity (Tsujimoto, 2008).

Research has shown that PFC plasticity may play a role in cognitive decline, this includes synaptic and certain genes' alterations (Rapanelli et al., 2010). This genetic plasticity has been linked to learning abilities as a study revealed that there is higher plasticity of PFC during learning compared to after learning (Rapanelli et al., 2010). Amongst other genes, Neuronal pentraxin-2, tyrosine hydroxylase, and FBJ murine osteosarcoma viral oncogene homolog B genes have been slightly implicated in cognitive impairments (Xiao et al., 2017).

2.8.1 Neuronal pentraxin-2

Neuronal pentraxin-2 (Nptx2) is a protein-coding gene and forms part of the neuronal pentraxins family which includes Nptx1 and NptxR, its expression is prompted by neuronal activity (Libiger et al., 2021, Boiten et al., 2020). Neuronal pentraxins are synaptic proteins that are associated with C-reactive proteins and are mainly expressed in the brain, spinal cord, and dorsal root ganglia (Xiao et al., 2017, Shao et al., 2020). It is responsible for the transmission of neurotransmitters and the formation of excitatory synapses, therefore its aberrant regulation can cause synaptic dysfunction which has been associated with PD pathology and progression (Nilsson et al., 2022). Occasionally Nptx2 takes part in the clustering of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors. The clustering of AMPA-type glutamate receptors leads to non-apoptotic death of DArgic neurons. In PD when this gene is upregulated, it may implicate its involvement in the pathology of PD (Moran et al., 2008).

The dysregulation of this gene in the human brain has been linked with cognitive dysfunction in Alzheimer's disease (Xiao et al., 2017). Not only in Alzheimer's, but in PD it has also been recently proven that its downregulation is linked with cognitive dysfunction (Nilsson et al., 2022). After L-dopa treatment, the Nptx2 gene has been found to be over-expressed in the striatum and it is predicted to be associated with the development of LID (Charbonnier-Beaupel et al., 2015). This gene has been found in Lewy bodies in the substantia nigra and expression of its mRNA was found upregulated in the frontal cortex and substantia nigra of PD patients (Nilsson et al., 2022).

2.8.2 Tyrosine hydroxylase

Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the synthesis of catecholamines such as DA and noradrenaline, although present in both the central nervous system and the sympathetic nervous system, this enzyme is selectively expressed in the central nervous system (Dunkley and Dickson, 2019). TH catalyzes the conversion of L-tyrosine to L-dopa. TH gene mutations have been linked with the development of LID in PD, and it could result in TH deficiency which has been found in patients who are in chronic L-dopa and had LID (Pons et al., 2013). This could mean L-dopa administration downregulates TH. TH gene downregulation has been linked with some cognitive declines. Changes in the activity of the TH gene are associated with the pathology of diseases such as PD.

TH usually compensates for the lost dopamine in PD, this then results in reduced TH in the nigrostriatal pathway. Downregulation of TH has been found in post-mortem brains of PD and this has been linked to DA deficiency which then results in neurodegeneration (Nagatsu et al., 2019, Blanchard-Fillion et al., 2001). TH has been found more decreased in brain regions where catecholamines are more dominant (Blanchard-Fillion et al., 2001). Progression of PD can be a result if TH loses its ability to produce catecholamines.

2.8.3 FBJ murine osteosarcoma viral oncogene homolog B

FBJ murine osteosarcoma viral oncogene homolog B (FosB) is a protein that is encoded by the FosB gene. The FosB gene also encodes for ΔFosB and is regulated by the response of cyclic adenosine 3',5'-cyclic monophosphate. ΔFosB is shortened version of the FosB gene, it is usually triggered by stimuli such as chronic exposure to drugs (Alibhai et al., 2007, Beck et al., 2021). ΔFosB gene has the ability to change the regulation of transcription which then leads to a long-term response to drug exposure and addiction, this gene is known to be highly expressed in response to stress (Beck et al., 2019). In PD brains of animal models, high expression of the ΔFosB gene has been linked with the presence of LID (Beck et al., 2021). It is still unclear whether this gene play role in cognitive decline associated with chronic exposure to L-dopa. Amongst other responsibilities, ΔFosB has been associated with cognitive functions and social behaviors (Manning et al., 2019). Post-neurotoxin administration, expression of the FosB gene was found to be unchanged (Beck et al., 2019)

In the striatum of PD subjects that have been treated with L-dopa, the FosB gene has been found to be accumulated (Beck et al., 2021). Although FosB itself is a stable protein, however, once it is triggered it lasts long in the brain. FosB gene has been linked with LID and growing evidence suggests that it is one of the causes of the development of LID and blocking it might ease LID (Beck et al., 2019). ΔFosB gene has been found to be upregulated in subjects that have a drug addiction and it also responds to other chronic stimuli (Beck et al., 2019). Since LID are also a result of drug exposure (prolonged L-dopa therapy), this gene has been found to be upregulated in the striatum of primate models and its upregulated has been linked with the severity of LID (Ulery et al., 2006, Andersson et al., 1999).

PD research has implicated that striatal overexpression of ΔFosB may be playing a key role in the mechanisms associated with the development of LID. According to Andersson and co-workers, this gene may have a potential therapeutic role in managing LID in advanced PD patients through gene therapy strategies (Andersson et al., 1999).

3. Aims

- To investigate the role played by the expression of Nptx2, TH, and FosB genes in the cognitive decline of L-dopa-Induced dyskinesia.
- To investigate the association between neuroinflammation and serotonin on the pathology of L-dopa-Induced dyskinesia.

4. Objectives

- Previously stored samples (hippocampus, prefrontal cortex, and striatum) harvested from male Sprague Dawley rats were used to determine gene expression of Nptx2, TH, FosB, TNF-α, and IL-1β.
- To determine gene expression Nptx2, TH, and FosB in the prefrontal cortex and correlate this with the cognitive decline seen in PD.
- To link the Nptx2, TH, and FosB genes with previously collected behavioral data (MWMT, NORT) and associate results with cognitive decline.
- To analyze and link previously collected behavioral data (AIMS) with serotonin concentration in the hippocampus and striatal neuroinflammation seen on LID.

5. General methodology

An animal model was used in this study, which assisted in evaluating PD-like behavioral symptoms using different behavioral tests. The current ethically approved study (AREC/00003538/2021) used harvested samples from a co-study with the following general methodology: Upon ethical approval from the University of KwaZulu-Natal Animal Ethics Committee, 72 male Sprague Dawley rats were obtained from Biomedical Resource Unit. They were acclimatized to their new environment and were tested for balance and coordination by Beam Walking Test (BWT). They were then grouped into 2 phases (PRE-LID and LID group). Each phase had 3 different groups (control, untreated, and treated). Animals were then taken for stereotaxic surgery where the control group was lesioned with saline and both treated and untreated groups were lesioned with neurotoxin 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle to induce Parkinsonism. In the PRE-LID phase, the treated group received 14 days of L-dopa subcutaneously, untreated group was given saline. The treated group from the LID phase received L-dopa for 28 days.

An hour after treatment, all animals were tested for balance and coordination again by BWT. Animals were also tested for spatial learning and recognition memory by behavioral tests Novel Object Recognition Test (Schallert et al.) and Morris Water Maze Test (MWMT) respectively. Animals from pre-LID phase (14 days) did not exhibit any LID while animals from the LID phase were tested for dyskinesias since they were on L-dopa treatment for a prolonged period (28 days) and were actually displaying LID. This was tested by measuring abnormal involuntary movements score (AIMs).

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Animals were sacrificed, and brain tissue was harvested followed by dissection of the hippocampus, prefrontal cortex, and striatum for neurochemical analysis. Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of serotonin in the hippocampus. Reverse transcription-polymerase chain reaction (PCR) was used to determine the gene expression of FosB, Nptx2, and TH genes in the prefrontal cortex and the gene expression of Tnf-α and IL1-β in the striatum.

6. Study design

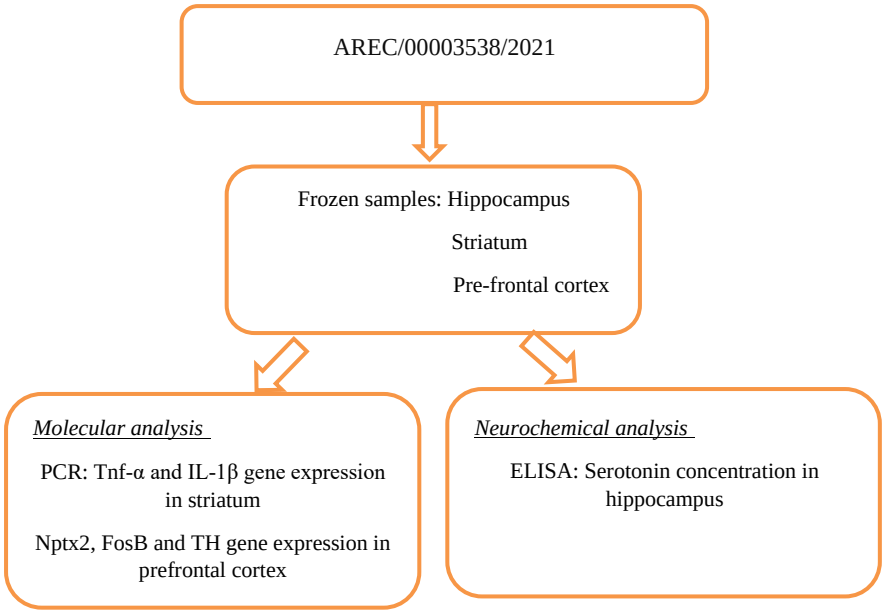

Figure 1 shows the experimental design for the methodology of mimicking parkinsonism and treating with L-dopa in a rat model.

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Chapter 2: Manuscript 1

Investigation of gene expression in the pre-frontal cortex associated with L-dopa-induced cognitive decline in a Parkinsonian rat model

Abstract

Cognitive dysfunction is one of the non-motor symptoms of Parkinson's disease (PD), a neurological disorder characterized by continuous degeneration of dopaminergic neurons in the substantia nigra projecting to the striatum. The prefrontal cortex (PFC) is one of the brain structures responsible for controlling cognitive functions, this means changes in the PFC might significantly cause a decline in cognition. This study aimed to investigate variances in the expression of the FosB, Nptx2, and TH genes caused by abnormal gene regulation and its association with levodopa (L-dopa)-induced cognitive decline in the PFC. Quantitative PCR was performed on PFC tissue obtained from male Sprague Dawley rats induced with 6-hydroxydopamine (6-OHDA) and treated with L-dopa for 14 and 28 days. Gene expression was correlated with cognitive decline as determined by the Morris Water-Maize and Novel Object Recognition behavioral tests. The molecular results showed significant downregulation of all these genes (FosB, Nptx2, and TH genes) in the groups that were treated with L-dopa for an extended duration (28 days). Behavioral results from the Novel Object Recognition test showed that a lesion with the neurotoxin 6-OHDA caused impaired object recognition memory and levodopa administration was not able to restore recognition memory. Morris Water Maze test results from the pre-LID phase (14 days) showed the ability of levodopa in improving spatial memory and learning. Prolonged levodopa therapy (28 days) resulted in spatial memory decline. Our data show a correlation between the downregulation of FosB, Nptx2, and TH gene expression and levodopa-related cognitive decline.

Keywords: Parkinson's disease, L-dopa, Cognition, FosB, Nptx2, and TH gene.

1. Introduction

Parkinson's disease (Axelsen and Woldbye) is a neurodegenerative disorder that causes the loss of dopaminergic neurons from the substantia nigra (Labbé et al., 2016). Although its pathology is not well-understood, PD universally affects 2% of elderly people, and in rare cases, it can affect young adults with a prevalence of 0.3% (Raza and Anjum, 2019). Clinically, PD patients present with motor impairments such as bradykinesia, postural instability, rigidity, tremor, and gait dysfunction (Raza and Anjum, 2019). Non-motor symptoms are usually neuropsychiatric which may include cognitive dysfunctions and depression, others may be autonomic such as bladder impairments, and sleeping disturbances such as insomnia have also been outlined as non-motor symptoms of PD (Sveinbjornsdottir, 2016). As the disease progresses, about 80% of PD patients present with cognitive decline and dementia (Biundo et al., 2016).

Levodopa (L-dopa) remains the first-line treatment in PD management, although its prolonged consumption eventually results in undesirable motor complications known as L-dopa-induced dyskinesia (LID) (Haddad et al., 2018). The pathology of LID is not well understood, striatal neurodegeneration of dopaminergic cells in pre-synapses has been implicated as one of the main contributing factors (Yoo et al., 2019). In vivo studies have implicated irregularities in the pre-frontal cortex (PFC) of PD and LID patients, where there was an observation of an enlarged density of grey matter (Yoo et al., 2019). The grey matter of the PFC is known to be one of the key facilitators of cognitive and executive functions, this means dysfunction of the PFC may result in cognitive impairments (Friedman and Robbins, 2022). The dopaminergic and noradrenergic neurons give impulses to the PFC and have a central role in mediating cognitive functions such as memory (Lee and Goto, 2015). A recent study showed a link between LID pathology and continuous cognitive decline associated with the frontal lobe (Yoo et al., 2019). This study showed that striatal alterations and plasticity of the cortex trigger an unusual cognitive loop in the PFC, this results in PD patients who have this loop being more susceptible to LID development and cognitive dysfunction compared to those who don't have it (Yoo et al., 2019).

Tyrosine hydroxylase (TH) is a rate-limiting enzyme that has been predicted to play a key role in the functioning of PFC, this is because it catalyzes tyrosine to dopamine which supports PFC in the execution of cognitive functions (Lee and Goto, 2015, Daubner et al., 2011). Downregulation of the TH gene may result in impaired memory. It has been reported that when it comes to the TH gene, Sprague-Dawley rats may have numerous and different polymorphisms (Murru et al., 1997).

FBJ murine osteosarcoma viral oncogene homolog B (FosB) gene produces a full-length protein, FosB, and a shortened form known as delta FosB (ΔFosB) (Solecki et al., 2008). The FosB gene has been suggested to play a central role in memory in the hippocampus (Corbett et al., 2017).

Neuronal Pentraxin 2 (Nptx2), also known as neuronal activity-regulated protein is a glycoprotein that facilitates the formation of excitatory synapses, it also plays a role in normal learning and memory (Tang et al., 2019). Nptx2 is reduced in the serum of patients with dementia and this has been thought to be associated with cognitive decline (Shao et al., 2020). This association, however, is not dependent on factors such as age and gender. It was revealed that Nptx2 may be a key biomarker for dementia although more research needs to be conducted for clear pathogenesis of this glycoprotein in dementia (Shao et al., 2020). Nptx2 is one of the overexpressed genes in the substantia nigra of PD (Moran et al., 2008)

Cognitive decline associated with PD and LID has no known mechanisms, but growing evidence has implicated genetic alterations in PFC (Planas-Ballvé and Vilas, 2021). This study aimed to investigate the expression of Nptx2, TH, and FosB genes in the PFC and their association with LID-induced cognitive decline. The current study makes use of PFC tissue harvested from 6-hydroxydopamine-induced and L-dopa-treated male Sprague Dawley rats in the determination of gene expression of Nptx2, TH, and FosB.

2. Methods and materials

2.1 Ethical clearance

Upon ethical approval from the University of Kwazulu-Natal Animal research committee (AREC/00003538/2021) and the South African Department of Agriculture, Land Reform and Rural Development (DALRRD) – 12/11/1/5/2 (1684JD), 72 rats were obtained from the Biomedical Resource Unit of the same institution.

2.2 Animal grouping

A total of 72 rats were acclimatized and grouped into 2 phases which were the pre-LID phase (14 days of L-dopa treatment) and the LID phase (28 days of L-dopa treatment). Each phase had 36 animals and both these phases consisted of 3 groups of animals (n=12 per group): the control group, which was lesioned with saline, an untreated group which was lesioned with 6-OHDA, and the test group lesioned with 6-OHDA and treated with L-dopa.

2.3 Stereotaxic surgery

Before stereotaxic surgery, ketamine (125mg/kg) and xylazine (10mg/kg) was administered intraperitoneally, to serve as anesthesia (Veilleux-Lemieux *et al.*, 2013). The function of ketamine was to keep a rat unconscious throughout the surgery while xylazine acted in keeping muscles relaxed while enhancing heart rate so that blood pressure does not drop during surgery (Veilleux-Lemieux *et al.*, 2013). After xylazine and ketamine have fully stabilized, rats were put in a stereotaxic frame where an incision was made on the scalp followed by intracranial administration of 6-OHDA in the brain. Both treated and untreated groups were unilaterally injected with 6-OHDA (8µg/µl) that was dissolved in 0.2% ascorbic acid using a Hamilton syringe (Dalla Vecchia *et al.*, 2018) according to the following coordinates from lambda: anterior 4.7 mm, lateral 1.6 mm, and ventral 8.4 mm below the skull (Paxinos

and Watson, 1986). For adequate diffusion of 6-OHDA in nearby tissues, the needle was kept in place for 8 minutes before it was slowly removed (Mabandla *et al.*, 2015). The animals from the control group were injected with saline through the same procedure. After surgery, rats were given analgesics (0.05mg/kg) subcutaneously. Rats were then placed in cages with a heating pad to prevent hypothermia.

2.4 Treatment

After a week of lesion stabilization, animals from the test group were subcutaneously injected with L-dopa (50mg/kg) mixed with benserazide, which served to prevent the peripheral breakdown of L-dopa. This was done twice (9 am and 4 pm) a day for 14 and 28 consecutive days (Ndlovu et al., 2016). Animals from the control group were handled at times corresponding to L-dopa treatment, while animals from the untreated control group were given saline subcutaneously.

2.5 Behavioral tests

2.5.1 Novel object recognition test

This test was done to assess the cognition of rats through novel object identification, which was determined by a rat's exploratory behavior (Antunes and Biala, 2012, Schallert et al., 1992) An open field square wooden box (100×100×50 cm) was used and two identical objects were put in the field (Moscardo et al., 2012). The test constituted of 3 stages which were habituation, training, and test stage (Batool et al., 2016). It was carried out in 4 days, where the first 3 days were for familiarizing animals with the test set-up where the animal was separately placed for these 3 days while on the 4th day, it was tested. For the first 3 days, animals were put in a field without the object for 15 minutes (Ngoupaye et al., 2017). On the 4th (L'Episcopo et al.) day, rats were put in a field that now had 2 identical objects for 5 minutes, and it was allowed to explore them. After an hour, 1 of the objects was replaced by a novel object. Here, the time taken by each rat to explore objects was recorded. If the rat spent more time studying a novel object it meant it still had a complete recognition memory. A discrimination index (DI) for each animal was calculated which is defined as (N - F)/(N + F) where N was the time taken to by the rat in studying the novel object while F was the time spent on a familiar object (Ngoupaye et al., 2017).

2.5.2 Morris Water Maze Test (MWMT)

This test was used to assess spatial learning and memory (Da Cunha et al., 2007). A room where the test occurred was warmed before the test started. The apparatus included a spherical pool with a black interior that is half-filled with warm water. A small transparent platform/stage was located inside the pool. Rats were expected to search and find this platform that was placed just 1cm below the water's surface. The pool was divided into 4 quadrants, where each quadrant had a cue. In one of these quadrants, there was a platform. The rats were put in the other 3 quadrants except for the one with the platform (D'Hooge and De Deyn, 2001). For spatial learning, the rat needed to learn to be able to locate a hidden platform with the help of cues while recording the time it took a rat to find the platform. This

time was defined as the ability of a rat to learn. For spatial memory, a platform was taken out of the pool, and the ability of a rat to remember was measured by the time it spent on the quadrant that had the platform (Cassim et al., 2015). After water exposure, the rats were towel-dried and placed in a bedded cage next to a heating pad.

2.6 Euthanasia

After 14 days of treatment followed by behavioral testing, rats from the PRE-LID phase were decapitated using a guillotine (n=12 per group) and after 28 days of treatment followed by behavioral tests, LID phase rats (n=12 per group) were sacrificed. Surgical scissors were used to open the skull. Once open, brain tissue was extracted and kept in a frozen 0.9% saline slush before the striatum, prefrontal cortex, and hippocampus were dissected from it. All the collected samples were moved into Eppendorf tubes and were snap-frozen in liquid nitrogen. The frozen tissue in nitrogen was then stored at -80^{0}C in the bio-freezer until the day of neurochemical analysis.

2.7 Molecular analysis

2.7.1 Reverse transcription PCR (RT-PCR)

2.7.1.1 RNA extraction

Following the manufacturer's guidelines, a Quick-RNA Miniprep Kit from Zymo Research was used to extract RNA from the tissue (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). Briefly, at room temperature, 96 ml of 100% ethanol was added to 24 ml of RNA wash buffer. Frozen PFC and striatum tissues were homogenized in an RNA lysis buffer. Debris was removed by centrifugation and the supernatant was transferred into a nuclease-free tube. The samples were filtered again by centrifugation to remove most of the genomic DNA, flow-through was saved and 95% of ethanol was added to it (at the ratio of 1:1). 400ul of RNA prep buffer was added to the mixture and centrifuged, the flow-through was discarded. 700ul of RNA wash buffer was added to the tube and centrifuged. Flow-through was discarded and 400ul of RNA wash buffer was added and the mixture was centrifuged for 1 minute. Lastly, 100ul of DNase-free water was directly added to the tube and it was centrifuged. Unless stated, all centrifuge steps were done at 10000xg for 30 seconds.

2.7.1. 2 cDNA synthesis

cDNA was synthesized from RNA based on the supplier's guidelines from BioLabs Inc. ProtpScript II first-strand cDNA synthesis kit (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). The reaction tube had 1µl of RNA, 2µl of d(T)23 VN, 10µl of ProtoScript II Reaction Mix, and 2µl of ProtoScript II Enzyme Mix. 5µl of nuclease-free water was added which summed up the reaction to 20µl. The tube was vortexed and incubated at 42^{0}C for 1 hour. The enzyme was then inactivated at 80 C for 5 minutes.

2.7.1.3 Gene expression

Gene expression was done using the SYBR GREEN Quantitative PCR Master mix (Sigma-Aldrich, South Africa), and primers were obtained from Inqaba Biotechnical Industries (Pty) Ltd, South Africa.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Primer sequence specifications for all genes (Nptx2, TH, FosB, Tnf-α, IL-1β, and GAPDH) are in table 1 below. A PCR tube mixture contained a total of 10µl reaction volume: 5µl of dye SYBR GREEN (Sigma-Aldrich, South Africa), 2µl of synthesized cDNA, 1µl of forward primer, 1µl of reverse primer, and 1µl of nuclease-free water. PCR was run using a LightCycler® 96 instrument (Roche Life Science, South Africa). Parameters of the PCR were: Pre-incubation at 95^0C for 600 seconds, 3-step amplification at (i) 95^0C for 15 seconds, (ii) 60^0C for 30 seconds with 55 cycles, (iii) 72^0C for 30 seconds, melting was done at (i) 95^0C for 10 seconds, (ii) 65^0C for 60 seconds, (iii) 97^0C for one second and cooling was done at 37^0C for 30 seconds.

Table 1: The primers sequences of the following genes: Nptx2, TH, FosB, and GAPDH

Primer	Forward primer	Reverse primer
Nptx2	5'- TCC GGG CAC AAG AGA TCA TC-3'	5'- GAT GTT TCC AGG CAT GTT CGT-3'
TH	5'-TCG GAA GCT GAT TGC AGA GA-3'	5'-TTC CGC TGT GTA TTC CAC ATG-3'
FosB	5'GTG AGA GAT TTG CCA GGG TC-3'	5'-AGA GAG AAG CCG TCA GGT TG-3'
GAPDH	5′-GGCATTGCTCTCAATGAC AA-3′,	5′-ATGTAGGCCATGAGGTCC AC-3′

3. Statistical analysis

3.1 Delta-delta Ct method of analysis

The delta-delta Ct method (2^ - (ΔΔCt) of results analysis was employed. This method was first introduced in 2009 by Bustin and colleagues calling it Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). The method was performed as follows:

- Calculation of ΔCt = Ct of a gene of interest – Ct of GAPDH
- Calculation of average ΔCt for the control group (saline + handling)
- Calculation of ΔΔCt = ΔCt of each sample – ΔCt of control (averaged)
- Lastly, 2^-(ΔΔCt)

For statistical analysis, GraphPad Prism version 8 was used to analyze results from the delta-delta Ct method (GraphPad Software Inc. California USA). Data were tested for normality by the Shapiro-Wilk test. One-way analysis of variance (Cools et al.) Kruskal-Wallis non-parametric test was used, followed by Dunn's post hoc multiple comparison tests. P values were considered statistically significant if $p<0.05$. All data were expressed as mean ± SEM.

Table 2 shows asterisks in the graph and their p-value descriptions

Asterisk	Description
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
****	$P \leq 0.0001$

4. Results

4.1 Behavioural results

4.1.1 Novel object recognition test

Assessment of recognition memory was done using the ability of an animal to differentiate between familiar and novel objects, this is called the discrimination index. Two phases were compared: the PRE-LID phase which was on L-dopa treatment for 14 days, and the LID phase which was on treatment for 28 days. There was a significant decline in recognition ability after the 6-OHDA lesion in both phases (PRE SALINE vs PRE 6-OHDA, p=0.0012 and LID SALINE vs LID 6-OHDA, p=0.0001). There was a significant L-dopa effect seen exacerbating recognition ability in the PRE-LID phase (PRE 6-OHDA vs PRE L-DOPA, p=0.0033). In the LID phase, there is a trend suggesting the ability of L-dopa to slightly ameliorate impaired recognition caused by 6-OHDA (LID 6-OHDA vs LID L-DOPA; Figure 1).

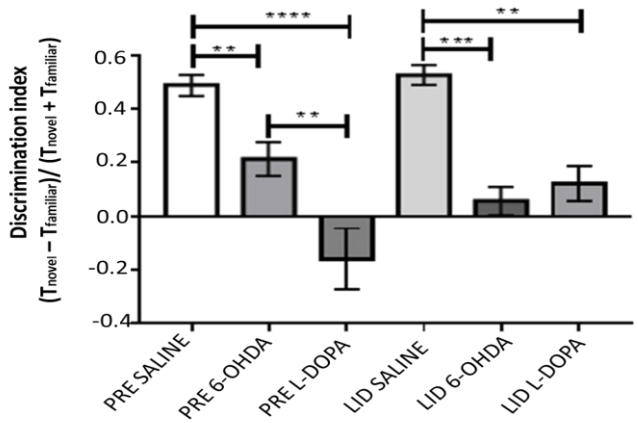

Figure 1: Graph showing the discrimination index assessed using the Novel Object Recognition Test (Schallert et al.), n=11 per group.

4.1.2 Morris Water Maze Test

4.1.2.1 Spatial learning

Spatial learning was assessed using Morris Water Maze Test. There was a significant learning impairment after the 6-OHDA lesion in both phases as indicated by the amount of time spent trying to locate the hidden platform (PRE SALINE vs PRE 6-OHDA, $p<0.0001$ and LID SALINE vs LID 6-OHDA), $p<0.0001$. L-dopa was observed to improve this impairment (PRE-6-OHDA vs PRE L-DOPA, $p<0.0001$ and LID 6-OHDA vs LID L-DOPA), $p<0.0001$ (Figure 2a.

4.1.2.2 Spatial memory

Spatial memory was tested by the time an animal spent in a target quadrant. Post 6-OHDA lesion animals from both phases had impaired memory as they spent a shorter time in a target quadrant (PRE SALINE vs PRE 6-OHDA, $p<0.0001$, and LID SALINE vs LID 6-OHDA, $p<0.0001$). Administration of L-dopa alleviated memory decline in the PRE LID phase (PRE 6-OHDA vs PRE L-DOPA, $P<0.001$. In the LID phase, however, L-dopa exacerbated the memory impairment (PRE-L-DOPA vs LID L-DOPA, $p<0.0001$) (Figure 2b).

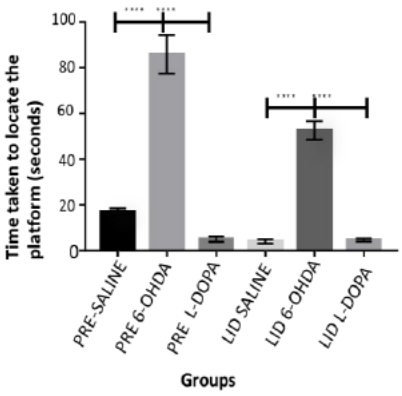

Figure 2a: Graph representing time taken by animals to locate a hidden platform (n=11)

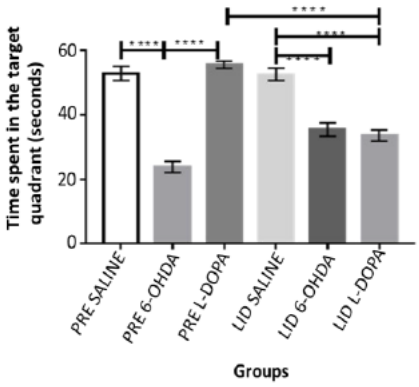

Figure 2b: Graph showing the amount of time spent by animals on the target quadrant after the platform had been removed (n=11)

4.2Molecular results

4.2.1 Expression of the Nptx2 gene in the PFC

There was a significantly reduced expression of the Nptx2 gene in the LID-L-dopa group as compared to LID-SALINE (*p=0,0395). There is a significant downregulation of the Nptx2 gene in LID L-dopa vs PRE-SALINE (**p=0,0098). (Figure 3)

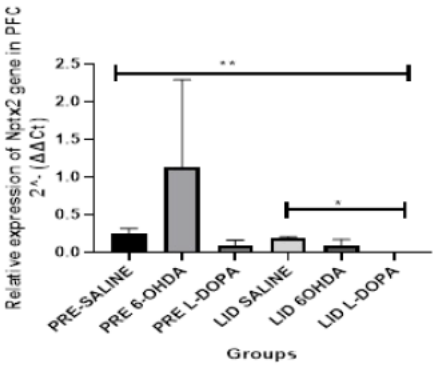

Figure 3: Expression of the Nptx2 gene in the pre-frontal cortex.

4.2.2 Expression of Th gene in the PFC.

A trend of upregulated TH gene is observed in the PRE-SALINE vs PRE 6-OHDA groups. This suggests that 6-OHDA may have induced the upregulation of this gene. An L-dopa effect is seen as determined by the significant downregulation of the TH gene in the L-dopa group, PRE-6-OHDA vs PRE-L-dopa (*p=0,0458). TH gene was overexpressed in the early stages of the disease and as the disease progressed, it was downregulated, PRE-6-OHDA vs LID 6-OHDA (*p=0,0319). Long-term L-dopa seems to be further downregulating the expression of the TH gene as there is a significant downregulation of the TH gene in the LID L-DOPA group compared to LID SALINE (*p=0,0149) (Figure 4).

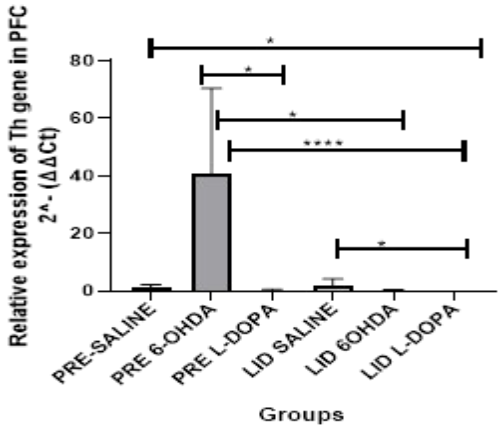

Figure 4: Graph showing the expression of the TH gene in the PFC.

4.2.3 Expression of FosB gene in the PFC.

FosB gene expression was significantly reduced in the prolonged L-dopa therapy group compared to the saline group LID SALINE VS LID L-DOPA (*p=0,0219) (Figure 5).

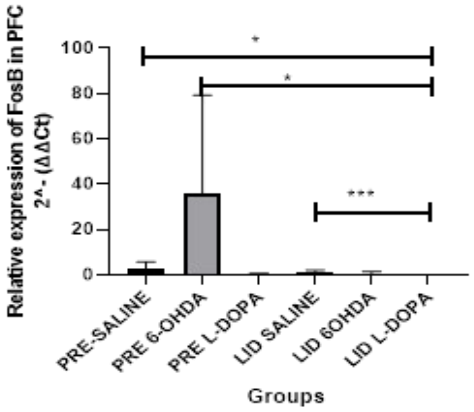

Figure 5: Graph showing the expression of the FosB gene in the pre-frontal cortex.

5. Discussion

The current study aimed to investigate changes in the PFC expression of Nptx2, FosB, and TH genes and correlate this with L-dopa-induced cognitive impairment seen in PD. We compared the effect of short-term L-dopa (PRE-LID phase/14 days of L-dopa) and long-term L-dopa (LID phase/28 days of L-dopa) administration on the expression of these genes. Behavioral tests showed that nigrostriatal lesions with 6-OHDA led to a decline in spatial learning, spatial memory, and recognition memory. L-dopa administration exacerbated recognition memory. Short-term L-dopa treatment (14 days) ameliorated both spatial learning and spatial memory while 28 days caused a decline in spatial memory but not spatial learning.

Our NORT results of the PRE-LID phase (Figure 1) showed a recognition impairment caused by nigrostriatal lesion with 6-OHDA (PRE SALINE vs PRE 6-OHDA) and an L-dopa effect exacerbated this impairment (PRE 6-OHDA vs PRE-L-DOPA). The results of the LID phase showed that the neurotoxin 6-OHDA caused a decline in recognition in the LID 6-OHDA group. These results align with cognitive studies in PD where it was reported that a nigrostriatal lesion with 6-OHDA causes cognitive decline and L-dopa is linked with its progression (Yoo et al., 2019, Haghparast et al., 2018). An L-dopa-induced cognitive impairment is observed in the LID L-DOPA group compared to LID SALINE.

Spatial learning results from Morris Water Maze Test are demonstrated in figure 2a and they show that animals from both PRE 6-OHDA and LID 6-OHDA presented with a spatial learning impairment, and

the L-dopa effect is seen to ameliorate this impairment in both PRE-L-DOPA and LID L-DOPA, respectively. These results are in agreement with what was reported in 2009 where L-dopa was shown to ameliorate learning deficits (Ambrée et al., 2009). A 6-OHDA effect (Figure 2b) is observed as rats from both PRE-6-OHDA and LID 6-OHDA spent less time on the target quadrant compared to controls (PRE SALINE and LID SALINE). This suggests a spatial memory decline and these are in line with what has been reported regarding this neurotoxin causing spatial memory impairment (Matheus et al., 2016). Short-term L-dopa therapy (14 days) was able to ameliorate the impairment while continued L-dopa (28 days) exacerbated spatial memory decline. Short-term L-dopa has been shown to enhance spatial memory (Cools et al., 2002), but continued L-dopa may cause confusion and lead to acute memory loss. L-dopa has also been reported to cause cognitive deficits in aged rats (Hernández et al., 2014).

Molecular results from the LID phase (Figure 3) show significant downregulation of the Nptx2 gene in animals that were on long-term L-dopa treatment compared to normal controls (LID SALINE vs LID L-DOPA). This suggests that the administration of L-dopa reduces the expression of the Nptx2 gene in the PFC. There could be a link between the downregulation of this gene and cognitive decline since our behavioral results suggest that continued L-dopa worsened recognition inability and spatial memory. Studies have reported that short-term L-dopa ameliorates cognition while chronic L-dopa further causes spatial memory impairment (Stansley and Yamamoto, 2015, Cools, 2006). The PRE-LID phase shows no significant expression of the Nptx2 gene within the groups. Although our behavioral results indicated that short-term L-dopa worsened recognition ability while improving spatial learning and memory, it does not have any effect on the expression of Nptx2 when it is consumed for a short period (14 days in our case). There is a trend of increased expression of Nptx2 in PRE-6OHDA, this could mean introducing neurotoxin 6-OHDA causes high expression of this gene, but as the time progresses (LID-6-OHDA), we do not see this trend which could be suggesting the adaptation of brain over a long exposure to the neurotoxin.

Nptx2 is a neuronal activity-regulated protein that is encoded by the Nptx2 gene and it stimulates the formation of excitatory synapses and may play a role in the regulation of learning and memory (Tang et al., 2019). Neuronal activity usually triggers the transcription of the Nptx2 gene, in PD this gene is known to be highly upregulated especially in the substantia nigra and cerebral cortex (Chapman et al., 2020, Moran et al., 2008). An Alzheimer's study reported a serum and cortical downregulation of this gene, the downregulation was linked with cognitive deficits assessed by psychometric tests (Xiao et al., 2017, Shao et al., 2020). Our results show the downregulation of this gene in continued L-dopa therapy and a positive correlation with the decline in cognitive functions.

In the PRE LID phase (Figure 4), we report a significant downregulation of the TH gene after L-dopa (PRE 6-OHDA vs PRE L-DOPA) which may be suggesting an L-dopa effect in downregulating the TH

gene. Since L-dopa replaces dopamine lost due to the 6-OHDA lesion, this could mean that TH no longer needs to overproduce in compensating dopamine because L-dopa gets converted to dopamine by an enzyme aromatic L-amino-acid decarboxylase. This is consistent with previous research where L-dopa has been shown to have the ability to reduce tyrosine levels (Riederer, 1980).

We note a significant downregulation of the TH gene in the LID Phase LID L-DOPA group compared to the LID SALINE group. This also may be because L-dopa elevates levels of dopamine in their neurons, and this may inhibit TH through feedback inhibition. TH has a significant role in the PFC as it is a rate-limiting enzyme in the production of catecholamines such as dopamine and noradrenaline, these are responsible for cognitive functions such as memory and cognition (Grace et al., 1997, Arnsten, 1997, Fernstrom and Fernstrom, 2007). It has been shown that there is an existing connection between working memory and the expression of the TH gene (Lee and Goto, 2015). Here we show that the TH gene gets downregulated by L-dopa, and this may be linked with cognitive declines as determined by NORT where long-time L-dopa further caused recognition impairment.

In the LID phase (Figure 5), we note a significantly downregulated FosB expression in the LID L-DOPA group compared to the LID SALINE group. Long-term L-dopa has been reported to lower the expression of FosB (Park et al., 2014). A shortened splice variant of the FosB gene which is ΔFosB has been reported to play a role in LID pathology (Du et al., 2015). An LID study reported a high striatal expression of ΔFosB in subjects that were on chronic L-dopa and developed LID. Our study, however, found a downregulated FosB gene in the PFC of rats that were on chronic L-dopa compared to control rats. Our results showed an L-dopa-induced PFC impairment that resulted in the downregulation of Nptx2, FosB, and TH genes in the LID L-DOPA group, and because PFC plays a central role in cognitive functions, changes to the expression of these genes may be a possible contributing factor in L-dopa induced cognitive decline (Cools et al., 2002).

In addition to its common motor symptoms, PD is also characterized by non-motor symptoms such as cognitive deficits (Owen, 2004). These cognitive deficits are usually associated with numerous changes such as abnormal gene regulation in the PFC which is known to be responsible for cognitive control, decision-making, attention, and impulse inhibition (Lee and Kesner, 2003). This means that any damage to this tissue may result in cognitive decline (Bosboom et al., 2004). In animal models, neurotoxin 6-OHDA mimics PD and behavioral tests have shown that this neurotoxin causes cognitive deficits (Kadowaki Horita et al., 2013).

A recent study has shown that PD patients that were on chronic L-dopa therapy and had LID developed cognitive decline quicker than PD patients that did not have LID (Yoo et al., 2019). It was found that patients that had LID had increased dementia compared to non-LID patients. They concluded that LID development may be closely linked to a decline in frontal lobe cognitive functions (Yoo et al., 2019). In vivo results from neuroimaging studies have brought to light that there are brain structural

irregularities in PD patients on chronic L-dopa, this includes PFC which has been observed to have a heightened density of grey matter (Yoo et al., 2019). Here, we carefully outline the gene expression changes in the PFC that may be caused by long-term L-dopa and correlate how this may be associated with cognitive dysfunction.

A recent study investigated L-dopa-induced genetic expression changes associated with cognitive decline in the PFC as most studies focus on the striatum (Radlicka et al., 2021), this study, however, does not include FosB, Nptx2, and TH genes. The striatum is known to be responsible for the regulation of voluntary movement whereas the PFC plays a role in cognitive control (Cools et al., 2019, Abd Wahab et al., 2019). Cognitive deficits develop as the disease progresses and the effect of L-dopa in alleviating cognitive decline remains unclear as some studies have reported its ability to ameliorate memory decline but was not able to improve learning inability (Aarsland et al., 2010, Simioni et al., 2017, Ghilardi et al., 2007).

Radlicka and colleagues suggested that it is possible that L-dopa affects the striatum and frontal cortex differently in rats, they suggested that the response may be dependent on specific mechanisms regulating each tissue (Radlicka et al., 2021). If the cells in the PFC respond differently to L-dopa than those on the striatum, it would be a reasonable explanation as to why the Nptx2, FosB, and TH genes are downregulated in the PFC after chronic L-dopa therapy. PD patients have been reported to present with both positive and negative cognitive responses to L-dopa, the feedback is dependent on the polymorphism of certain genes and the dopamine availability in the PFC and mesolimbic system (Calabresi et al., 2015).

6. Conclusion and recommendations

Overall, our results suggest a downregulation of Nptx2, FosB, and TH genes in the PFC caused by continued L-dopa therapy. Together with our behavioral results, we can conclude that the downregulation of these genes is associated with L-dopa-induced recognition impairment but may not be associated with spatial learning and memory. Nptx2, FosB, and TH genes are usually studied in the striatum, there is very minimal to no information about the role played by the expression of these genes in the PFC and how prolonged L-dopa therapy affects their expression. Future studies may investigate the relationship of similar patterns of Th, FosB, and Nptx2 genes downregulation in the PFC.

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Bridging

Previous studies have shown that long-term use of L-dopa may result in aberrant gene expression in the pre-frontal cortex which consequently induces cognitive decline. We investigated the effect of chronic L-dopa on FosB, Nptx2, and Th genes in the pre-frontal cortex and found that it significantly downregulated these genes. The results of gene expression corresponded with cognitive behavioral results where rats that were on long-term L-dopa treatment displayed cognitive impairment. It has also been shown that chronic L-dopa treatment eventually results in L-dopa-Induced Dyskinesias. The following study aimed at investigating whether neuroinflammation, through expression of inflammatory cytokines TNF-α and IL-1β, as well as serotonin deficiency play a role in the development of these dyskinesias.

Chapter 3: Manuscript 2

Role of serotonin deficiency and neuroinflammation in the pathology of L-dopa-Induced Dyskinesia

Abstract

Levodopa-Induced dyskinesias (LID) are abnormal involuntary movements that are experienced by Parkinson's disease (PD) patients as a result of long-term consumption of levodopa (L-dopa) therapy which is a gold-standard in the management of PD. LID have no known pathology although factors such as neuroinflammation (aberrant gene expression of pro-inflammatory cytokines) and dysregulation of serotonin have been implicated in their development. This study aimed to investigate the role of serotonin and neuroinflammation in the pathology of LID. Male rats were used as models for PD where they were injected with 6-OHDA to mimic PD and treated with L-dopa. Striatum and hippocampal tissue was harvested for molecular and neurochemical analysis respectively. Our results suggest that prolonged L-dopa consumption results in serotonin deficits and this deficit is directly proportional to the development of LID. We also found that TNF-α gene expression is triggered by long-term L-dopa administration which may be associated with the development of LID. There was no dysregulation in IL-1β gene expression, suggesting that this gene may not be implicated in the development of LID. Our data suggest that prolonged L-dopa therapy may lead to serotonin deficiency and upregulation of TNF-α gene expression which may play a contributory role in the development of LID.

Keywords: Parkinson's disease, L-dopa-Induced Dyskinesia, TNF-α, IL-1β, Serotonin

1. Introduction

Levodopa (L-dopa) is the gold standard in managing motor symptoms of Parkinson's disease (Axelsen and Woldbye), which assists in improving the quality of life in patients (Gandhi and Saadabadi, 2020). L-dopa is a biological precursor of dopamine (DA) (Michalicha et al., 2021), and it is used as a DA replacement agent (Gandhi and Saadabadi, 2020). L-dopa-Induced Dyskinesia (LID) is a major complication in patients that have PD and are on chronic L-dopa therapy (Palafox-Sanchez et al., 2019). LID is defined as abnormal involuntary movements that are experienced by 50% of PD patients who consume L-dopa for a prolonged period (Chaudhuri et al., 2019). The pathology of LID is not well understood; however, a few possible factors may be contributors to its development. These include impairments between motor cortex and striatum connectivity which results in basal ganglia dysfunction hence the development of involuntary movements (Pandey and Srivanitchapoom, 2017). Another possible contributor is the period patients have been on L-dopa therapy for, the severity of dyskinesia is directly proportional to the duration of L-dopa consumption (Cenci, 2016). Dysfunction of pre-and post-synapses leads to failure of nigrostriatal DA transmission and causes DA fluctuation in the brain, this leads to an irregular response from dopaminergic neurons (Metman et al., 2000).

Neuroinflammation has also been implicated as one of the main biomarkers in the pathology of LID (Santos-Lobato et al., 2021). Cell culture studies have reported that high doses of L-dopa can be lethal to dopaminergic neurons and it must only be taken when other anti-Parkinson drugs are no longer effective (Carta et al., 2017). L-dopa causes oxidative stress in the brain, this triggers the inflammatory response of microglial cells which are the first line of defense in the central nervous system (Carta et al., 2017). When activated, microglial cells release pro-inflammatory cytokines such as TNF-α and IL-1β (dos Santos Pereira et al., 2021). Emerging evidence has shown that there is an activation of microglial cells in the striatum of rats that were experiencing LID (dos Santos Pereira et al., 2021). What is not clear is whether cytokine release by activated microglia could be directly proportional to the severity of dyskinesia.

Amongst other factors, serotonin has also been implicated in the development of LID. L-dopa gets converted to DA by the remaining dopaminergic neurons hence replacing lost synaptic DA levels (Carta and Bezard, 2011). However, as the disease progresses these remaining dopaminergic neurons also degenerate, which means no dopaminergic neurons are remaining to convert L-dopa to DA (Carta and Tronci, 2014). Serotonin neurons have been reported to play an active role in the conversion of L-dopa to dopamine (Lopez et al., 2001). This is because serotoninergic neurons have a similar enzymatic apparatus as DArgic ones (Arai et al., 1995). The challenge with serotonergic neurons is that the feedback mechanism to regulate DA levels on synapses is absent. The lack of this mechanism results in an uncontrollable synaptic DA release, which is thought to be a contributing factor to the development of LID (Carta and Tronci, 2014).

This study aimed to investigate the effects of short-term and long-term L-dopa administration on striatal and hippocampal serotonin levels, as well as TNF-α and IL-1β gene expression as possible contributors of LID pathology, with correlation to AIMS scores of each group.

3. Methods and materials

3.1 Ethical approval

This study was approved by the University of KwaZulu-Natal Animal Ethics Research Committee (AREC/00003538/2021) and the South African Department of Agriculture, Land Reform and Rural Development (DALRRD) – 12/11/1/5/2 (1684JD).

3.2 Animal grouping

72 Male Sprague-Dawley (SD) rats were obtained from the Biomedical Resource Unit of the University of KwaZulu-Natal. The rats were acclimatized for 7 days in an environmentally controlled facility and provided with food and water *ad libitum*. Rats were randomly divided into 2 phases: the PRE-LID phase (14 days of L-dopa treatment) and the LID phase (28 days of L-dopa treatment). Both these phases consisted of 3 groups of animals (Saline, 6-OHDA, and L-dopa-treated), with n=12 per group.

3.3 Stereotaxic surgery

After acclimatization, all animals underwent stereotactic surgery where they were lesioned in the nigrostriatal pathway. Prior to surgery, 125mg/kg of ketamine and 10mg/kg of xylazine were administered intraperitoneally, to serve as anesthesia (Veilleux-Lemieux, 2013). After stabilization of anasthesia, rats were placed in a stereotaxic frame and incision was made in their scalp. These coordinates were used: 4.7 mm anterior to lambda and mediolateral – 1.6 mm from the midline and dorsoventral, -8.4 mm below the skull (Howells et al., 2005). 6-OHDA and L-dopa groups of both PRE-LID and LID phases were unilaterally injected with 6-OHDA (8µg/µl) in 0.2% of ascorbic acid (Dalla Vecchia *et al.*, 2018) by Hamilton syringe in the nigrostriatal pathway. The syringe was kept for 8 minutes for well diffusion of 6-OHDA, then it was gently removed (Mabandla *et al.*, 2015). The animals from the saline groups of both phases were lesioned with saline using the same procedure. After surgery, rats were subcutaneously injected with an analgesic (0.05mg/kg). The lesion was allowed to stabilize for one week (Ndlovu et al., 2016).

3.3 Treatment

After the week of lesion stabilization was over, the L-dopa group of animals from the PRE-LID phase was subcutaneously injected with L-dopa (50mg/kg) for 14 consecutive days while the same group from the LID phase received the exact treatment for 28 consecutive days. This was done at 9 am and 4 pm (Ndlovu et al., 2016). Animals from the saline groups were handled only, while the 6-OHDA groups were subcutaneously injected with saline.

3.4 AIM score

Each rat was kept in a crystal-clear plastic cage for about 10 minutes before the L-dopa injection. L-dopa was administered for 28 consecutive days and dyskinesias were measured and recorded at 9 am and 10 am daily from day 21 to day 28 (Ndlovu et al., 2016). The dyskinesias' severity was measured using abnormal involuntary movements score (AIMs) (Ndlovu et al., 2016). A scale of 0 to 4 was used to rate the severity of AIMs in the axial limb and orolingual, however, locomotive AIMs assessment will differ from these (Ostock et al., 2011). The scale was elaborated to be: 0 = not present, 1= occasional, 2 =frequent, 3 =continuous but interrupted by sensory distraction, and 4= continuous, severe, and not interrupted by sensory distraction (Ndlovu et al., 2016). Various AIM subtypes were assessed visually: (i) Axial dystonia which is contralateral twisted posturing of the neck and upper body. (ii) Abnormal orolingual movements which are stereotyped jaw movements and contralateral tongue protrusion. (iii) Abnormal forelimb movements which are seen to be repetitive rhythmic dystonic posturing of the contralateral forelimb and/or grabbing movements of the contralateral paw (Ostock et al., 2011). The AIMs overall was determined from collected data of each rat and the total of each rat in a group was summed to determine the total of each group (Ndlovu et al., 2016).

3.5 Euthanasia

After 14 and 28 days of L-dopa treatment, rats from the PRE-LID phase and LID phase, respectively, were decapitated using a guillotine. Surgical scissors were used to open the skull. Once open, brain tissue was extracted and kept in a frozen 0.9% saline slush before the striatum and hippocampus were dissected from it. All collected samples were placed in Eppendorf tubes, snap-frozen in liquid nitrogen, and then transferred to a -80 C in the bio-freezer for storage before neurochemical analysis.

3.6 Reverse transcription PCR (RT-PCR)

3.6.1 RNA extraction

Following the manufacturer's guidelines, a Quick-RNA Miniprep Kit from Zymo research was used to extract RNA from the tissue (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). Briefly, at room temperature, 96 ml of 100% ethanol was added to 24 ml of RNA wash buffer. Frozen PFC and striatum tissues were homogenized in an RNA lysis buffer. Debris was removed by centrifuging and the supernatant was transferred into a nuclease-free tube. It was filtered again by centrifugation to remove most of the genomic DNA, flow-through was saved and 95% of ethanol was added to it (at the ratio of 1:1). 400ul of RNA prep buffer was added to the mixture and it was centrifuged, flow-through discarded. 700 ul of RNA wash buffer was added to the tube and centrifuged. Flow-through was discarded and 400ul of RNA wash buffer was added and the mixture was centrifuged for a minute. Lastly, 100 ul of DNase-free water was directly added to the tube and it was centrifuged. All centrifuges were done at 10000xg for 30 seconds unless specified.

3.6.2 cDNA synthesis

cDNA was synthesized from RNA based on the supplier's guidelines from BioLabs Inc.ProtpScript II first-strand cDNA synthesis kit (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). The reaction tube had 1µl of RNA, 2µl of d(T)23 VN, 10µl of ProtoScript II Reaction Mix, and 2µl of ProtoScript II Enzyme Mix. 5µl of nuclease-free water was added which summed up the reaction to 20µl. The tube was gently vortexed and incubated at 42^0C for an hour. After which, the enzyme was heat-inactivated at 80 C for 5 minutes.

3.6.3 Gene expression

Tumour Necrosis Factor-Apha and IL-1β gene primers were ordered from Inqaba Biotechnical Industries (Pty) Ltd, South Africa. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Primer sequence specifications for all genes (Tnf-α, IL-1β, and GAPDH) are in table 1 below. A PCR tube mixture contained a total of 10µl reaction volume: 5µl of dye SYBR GREEN (Sigma-Aldrich, South Africa), 2µl of synthesized cDNA, 1 µl of forward primer, 1 µl of reverse primer, and 1 µl of nuclease-free water. PCR was run using a LightCycler® 96 instrument (Roche Life Science, South Africa). Parameters of the PCR were: Pre-incubation at 95^0C for 600 seconds, 3-step amplification at (i) 95^0C for 15 seconds, (ii) 60^0C for 30 seconds with 55 cycles, (iii) 72^0C for 30 seconds, melting was done at (i) 95^0C for 10 seconds, (ii) 65^0C for 60 seconds, (iii) 97^0C for one second, lastly cooling was done at 37^0C for 30 seconds.

Table 1: The primers sequences of the following genes: TNF-α, IL-1β, and GAPDH

Primer	Forward primer	Reverse primer
TNF-α	5'- GTC TGT GCC TCA GCC TCT TC -3'	5'- CCC ATT TGG GAA CTT CTC CT
IL1-β	5'- AGG ACC CAA GCA CCT TCT TT-3'	5'- AGA CAG CAC GAG GCA TTT TT-3'
GAPDH	5′-GGCATTGCTCTCAATGAC AA-3′,	5′-ATGTAGGCCATGAGGTCC AC-3′

3.6.4. Delta-delta Ct method of analysis

The delta-delta Ct method (2^ - (ΔΔCt) of results analysis was employed to analyze gene expression. This method was first introduced in 2009 by Bustin and colleagues as Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). The method was performed as follows:

- Calculation of ΔCt = Ct of a gene of interest – Ct of GAPDH
- Calculation of average ΔCt for the control group (saline + handling)
- Calculation of ΔΔCt = ΔCt of each sample – ΔCt of control (averaged), and

- Calculation of 2^-(ΔΔCt), representing the expression of our genes of interest relative to the housekeeping gene GAPDH.

3.7 Enzyme-linked immunosorbent assay (ELISA)

Following the manufacturer's instructions, the concentration of serotonin was measured using a competitive ELISA kit by ElabSceince (Biocom Africa (Pty) Ltd, South Africa). Excess blood was removed from the tissue (hippocampus) by grounding it into small pieces and rinsing it with ice-cold PBS (0.01M. pH=7.4). Tissue was weighed and homogenized in PBS (1g of tissue: 9mL of PBS). Homogenate was centrifuged for 5 minutes at 5000xg to get supernatant. In a 96-well plate, the first two columns contained 50µl of standards in duplicates. In other well columns, 50µl of samples were added. 50µl of Biotinylated Detection Ab working solution was added to each well. The plate was incubated at 37^0C for 45 minutes. Each well content was decanted and 350µl of wash buffer was added to each well. This was socked for 120 seconds and repeated three times. 100µl of HPR conjugate solution was added to each well. The plate was sealed and incubated for half an hour at 37^0C. The plate content was aspirated and washed using a wash buffer five times. 9µl of Substrate reagent was added and the plate was incubated for 15 minutes at 37^0C. A stop solution of 50µl was added to each well and the plate was read at 450nm using SPECTROstarNANO micro-plate reader (BMG LABTECH).

5. Statistical analysis

For statistical analysis of serotonin results and all gene expression data (Bustin et al., 2009), GraphPad Prism version 8 (GraphPad Software Inc. California USA) was used. Data were tested for normality using the Shapiro-Walk normality test. One-way analysis of variance (Cools et al.) Kruskal-Wallis non-parametric test was used, followed by Dunn's post hoc multiple comparison test. P values were considered statistically significant if $p<0.05$. All data were expressed as mean ± SEM.

Table 2 shows asterisks and their P-value description

Asterisk	Description
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
****	$P \leq 0.0001$

5. Results

5..1 Behavioural results

5.1.1 Abnormal Involuntary movements score (AIMs)

The graph below is a demonstration of abnormal involuntary movements known as Levodopa-Induced Dyskinesia (LID) in a group that was on L-Dopa for 28 days. The scoring started from day 21 -28 of L-dopa treatment and there were no significant differences in the severity of LID between the 8 days of observation. There was however a trend suggesting consistency in the occurrence of LID. LID was not tested on the PRE-LID phase since the rats did not physically exhibit any sign of dyskinesia.

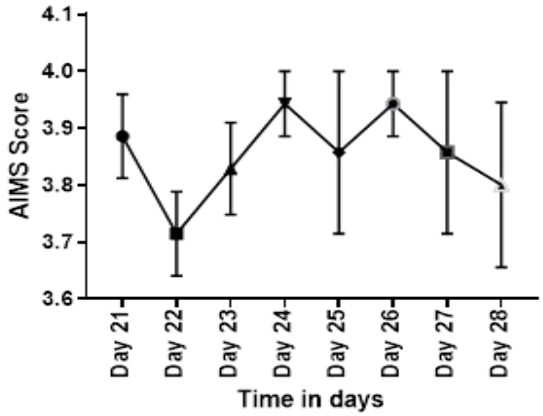

Figure 1: A line graph showing the occurrence of LID assessed using an AIM score for 8 days post-L-dopa in the LID L-dopa group.

5.2 Neurochemical results

5.2.1 ELISA concentration of hippocampal serotonin

There was a decrease in serotonin concentration in the LID L-DOPA group when compared to the LID 6-OHDA (**p=0.0091) and PRE-SALINE (***p=0.0002). A significant decrease in the concentration of serotonin is also noted in the PRE-L-DOPA (**p=0.0014), PRE-6-OHDA (**p=0.0022), and LID SALINE (**p=0.0014) groups compared with the PRE-SALINE group.

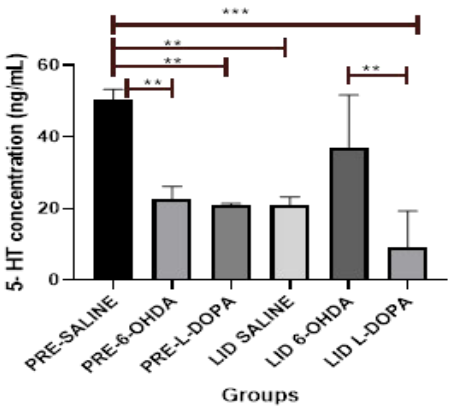

Figure 2: A graph showing the concentration of serotonin in the hippocampus.

5.2.2 ***Gene expression of IL-1β***

No statistical significance was observed between the groups; however, we noted a trend of increased gene expression in the PRE L-DOPA group which suggests an L-DOPA effect that resulted in the upregulation of IL-1β.

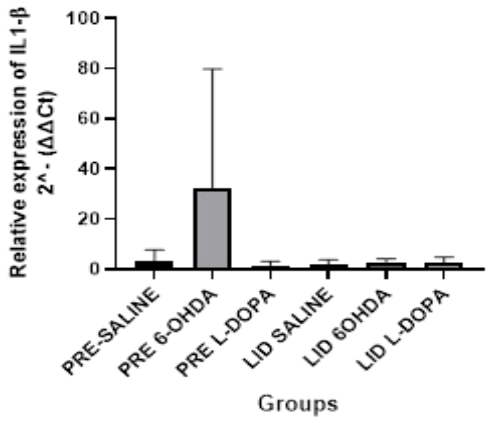

Figure 3. A graph illustrating the expression of the striatal IL-1β gene.

5.2.3 Gene expression of striatal Tnf-α

There was a significant overexpression of TNF-α gene in LID L-DOPA group compared to LID 6-OHDA (***p=0.0002). An upregulation of TNF-α is noted in the LID L-DOPA group compared to LID saline (***p=0.0002). A significant expression of TNF-α gene is seen in LID L-DOPA compared to PRE-L-DOPA (***p=0.0002).

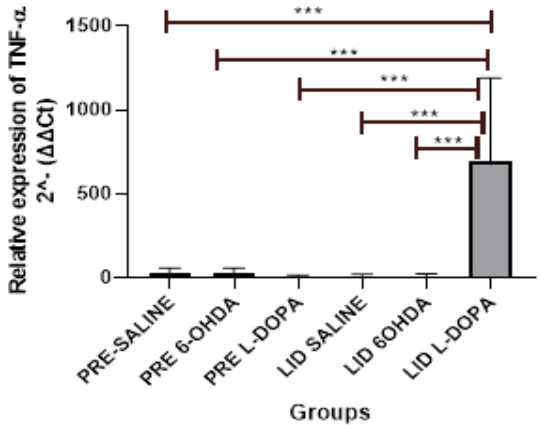

Figure 4. A graph showing the expression of TNF-α gene in the striatum.

6. Discussion

The current study aimed to investigate the implications of striatal neuroinflammation and hippocampal serotonin in LID pathology. We investigated alterations in serotonin, TNF-α, and IL1-β genes in animals that are on short-term L-dopa therapy (14 days) and those that were on long-term L-dopa therapy (28 days).

Fluctuations in other neurotransmitter systems (other than DAnergic systems) such as glutamatergic, serotonergic, and adrenergic systems have been reported to contribute to the development of LID (Zheng et al., 2021). Here, we specifically focused on the role that may be played by hippocampal serotonin in LID pathology. We found a significantly low concentration of serotonin in the PRE 6-OHDA compared to the PRE-SALINE group suggesting a 6-OHDA effect in reducing serotonin. Our results align with previous studies where 6-OHDA lesioned rats presented with reduced hippocampal serotonin transporters (Walker et al., 2020, Santiago et al., 2014). We report a significantly reduced serotonin in PRE-L-DOPA compared to PRE-SALINE, no significance was seen between PRE 6-OHDA and PRE L-DOPA, but there is a trend suggesting a possible further decrease of serotonin that may be induced by L-dopa administration. This is supported by an *in vitro* study which reported that L-

dopa can damage serotonergic cells (Stansley and Yamamoto, 2015). In the later stages of L-dopa treatment, we now observed a significant decrease in serotonin concentration in LID L-DOPA group compared to LID 6-OHDA group. This implies ability of L-dopa to reduce serotonin levels that may have been elevated by neurotoxin 6-OHDA although there is no remarkable significance, an increasing trend is seen in LID 6-OHDA compared to LID SALINE. Post-mortem studies have detected a serotonin deficit in PD brains (Kish et al., 2008). Serotonergic neuron denervation has been investigated and is suspected to start in the early stages of PD (Politis and Loane, 2011). An in vivo study reported that lesion with 6-OHDA did not affect serotonin levels, while administration of L-dopa reduced serotonin in the hippocampus (Navailles et al., 2010a). Looking at behavioral results, the AIM score graph (figure 1) shows a consistently severe LID in the LID-L-DOPA group, this suggests that serotonin deficit that is induced by continued L-dopa is directly proportional to the severity of LID.

Serotonergic neurons have been reported to have the ability to take up L-dopa and decarboxylate it to dopamine, (Stansley and Yamamoto, 2013, Miller and Abercrombie, 1999). Although L-dopa restores lost dopamine in the striatum, it has been reported that not only striatum dopamine is restored, but there has been an elevation of dopamine concentration in other brain areas that usually have little or no dopamine, including the hippocampus (Navailles et al., 2010b). This is because as L-dopa enters the central nervous system, it is converted to dopamine by aromatic amino-acid decarboxylase, an enzyme that is not specific to dopamine neurons only but can be found in other neurons such as those of serotonergic systems (Ugrumov, 2009). Moreover, dopamine released in extra-striatal brain areas which include the hippocampus has been reported to be released by serotonergic neurons (Kannari et al., 2001). This dopamine release serotonergic neurons lack the feedback mechanism to control the fluctuation of dopamine in the synapses (Carta and Tronci, 2014). The uncontrolled fluctuation of dopamine has been implicated in the development of LID, this was proven when serotonin neurons afferents were removed by serotonin agonist, and this resulted in the cessation of LID (Carta et al., 2007).

Our behavior results show that animals on chronic L-dopa experienced severe LIDs, these animals also present with a significant reduction of serotonin which is induced by L-dopa (figure 2). This data corresponds with previous studies that showed that since dopamine is an oxidant, its high concentrations (induced by L-dopa), can be neurotoxic to serotonergic neurons (Berman and Hastings, 1999). The cause of serotonin deficits induced by chronic L-dopa in our results may be because dopamine (converted from L-dopa) has the potential to inactivate a rate-limiting enzyme (tryptophan hydroxylase) which is responsible for the production of serotonin (Kuhn and Arthur, 1998). Overall, these results suggest that prolonged L-dopa consumption may results in serotonin deficits and this deficit may be directly proportional to the development of LID.

Amongst other factors, neuroinflammation has been implicated in the pathology of LID. Here we assessed if TNF-α and IL-1β genes contribute to the development of LID. Microglial cells are

responsible for the inflammatory response in the brain, they are the first line of defense in the central nervous system (Mosher and Wyss-Coray, 2014). In animal models, 6-OHDA lesion in the medial forebrain bundle has been reported to cause oxidative stress that leads to microglial activation, and when activated, they act by releasing pro-inflammatory cytokines such as TNF-α and IL-1β in response to neuroinflammation (Carta et al., 2017). Biochemical data from the post-mortem brain in previous research has revealed that the continuation of L-dopa therapy elevates neuroinflammation in the striatum (Bortolanza et al., 2015).

The aim here was to look at the poorly understood pathology of LID and how neuroinflammation may be implicated in this pathology. The main goal was to assess the effect of long-term consumption of L-dopa therapy in relation to the levels of pro-inflammatory cytokines TNF-α and IL1-β. A significantly high expression of the TNF-α gene in the LID L-DOPA group as compared to the PRE-L-DOPA is noted. This may mean that long-term consumption of L-dopa causes striatal neuroinflammation that results in microglial activation, which is the first line of defense in the central nervous system. This is supported by a recent study that showed that continued L-dopa administration elevates TNF-α concentration in the striatum (Zheng et al., 2021). TNF-α is normally non-toxic to dopaminergic neurons but its aberrant upregulation or downregulation may have a detrimental effect on these neurons (McGuire et al., 2001). TNF-α is said to be further upregulated by L-dopa therapy which is then assumed to be one of the reasons for the development of LID (Pisanu et al., 2018). This could explain why we have severe LID in our behavioral results (figure 1). Another significantly low concentration of TNF-α in LID 6-OHDA compared to LID L-DOPA is seen. This result may mean that long-term L-dopa further causes the elevation of TNF-α that was initially triggered by the introduction of 6-OHDA. Evidence from animal studies showed high expression of the TNF-α gene in dyskinetic animals, this upregulation and severity of LID were ameliorated after animals were given anti-inflammatory cytokines (Barnum et al., 2008).

Looking at IL-1β results (figure 3), there is no significant expression of this gene, there is however a trend of possible upregulation in the PRE-6-OHDA group. This result suggests that IL-1β may have no part in the development of LID. Or it could be because the response of microglia is dependent on the nature of the external stimulus entering the brain, they have the flexibility to take up various phenotypes dependent on the stimulus and consequently respond to each stimulus according to its nature and intensity (Joers et al., 2017, Olah et al., 2011). If microglia are able to take up different phenotypes, it suggests that the term 'activated' limits the diverse functions these cells can perform. Microglia can either be activated and produce a pro-inflammatory cytokine phenotype or they can undertake the state of being dystrophic which will limit them to provide neuroprotection (Olah et al., 2011). This could be the case with the lack of IL-1β gene expression in our results.

7. Conclusion and recommendation

Overall, our data suggest that long-term consumption of L-dopa causes aberrant upregulation of the TNF-α gene as compared to short-term L-dopa, therefore this gene may be a possible contributing factor towards the severity of LID demonstrated by our behavioral results. The lack of gene expression dysregulation in IL-1β results suggest that IL-1β may not play a contributory role in LID pathology. Serotonin results suggest that prolonged L-dopa consumption results in serotonin deficits which may be directly proportional to the development of LID. Future research may focus on looking at role of L-dopa alone in the expression of IL-1β and TNF-α and compare this with role of L-dopa combined with other drugs.

8. References

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Chapter 4: Synthesis, conclusions, and recommendations

1. General synthesis

Parkinson's disease (Axelsen and Woldbye) is a neurological disorder that is a result of the death of brain cells that are responsible for movement. Its symptoms include slowness of movement, tremors, and rigidity, these are collectively categorized as motor symptoms while non-motor symptoms include depression, insomnia, and cognitive dysfunction. Although it has no known pathogenesis, neurons responsible for dopamine (DA) transmission between cells have been implicated to be the key players in the pathology of PD. DA is responsible for reward and movement in the central nervous system, it also plays role in the regulation of cognitive function. The main source of DA in the brain is the substantia nigra hence its cells degenerate during the progression of PD.

No cure has been successful in reversing the progression of PD, however, DA replacement with levodopa (L-dopa) has been the gold standard in managing motor symptoms. It is still unclear whether L-dopa is also able to alleviate non-motor symptoms. Although it is a gold-standard, long-time consumption of L-dopa results in motor complications of abnormal involuntary movements known as L-dopa-induced dyskinesia (LID). The mechanism involved in the development of LID is still not known, however striatal neuroinflammation, and serotonin deficits have been implicated in the pathology of LID. Here we investigated neuroinflammation and serotonin in subjects that were on chronic L-dopa where we found significant upregulation of the TNF-α gene and no significant changes in the IL-1β gene, we also observed a decline in serotonin levels in rats that were on chronic L-dopa.

Cognitive decline is a huge complication seen in PD as well with little to no known pathophysiology. This study also aimed and looked at how chronic L-dopa causes genetic alterations in the pre-frontal cortex (PFC) and eventually leads to cognitive dysfunction. We looked at FosB, Nptx2, and TH genes in the PFC. We correlated the results of our genes' expression with behavioral tests for cognition, where we found that the genes were highly expressed in groups that were on chronic L-dopa treatment and these groups had impaired cognition.

2. Conclusions

Overall, this study showed that although L-dopa is effective in managing motor symptoms of PD, its long-term usage may have harmful outcomes for PD patients. Our data suggest that continued L-dopa administration may result in the abnormal regulation of the expression of FosB, Nptx2, and TH genes in the PFC which correlates to neurocognitive deficits. We have also shown that its prolonged consumption triggers neuroinflammation through upregulation of the TNF-α gene, and this may be implicated in the development of LID as shown by the AIM score. Our data show that IL-1β gene expression may not be affected by continued L-dopa treatment.

Appendices

Appendix 1: Ethical approval certificate

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

23 February 2022

Ms Nombulelo Agata Mthembu (214505143)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Ms Mthembu,

Protocol reference number: AREC/00003538/2021
Project title: Role played by serotonin receptors and inflammatory cytokines in LID pathology in a Parkinsonian rat model.

Full Approval – Research Application

With regard to your revised application received on 12 September 2021, the Animal Research Ethics Committee has accepted the documents submitted and **FULL APPROVAL** for the protocol has been granted.

This approval covers the following student co-investigators studies:

1. Nthlane, Refentshe Amandu'S (216006515) Title: FosB expression as a possible link to L-dopa addiction in early phase treatment of Parkinsonism.
2. Ngalonkulu, Ongeziwe (218022342) Title: Role of Th gene expression in development of LID-induced cognitive decline in Sprague-Dawley rats
3. Mqadi, Andiswa (217005099) Title: Comparison of Nptx2 expression between pre-LID and post-LID phases in 6-OHDA-induced Parkisonism

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

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 22 February 2023.

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

Yours faithfully [redacted]

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee
/kr
cc Supervisor: Dr Zama Msibi
cc BRU Manager: Dr Jaca

Animal Research Ethics Committee (AREC)
Ms Karen Reinertsen (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8850 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Appendix 2: DAFF approval letter

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Gololo • Tel: +27 12 319 7622 • Fax: +27 12 319 7470 • E-mail: HenryG@ealrd.gov.za
Reference: 12/11/1/5/2 (1684.ID)

Responsible person(s): Miss Nombulelo Agata Mthembu
Institution: Department of Human Physiology, University of KwaZulu-Natal
Email: Mslbiz2@ukzn.ac.za

Dear Miss Mthembu

CONDITIONS FOR RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Title of research project / study: "Investigating the role of serotonin receptors and inflammatory cytokines in LID pathology in a Parkinsonian rat model"

Your application, requesting permission under Section 20 of the Animal Diseases Act, 1984 (Act no 35 of 1984) to perform the research project or study stipulated above, refers.

1. Based on the information provided in your application, the Director of Animal Health has no objection to this study. The study may continue if statement 1.1 to 1.6 hereunder are, and remain, accurate. **Should the scope of your research project change in any way you are required to inform the Section 20 Secretariat and may not proceed with any activities until written permission to do so have been granted by the National Director: Animal Health.**

   1.1. No work will be done with controlled and notifiable animal diseases (list can be obtained / requested from this office), which includes any animal diseases which do not occur in South Africa;
   1.2. No imported material of animal origin or imported animal pathogens will be utilized in the study;

Appendix 3: Animal training certificate

UNIVERSITY OF KWAZULU-NATAL

September 13, 2019

Dear Dr Singh
Chair: Animal Research Ethics Committee
c/o School of Life Sciences

RE: COMPETENCE TRAINING ON RATS ONLY

This letter confirms that Miss Nombulelo Mthembu: 214505143 has undergone an evaluation for invasive procedures on the 13 September 2019 and shows competence regarding the following:

a. Animal handling
b. Intraperitoneal injection
c. Subcutaneous injection

Kind regards

[redacted]

Dr Juna
Veterinarian

To Reduce Replace and Refine Animal Research

Appendix 4: cDNA synthesis manual

For life science research only.
Not for use in diagnostic procedures.

Roche

cDNA Synthesis System

Version: 24
Content version: October 2017

For the synthesis of double-stranded cDNA from total RNA or mRNA

Cat. No. 11 117 831 001 1 kit
up to 10 reactions

Store the kit at −15 to −25°C

sigma-aldrich.com

Appendix 5: RNA isolation manual

ZYMO RESEARCH

RNA Purification

Quick-RNA™ Miniprep Kit

RNA from any sample

Highlights

- Spin-column purification of total RNA (including small/microRNAs) from cells and tissue.
- You can opt to isolate total RNA (≥ 17 nt) or isolate small (17-200 nt) and large RNAs (> 200 nt) into separate fractions.
- DNA-free RNA is ready for Next-Gen Sequencing, RT/qPCR, etc. *DNase I is included.*

Catalog Numbers:
R1054, R1055

Scan with your smart-phone camera to view the online protocol/video.

Appendix 6: SYBR green manual

BIO-RAD

iTaq™ Universal SYBR® Green Supermix

Catalog #	Supermix Volume	Kit Size
172-5120	2 ml (2 x 1 ml vials)	200 x 20 µl reactions
172-5121	5 ml (5 x 1 ml vials)	500 x 20 µl reactions
172-5122	10 ml (10 x 1 ml vials)	1,000 x 20 µl reactions
172-5124	25 ml (5 x 5 ml vials)	2,500 x 20 µl reactions
172-5125	50 ml (10 x 5 ml vials)	5,000 x 20 µl reactions

For research purposes only.

Storage and Stability
Guaranteed for 12 months in a constant temperature freezer at –20°C protected from light. For convenience, this supermix can be stored at 4°C short-term or refrozen up to ten times.

Kit Contents
iTaq™ Universal SYBR® Green supermix is a 2x concentrated, ready-to-use reaction master mix optimized for dye-based quantitative PCR (qPCR) on any real-time PCR instrument (ROX-independent and ROX-dependent). It contains antibody-mediated hot-start iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR® Green I dye, enhancers, stabilizers, and a blend of passive reference dyes (including ROX and fluorescein).

Instrument Compatibility
This supermix is compatible with all Bio-Rad and ROX-dependent Applied Biosystems real-time PCR instruments, and with the Roche LightCycler LC480, QIAGEN Rotor-Gene Q, Eppendorf Mastercycler EP realplex, and Stratagene Mx real-time PCR systems.

Reaction Mix Preparation and Thermal Cycling Protocol
1. Thaw iTaq™ Universal SYBR® Green supermix and other frozen reaction components to room temperature. Mix thoroughly, centrifuge briefly to collect solutions at the bottom of tubes, and then store on ice protected from light.

2. Prepare (on ice or at room temperature) enough assay master mix for all reactions by adding all required components except the DNA template according to the following recommendations (Table 1).

Table 1. Reaction Setup*

Component	Volume per 20 µl Reaction	Volume per 10 µl Reaction	Final Concentration
iTaq™ Universal SYBR® Green supermix (2x)	10 µl	5 µl	1x
Forward and reverse primers	Variable	Variable	300–500 nM each
DNA template	Variable	Variable	cDNA: 100 ng–100 fg Genomic DNA: 50 ng–5 pg
H_2O	Variable	Variable	—
Total reaction mix volume	*20 µl*	*10 µl*	—

* Scale all components proportionally according to sample number and reaction volumes.

3. Mix the assay master mix thoroughly to ensure homogeneity and dispense equal aliquots into each qPCR tube or into the wells of a qPCR plate. Good pipetting practice must be employed to ensure assay precision and accuracy.

4. Add DNA samples (and DNase-free H_2O if needed) to the PCR tubes or wells containing assay master mix (Table 1), seal tubes or wells with flat caps or optically transparent film, and vortex 30 seconds or more to ensure thorough mixing of the reaction components. Spin the tubes or plate to remove any air bubbles and collect the reaction mixture in the vessel bottom.

5. Program thermal cycling protocol on the real-time PCR instrument according to Table 2.

6. Load the PCR tubes or plate onto the real-time PCR instrument and start the PCR run.

7. Perform data analysis according to the instrument-specific instructions.

Appendix 7: ELISA manual

Elabscience®

(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !)

Catalog No : E-EL-0033
Product size: 96T/48T/24T/96T*5

ST/5-HT(Serotonin/5-Hydroxytryptamine) ELISA Kit

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Phone: 240-252-7368(USA)
Fax: 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please refer to specific expiry date from label on the side of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.