MOLECULAR TARGETS FOR CENTRAL NERVOUS SYSTEM DISORDERS

INVESTIGATION OF NEUROPROTECTIVE TARGETS FOR PARKINSON'S DISEASE AND THEIR ROLE IN PATHOPHYSIOLOGY WITH A SECONDARY LOOK AT A MOLECULAR TARGET FOR SCHIZOPHRENIA

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A Thesis

Submitted to the School of Graduate Studies in Partial Fulfilment of the

Requirements for the Degree

Doctor of Philosophy

DOCTOR OF PHILOSPHY (Health Science – Neuroscience) (2019) McMaster University, Hamilton, Ontario

TITLE: Investigation of neuroprotective targets for Parkinson's disease and their role in pathophysiology with a secondary look at a molecular target for schizophrenia

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NUMBER OF PAGES: 186

LAY ABSTRACT

Brain diseases like Parkinson's disease (PD) and Schizophrenia (SZ) are difficult to treat because their cause has not been discovered. PD shows degeneration of cells in the brain but the cause for degeneration is unknown. This makes developing treatments to protect cells from dying difficult. Two pathways are suggested to cause cell death in PD. This thesis proposes that both pathways are responsible for degeneration through a combined effort. Here, both pathways are shown to lead to cell death resembling PD and specific molecules are suggested as targets for developing protective treatments. Like PD, SZ has symptoms that cannot be treated because the cause is unclear. A protein was investigated for producing SZ-like symptoms and found to have potential for treatment design. This thesis aims to understand molecular changes in the brain leading to PD, with a look at SZ and how they can be used for better treatment design.

ABSTRACT

Disorders of the central nervous system (CNS) continuously pose problems for current therapeutics. In part, this is due to the uncertainty of underlying pathophysiological changes that give rise to specific disorders. Parkinson's disease (PD) specifically is a neurodegenerative CNS disorder with unknown origins of dopaminergic degeneration in the substantia nigra. Current therapies are reactive in nature and no existing neuroprotective therapies are available. Two hypotheses have been proposed to contribute to dopaminergic degeneration in PD: endoplasmic reticulum (ER) stress and oxidative stress. This thesis investigates molecular targets involved in each of these responses (mesencephalic astrocyte-derived neurotrophic factor (MANF) and cyclin-dependent 5 (CDK5)/p25 respectively) to support a multi-hit hypothesis in PD neural degeneration. Using behavioural and biochemical analysis, a reduction in MANF was found to participate in the ER stress hypothesis and CDK5/p25 hyperactivation is a viable neuroprotective target related to the oxidative stress hypothesis. Both pathways are evidenced in PD pathology and this thesis proposes specific targets for both pathways in the development of necessary neuroprotective therapies. Subsequently, included in this thesis is a chapter about the unmet pharmacological alleviation of negative and cognitive symptom domains in another CNS disorder of unknown pathophysiology: schizophrenia (SZ). These untreated symptoms are thought to be caused by irregularities in the signalling of multiple neurotransmitter systems. This chapter investigates the role of synapsin II, a protein involved in regulating signalling of multiple neurotransmitters, in manifesting negative and cognitive SZ symptoms and analyzes brain glucose metabolism. Reduced synapsin II levels were consistently implicated in the underlying physiology, and therefore synapsin II is proposed as a potential pharmacological target for these unmedicated symptomologies. Overall this thesis uses interrelated studies to propose novel molecular targets to address unmet therapeutic needs based on evidence of their involvement in the pathophysiology of PD and SZ.

ACKNOWLEDGEMENTS

My experience as a graduate student has been nothing short of fortunate and fulfilling. I have learned so much over the course of my studies and received incredible guidance and support from so many individuals. These acknowledgements only begin to express my appreciation and gratitude to everyone that supported me in this journey.

First and foremost, I would like to thank my supervisor, Dr. Ram Mishra. Learning from you has been a pleasure and an honour. I was beyond fortunate to become a part of your lab 4 years ago. During this time, you have continuously opened doors for me wherever possible and provided me with opportunities that I never dreamed possible. These opportunities have helped me grow and develop both inside and outside of my research and I am grateful for your continued encouragement. Through your mentorship I have learned so much about critical analysis, research, scientific rigor, and quality mentoring skills. I am so grateful for everything you have done for me.

I would also like to thank my supervisory committee members, Dr. Laurie Doering, and Dr. Benicio Frey. You have continuously provided me with guidance and scientific insight for my projects. Your support in my progress has been instrumental in developing many critical skills that I can use moving forward. I thank you for your mentorship, kindness, and your continued commitment to helping me become a better researcher.

The support from the many Mishra lab members over the years has been unwavering. My success is largely a result of the incredible environment and people I have been surrounded by. I would like to personally thank Ritesh Daya, for believing in me from the start, taking the time to answer my never-ending questions, providing me with mentorship that I can only hope to use going forward and being a valued friend. Your mentorship from day one has inspired me and I am sincerely grateful. To so many of the other Mishra lab members: Luke Molinaro, Jay Bhandari, Patricia Hui, Shreya Prashar, Roohie Sharma, Sharon Thomson, Aaron Edward, Sharnpreet Kooner, Hetashree Joshi, Omar Shawaf, Kosalin Akilan, Joella Ho, Brett McIntyre, Dima Malkawi, Khaled Nawar, Judy Tran, Brendan Fera, Vidhi Patel, Sumitha Sivagnanasundram and Aurore Latragna. Working with you in the lab has been a wonderful experience, friendships have been made and your support will always go appreciated.

Many fellow graduate students have also given me so much support and peer feedback. I cannot thank you all enough for helping me through the tough times and celebrating the highs with me. Lisa Dyce you never cease to amaze me in all that you do for everyone around you. Without our long nights working or otherwise I never could have made it this far. To Crystal Mahadeo, Rachelle Ho, Saurabh Shaw, Kathryn Reynolds, Gabriella Mattina, Gesine Alders and Monica Akula. Thank you for your friendship, help with learning new methodologies and working with me in SOMA to run so many events.

Finally, to my friends and family. Everything would never have been possible without your love and unwavering support. To my girls, Nicole Coomber, Julie Vollick, Shealynn Attridge and Sarah Gaffney and the rest of the crew, Nathan Melo, Clayton Fenton and Brandan Lee, thank you for always being an incredible support system and listening to me blather on about science even when I wasn't asked. I am blessed to have you all there for so many important events throughout my life. To my parents, nothing can ever express to you my appreciation for everything you have done for me. Making you proud has helped drive my and you are the source of my inspiration. From the cookies as "brain food" to taking my car for an oil change, every little thing you do for me does not go unnoticed and has helped me focus on my studies. Thank you a million times over for everything.

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LIST OF ABBREVIATIONS

[^{18}F]FDG – Fluorodeoxyglucose

5-CSRTT – 5-choice serial reaction time test

5-HT2A – 5-hydroxytryptamine receptor 2A

6-OHDA – 6-hydroxydopamine

8-ARM – 8-arm radial maze

8-OHdG - 8-hydroxy-2'-deoxyguanosine

aCSF – Artificial cerebrospinal fluid

AD – Alzheimer's disease

ADP – Adenosine diphosphate

ANOVA – Analysis of variance

AP-1 – Activator protein 1

APDs – Antipsychotic drugs

AP-α – Activation protein 2 – alpha

ARMET – Arginine-rich mutated early tumors

AS – Antisense oligonucleodite

ATF4 – Activating transcription factor 4

ATF6 - Activating transcription factor 6

ATP – Adenosine triphosphate

BAX – BCL2 associated X protein

BBB – Blood brain barrier

BCL2 – B cell lymphoma 2

BiP – Binding immunoglobulin protein

BOLD - Blood oxygenation level-dependent

BSA – Bovine serum albumin

cAMP – Cyclic adenosine monophosphate

CDK5 – Cyclin dependent 5

CHOP - C/EBP homologous protein

COMT - Catechol-o-methyl-transferase

CPM – Count per minute

CT – Computerized tomography

D2R – Dopamine D2 receptor

DARPP32 – Dopamine- and cAMP-regulated phosphoprotein

DBS – Deep brain stimulation

ddCT – Delta-delta Ct

DLPFC – Dorsolateral prefrontal cortex

DNA – deoxyribonucleic acid

DTT - Dichlorodiphenyltrichloroethane

EDTA - Ethylenediaminetetraacetic acid

EGR-1 – Early growth response factor 1

EGTA - Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra acetic acid

eIF2α - Eukaryotic initiation factor 2

ER – Endoplasmic reticulum

ERAD – Endoplasmic reticulum associated degradation

ERSE I – Endoplasmic reticulum stress response element 1

ERSE II – Endoplasmic reticulum stress response element 2

ETC – Electron transport chain

FDA – Food and Drug Administration

fMRI – Functional magnetic resonance imaging

FSRR – Fixed speed rotarod

GABA – Gamma-aminobutyric acid

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GPe – External globus pallidus

GPi – Internal globus pallidus

GRP78 – Glucose-regulated protein 78

GWAS - Genome wide association studies

HCL- Hydrochloric acid

IP – Intraperitoneal

IRE1 - Inositol-requiring enzyme 1

ITI – Inter-trial interval

JNK3 – C-jun N-terminal kinase 3

KO – Knockout

LB – Lewy Bodies

L-dopa – Levodopa or L-3,4-dihydroxyphenylalanine

LH – Limited hold

LRRK2 – Leucine-rich repeat kinase 2

MANF – Mesencephalic astrocyte-derived neurotrophic factor

MAO – Monoamine oxidase

MAO-B – Monoamine oxidase B

MDS – Movement Disorder Society

MDS-UPDRS – Movement Disorder Society – Unified Parkinson's disease rating scale

MEF2 - Myocyte enhancer factor 2

MM – Mismatched oligonucleotide

mPFC – Medial prefrontal cortex

MPP+ - 1-methyl-4-phenylpyridinium

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NADPH - Nicotinamide adenine dinucleotide phosphate

NMDA - N-methyl-D-aspartate

NTF – Neurotrophic factor

P4 – Parkinson's Foundation Prevalence Project

PANSS – Positive and negative syndrome scale

PBS – Phosphate buffered saline

PCP – Phencyclidine

PD – Parkinson's disease

PEA-3 – Polyoma enhancer activator 3

PERK - Protein kinase RNA-like endoplasmic reticulum kinase

PET – Positron emission tomography

PFA – Paraformaldehyde

PI3K – Phosphatidylinositol 3-kinase

PINK1 – PTEN induced kinase 1

PPI – Prepulse inhibition

Prx2 - Paired-related homeodomain protein 2

PVDF – Polyvinylidene difluoride

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RP – Reserve pool

Rpm – Rotations per minute

RRP – Readily releasable pool

RT-qPCR – Real time quantitative polymerase chain reaction

ScP – Scrambled peptide

SD – Stimulus duration

SDS – Sodium dodecyl sulfate

SDS-PAGE - Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

SEM – Standard error of the mean

shRNA – Short hairpin RNA

SN – Substantia nigra

SNc – Substantia nigra pars compacta

STN – Subthalamic nucleus

STR - Striatum

SynII – Synapsin II

SynIIa – Synapsin IIa

SynIIb – Synapsin IIb

SZ – Schizophrenia

T1 – Training stage 1

T12 – Training stage 12

TBS-T – Tris-buffered saline with tween

TH – Tyrosine hydroxylase

TP5 – Truncated peptide 5

UPDRS – Unified Parkinson's disease rating scale

UPR – Unfolded protein response

VGLUT1 – Vesicular glutamate transporter 1

VGLUT2 – Vesicular glutamate transporter 2

XBP1 – X-box binding protein 1

XBP1s – X-box binding protein 1 spliced

ACADEMIC ACHIEVEMENTS

Majority of the work presented within this thesis was designed, conducted and analysed by the author of the thesis, Ashley Bernardo, with supervision provided by Dr. Ram Mishra. Each chapter specifically indicates Author Contributions related to each finding. In addition to the papers included within the thesis, during graduate studies the Author was involved in knowledge dissemination of the findings at local, national and international conferences.

1. (2019). Decreased expression of MANF leads to motor dysfunction and alters ER stress pathways: MANF's role in Parkinson's disease pathophysiology. Canadian Association for Neuroscience, Toronto, Canada
2. (2019). Peptidomimetic: PAOPA [3(R)- [(2(S)pyrrolidinylcarbonyl)amino]-2-oxo-1 pyrrolidineacetamide] as a prototypal Dopamine receptor:D-2/D-4 Allosteric Modulator as novel drug lead for Parkinson Disease. World Congress on Parkinson's Disease and Related Disorders, Montreal, Canada
3. (2018). Development of Potential New Drugs Targeting Brain Cyclin Dependent Kinase 5 (CDK5). Psychiatry Neuroscience and Behavior Graduate Research Day, Hamilton, Canada
4. (2018). Inhibiting a devil in Parkinson's disease. Neuroscience Undergraduate Conference, Hamilton, Canada
5. (2017). Hyperactive Cdk5 Inhibitor Peptides -TP5 and Peptide A- Display Neuroprotective and Restorative Roles in the 6-OHDA Lesioned Model of Parkinson’s Disease. Canadian Association for Neuroscience, Toronto, Canada
6. (2017). Investigating the Devil in Neurodegeneration. McMaster Integrative Neuroscience Discovery and Study 10th Anniversary Event, Hamilton, Canada
7. (2017). Reduced Expression of Synapsin II in the MPFC Manifests Behavioural and Brain Metabolic Changes: Implications in the Pathophysiology of Schizophrenia. Southern Ontario Association for Neuroscience, St. Catherines, Canada
8. (2017). Development of potential new drugs targeting brain cyclin dependent kinase 5 (CDK5) for the treatment of Parkinson's disease. Society for Neuroscience, Washington D. C, United States
9. (2017). Schizophrenia Research: Behind the Laboratory. Emerging Professionals in Public Health, Hamilton, Canada
10. (2017). Neuroprotective and Restorative Roles of Novel Hyperactive CDK5 Inhibitor Peptides – TP5 and Peptide A - in the 6-OHDA Lesioned Model of Parkinson’s Disease. Psychiatry Research Day, Hamilton, Canada
11. (2017). Reduced synapsin II expression in the medial prefrontal cortex of rats manifests behavioural and brain metabolic changes: Implications in the pathophysiology of schizophrenia. Society for Neuroscience, Washington D.C, United States
12. (2017). Behavioural and Brain Metabolic Changes Provide Evidence for Reduced Synapsin II Levels in the Pathophysiology of Schizophrenia. Society of Biological Psychiatry, San Diego, United States
13. (2016). Research in Schizophrenia. The Schizophrenia Series: Unmasking Reality, Hamilton, Canada
14. (2015). Synapsin II Knockdown Produces Behavioural and Metabolic Changes Emulating Schizophrenic Phenotypes. Southern Ontario Neuroscience Association, Waterloo, Canada

Author has also contributed to the field through already published work:

1. Bernardo, Ashley, et al. “Synapsin II.” Encyclopedia of Signalling Molecules, 2nd ed., Springer International Publishing, 2017, pp. 5264–5274.
2. Thomson, S., Dyck, B., Daya, R., Ho, J., Bernardo, A., Tian, Y., Mishra, RK. Reduced expression of synapsin II in a chronic phencyclidine preclinical rat model of schizophrenia. 2019, Synapse. DOI:10.1001/syn22084

Each of the above achievements contributed to honors of the Ontario Graduate Scholarship 2017-2018, the Ontario Graduate Scholarship 2018-2019, Faculty of Health Sciences Graduate Programs Excellence Award 2019 and the Faculty of Healthy Sciences Graduate Programs Outstanding Achievement Award 2019.

Chapter 1: Introduction

Preface

This thesis has been divided into separate chapters for the sake of clarity and encompasses interrelated studies for the elucidation of molecular targets for potential therapeutic approaches in central nervous system disorders.

1.0 Introduction: Parkinson's Disease

1.1 Parkinson's Disease History

Dr. James Parkinson was a British medical practitioner alive from 1775-1824. He was the first to describe the disorder now known as Parkinson's disease (PD) in *An Essay on the Shaking Palsy*. In this published document he systematically discussed 6 case studies in immense detail. He discussed a differential diagnosis, etiology theories and both motor and non-motor symptoms (Obeso et al., 2017). Parkinson summarized the Shaking Palsy as "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured." It was over 50 years later that Jean-Martin Charcot, a French neurologist, designated the disease as "Parkinson's disease". Dr. Charcot was also very influential in refining and expanding Parkinson's early description and shared information about the disease internationally. He further separated the disorder from others including multiple sclerosis (Goetz, 2011). Both individuals were incredibly influential for the understanding and identification of PD.

1.2 Prevalence and Incidence

PD is the second most common neurodegenerative disorder and affects approximately 67 000 Canadians as of 2014 and estimates of 680, 000 individuals in the United States (Marras et al., 2018; Wong

et al., 2014). Prior to 2014, epidemiological studies of PD were outdated. Studies commonly referenced data from the 1970-1980s. Therefore, the Parkinson's Foundation established the Parkinson's Foundation Prevalence Project (P4) in 2014 to determine a more accurate prevalence across North America. Based on the United States Census Bureau population projections, P4 estimates the number of PD cases will rise to approximately 1 238 000 in the United States by 2030 (Marras et al., 2018). P4 was also tasked with determining if prevalence is uniform across geographies and if prevalence can tell us anything about the disease itself. Thus far, P4 has shown states with larger populations and/or larger numbers of elderly individuals have higher estimated numbers of people with a PD diagnosis. In general, literature agrees that the prevalence of PD increases with age (Pringsheim et al., 2014). Incidence of the disease also increases with age although may stabilize by 80+ years of age (Hirsch et al., 2016). A systematic review and meta-analysis in 2016 reported the highest-quality studies found an incidence rate of 17/100 000 person-years. Peak incidence rates were found between 70-79 years of age and consistently higher incidence rates were found in males compared to females (Hirsch et al., 2016). Additional evidence for PD affecting males over females was reported by Wong et al. showing higher prevalence rates reported in males than females regardless of living in a household or in a long-term care facility (Wong et al., 2014).

1.3 Sporadic and Familial Parkinson's Disease

PD cases are considered either sporadic/idiopathic, meaning there is no genetic component creating the disease, or familial, denoting cases that arise from established PD related genes. Majority of PD cases are sporadic and only 10-15% of cases are familial. While we do not know exactly how sporadic PD arises, environmental factors have been implicated in their manifestation. Environmental risks are mostly associated with occupational exposure to heavy metals or pesticides, including herbicides and insecticides. Heavy metals that have been suggested as chronic exposure risks include iron, copper, and lead (Ball et al., 2019). Gorell et al. reported occupations with 20 years or more exposure to copper, lead-copper, lead-iron or iron-copper increased the risk of PD (Gorell et al., 1997). Increased exposure to lead

over lifetime is also associated with a two-fold risk of developing PD (Coon et al., 2006). The literature on heavy metal exposure being linked to PD risk is highly debated whereas literature agrees that exposure to pesticides can increase the risk of developing PD (Ball et al., 2019; Hancock et al., 2008). Paraquat and rotenone are two well-established pesticides that can increase the risk of developing PD with increased exposure (Tanner et al., 2011). Exposure to these pesticides is so highly associated with PD that animal models utilize them by exposing rodents or other vertebrates and invertebrates to paraquat or rotenone. Other animal models harness attributes from familial cases of PD by genetically manipulating genes known to be associated with the disorder. Some of the most commonly associated genes include: SNCA (α-synuclein gene) (PARK1), Parkin (PARK2), PINK1 (PARK6), DJ-1 (PARK7), leucine-rich repeat kinase 2 (LRRK2) (PARK8) and ATP13A2 (PARK9). α-synuclein mutations are autosomal dominant. Different mutations can show different presentations. The A53T mutation leads to early onset of the disease and rapid progression while A30P mutations are associated with late disease onset. Duplications and triplications of the SNCA gene can also be found in familial cases (Schiesling et al., 2008). Parkin mutations are the most common genetic mutations leading to early onset of PD (occurring before age 50) and has a slow progression. There are over 100 known mutations in the Parkin gene that can lead PD. Parkin normally encodes for the ubiquitous parkin protein responsible for clearance of damaged mitochondria. Therefore, mutations in this gene lead to oxidative stress (Lohmann et al., 2003). The PINK1 gene is associated with early-onset PD and can have many types of mutations such as point mutations, missense mutations, nonsense mutations, insertions and deletions that cause the protein to lose its function. Normally, the PINK1 protein phosphorylates mitochondrial proteins and maintains mitochondria membrane potential. When PINK1 is not functioning properly, oxidative stress is produced (Gandhi et al., 2006; Valente, 2004). The DJ-1 gene is reported to have point mutations or deletions that interrupt its function as an antioxidant (Schiesling et al., 2008). LRRK2 is an autosomal dominant gene with 50 variants known to cause PD that present with a late-onset. LRRK2 mutations often lead to more apoptosis due to

increased kinase activity (Martin et al., 2014). Finally, ATP13A2 mutations are autosomal recessive and show early onset with rapid progression. Mutant forms of this protein remained retained in the endoplasmic reticulum (ER) and are not transported to lysosomes, which results in ER stress (Di Fonzo et al., 2007). Several other genes are also being investigated as genetic links to PD but current evidence for them is not as extensive as those discussed above.

1.4 Symptoms

PD presents with both motor and non-motor symptoms which independently vary widely among individuals. To aid diagnosis and monitor disease progression the Unified Parkinson's Disease Rating Scale (UPDRS) is used. The scale was first created in the 1980s. The Movement Disorder Society (MDS) sponsored a critique of the scale which revealed a lack of information about non-motor symptoms (Goetz 2007). MDS then sponsored a revision that successfully passed clinical testing. The updated scale, now referred to as MDS-UPDRS, incorporates the input from patients and caregivers and includes sections on non-motor experiences of daily living, motor experiences of daily living, motor examination and motor complications (Goetz et al. 2007, 2008).

1.4.1 Motor Symptoms

Motor symptoms are the major phenotypic hallmark of PD and include symptoms affecting locomotion, movement while at rest and specific movements with a purpose such as oral movements. The cardinal motor symptoms of PD include tremors, rigidity, akinesia, and bradykinesia as well as postural instability. Tremors found in patients occur at 4-6 Hz and first present at the distal part of the limb (Postuma et al., 2015). The most common tremor found at rest in the hand is the pill-rolling tremor, where the index finger and thumb rub together as if rolling a pill between them (Jankovic, 2008). Other tremors seen at rest can affect the lips, chin, tongue or legs (Hunker and Abbs, 1990). Rigidity is another motor symptom of PD and describes increased resistance and stiffness in a patient's muscles. This causes the

cogwheel phenomenon. Cogwheel phenomenon can be identified during a physical examination by muscle stiffness combined with muscle tremor that leads to "jerky" movement of a joint (Findley et al., 1981). Akinesia and bradykinesia are usually first noticed by a patient when they have difficulty performing daily routine activities. Slowness of movement extends to a patients locomotor ability and can lead to freezing, a phenomenon usually lasting less than 10 seconds (Jankovic, 2008). Freezing behaviour can been broken into subtypes described as start hesitation, turn hesitation, open space hesitation, hesitation in tight quarters and destination hesitation (Schaafsma et al., 2003). Finally, postural instability and locomotor impairments are major components of the motor symptoms found in PD. Postural instability usually presents after other motor symptoms and patients develop a stooped posture. Postural instability is one of the leading causes of increased fall risk in patients with PD and falls become more common in individuals with PD progression (Wood et al., 2002). Locomotion specific impairments include a shuffling gait and lack of arm swing while walking (Sveinbjornsdottir, 2016). In addition to these locomotor specific impairments, oral-motor disorders develop and create disturbances in speech. More than half of patients with PD develop quiet and hurried speech patterns and 40-80% have difficulty swallowing with limited saliva control (Perez-Lloret et al., 2012)(Kalf et al., 2011; Perez-Lloret et al., 2012).

1.4.2 Non-Motor Symptoms

The non-motor symptoms of PD are non-specific for the disorder and can range significantly among patients in their presentation time and severity. Non-motor symptoms often precede motor symptoms, and this is referred to as the premotor phase of the disease. The most commonly reported symptoms of the premotor phase (occurring prior to diagnosis) include gastrointestinal problems such as constipation, neuropsychiatric symptoms such as anxiety, depression and apathy, daytime sleepiness and loss of smell and/or taste (Pont-Sunyer et al., 2015; Shiba et al., 2000; Weintraub and Mamikonyan, 2019). Constipation is the most well-known risk factor for PD and is thought to be caused by slow colonic transit and potential degeneration of the dorsal vagal nucleus (Jost, 2010; Stocchi and Torti, 2017). The α-

synuclein model of PD has demonstrated reduced colon contractions prior to motor impairments suggesting α-synuclein aggregation within the enteric nervous system may be responsible for this prodromal phenomenon (Rota et al., 2019). Neuropsychiatric symptoms can precede and follow motor symptoms with increased severity often presenting after motor symptom onset. Over one third of patients state they experienced depression, apathy or anxiety prior to motor symptoms and an association between the rate of depression and severity of PD has been made (Fang et al., 2010; Menza and Mark, 1994; Reijnders et al., 2008; Starkstein et al., 2014; Weintraub and Mamikonyan, 2019). Early PD treatment can mitigate neuropsychiatric symptoms, but re-emergence of these symptoms can occur with increased disease progression. Patients receiving treatment report non-threatening visual hallucinations usually manifesting as familiar or unfamiliar people, animals or objects (Papapetropoulos et al., 2008). These hallucinations are often produced as a side effect of dopaminergic treatment however, a small percentage of patients report visual hallucinations prior to therapy (Holroyd et al., 2001; Pagonabarraga et al., 2016). In later stages of the disease, patients may begin to experience paranoid delusions thought to be associated with the high dose of dopaminergic treatment required to overcome motor symptoms (Sveinbjornsdottir, 2016). In addition to neuropsychiatric problems prior to the onset of motor symptoms, patients also experience sleep disturbances, cognitive decline, and sensory impairments. This often entails fractionated sleep and reported increased sleepiness during the day and prodromal rapid eye movement (REM) sleep behaviour disorder (Ondo et al., 2001; Porter et al., 2007; Tandberg et al., 1999). Major cognitive deficits such as dementia usually do not present until later in the disease however, cognitive deficits can be found in newly diagnosed untreated patients in domains such as episodic memory, executive function and verbal function (Elgh et al., 2009; Jankovic, 2008; Kandiah et al., 2009). Furthermore, sensory systems are affected before motor symptoms arise such as olfaction function (Sveinbjornsdottir, 2016). The degree of reduced sense of smell has also been able to predict cognitive decline in individuals with PD with worse baseline olfaction leading to more long-term cognitive deficits

(Fullard et al., 2017, 2016). The symptomology of PD is extremely heterogeneous and non-specific in the premotor phase therefore, diagnosis of the disease is difficult and requires specific diagnostic criteria.

1.5 Diagnosis

Wong et al. report the average age of the first symptom presentation is at 64.4 years of age. Approximately 1.9 years after the first symptom presentation, a PD diagnosis was made. In early onset cases, authors found almost 7 years between first symptom presentation and a diagnosis of PD. Wong et al. report that this is likely because the disease is associated with elderly populations and overlooked or ruled out by clinicians when diagnosing a younger patient (Wong et al., 2014). Efforts have been made to identify pre-diagnostic features of PD. 10 years prior to their diagnosis patients with PD reported both motor and non-motor symptoms including tremor and constipation more often than healthy controls. 5 years prior to diagnosis PD patients again describe both domains, specifically reporting tremors, constipation, balance impairment, fatigue, depression, and anxiety more often than sex and age-matched controls (Schrag et al., 2015). Non-motor symptoms are reported frequently pre-dating motor symptoms. Unfortunately, these pre-diagnostic identifiers are not specific enough for a PD diagnosis and an official PD diagnosis is only given once motor symptoms are evident (Jankovic, 2008).

The Canadian guidelines on Parkinson's Disease state that a diagnosis of PD should be suspected when a patient presents with the slowness of movement, rigidity, tremor and postural instability. Stage 2 of obtaining a PD diagnosis aims to rule out the possibility of other parkinsonian syndromes (Grimes et al., 2012). Parkinsonian syndromes are diagnosed if patients report falls early in the disease course, have a poor response to levodopa, show symptom symmetry at disease onset, does not report tremors, and show rapid disease progression. A PD diagnosis can be made once Parkinsonian syndromes are ruled out and often includes patients presenting with the following: unilateral onset of symptoms and persistent

asymmetry of clinical symptoms, good response to levodopa, tremor, slowness of movement and rigidity (Grimes et al., 2012; Jankovic, 2008).

When first diagnosed, a differentiating factor for PD is the mild unilateral motor symptom presentation. At this stage in the disease patients show good response to treatment and limited variability throughout the day. Within a few year of diagnosis, symptoms appear on the contralateral side. At this stage of the disease, medication response is moderate but still considered the "honeymoon stage". Continued disease progression leads to more pronounced motor disturbances such as gait impairments and requires more medication as there is an increasingly less drug response. Eventually, patients develop poor treatment response with major gait disturbances, difficulty swallowing, and uncontrollable tremors. Several non-motor symptoms are also present including cognitive dysfunction, dementia, psychosis, depression and anxiety as discussed above (Grimes et al., 2012; Jankovic, 2008).

1.6 Circuitry

PD is a neurodegenerative disorder that affects the basal ganglia of the brain. The basal ganglia is made up of several subcortical nuclei that controls functions such as procedural learning, eye movement, cognition, emotion and voluntary movement (Gerfen and Wilson, 1996). The basal ganglia controls movement through two different pathways termed the direct and indirect pathways. The direct pathway is responsible for facilitating movement while the indirect pathway is used to inhibit movement (Utter and Basso, 2008). Regulation of movement by these pathways is essentially controlled by regulating the activity of the thalamus, which is normally under inhibitory control. Movement is produced when this inhibition is released, and the thalamus can send excitatory signals to the motor cortex. The motor cortex then sends the signal to upper, then lower motor neurons which synapse at the neuromuscular junction to contract muscles of the body. The direct pathway initiates movement by reducing the inhibition of the thalamus allowing it to excite the motor cortex, while the indirect pathway increases inhibition of the

thalamus to reduce excitatory signals being sent to the motor cortex (Utter and Basso, 2008). Normally, the two pathways are in balanced resulting in smooth control of movement. Both pathways begin with dopamine input from the substantia nigra pars compacta (SNc), however, the SNc is the primary region of degeneration in PD. Degeneration results in hyperactivation and under stimulation of critical nuclei within the pathways and results in the hallmark motor symptoms of PD (Blandini et al., 2000).

1.6.1 The Direct pathway

The direct pathway involves dopaminergic projections from the SNc to the striatum where dopamine binds to D1 dopamine receptors. D1 receptors are G-protein coupled receptors that are stimulatory and increase intracellular cyclic adenosine monophosphate (cAMP) (Gerfen et al., 1995). Inhibitory GABAergic neurons from the striatum (STR) are activated and project to the internal globus pallidus (GPi) (Gerfen and Wilson, 1996; Utter and Basso, 2008). GABAergic neurons from the GPi normally inhibit the thalamus therefore, inhibition by the STR of inhibitory neurons in the GPi releases inhibition on the thalamus. This allows for excitation of the thalamus sending signals to the motor cortex and induces movement (Figure 1). In PD the loss of dopamine neurons in the SNc reduces dopamine levels in the STR and less dopamine is able to able to bind to D1 receptors. Without dopamine the inhibitory neurons of the STR are not excited therefore do not inhibit the GPi. GPi's inhibitory input to the thalamus can therefore continue and movement is supressed (Blandini et al., 2000) (Figure 2).

1.6.2 The Indirect pathway

The indirect pathway is facilitated when dopaminergic neurons from the SNc synapse at the STR. Dopamine binds to D2 dopamine receptors which are inhibitory GPCR's and reduce cAMP levels in the neuron(Gerfen et al., 1995). Inhibitory GABAergic projections from the STR innervate the external globus pallidus (GPe) (Utter and Basso, 2008). From here the GPe sends inhibitory inputs to the GPe and subthalamic nucleus (STN) which is the only excitatory nuclei in the basal ganglia. STN glutamatergic

outputs normally excite the GPi to increase the inhibition the GPi evokes on the thalamus(Kitai and Deniau, 1981). The increased inhibition of the thalamus does not allow excitatory signals to travel to the motor cortex and ultimately inhibits movement (Figure 1). In PD the loss of SNc neurons projecting to the STR diminishes dopamine binding to D2 receptors. Without this inhibitory signal the GABAergic neurons in the STR become hyperactive and over inhibit the GPe. This over inhibition effects GPe GABAergic neurons that are then unable to inhibit the STN. The STN has excitatory glutamatergic neurons that will then over stimulate the GPi and ultimately increase the GPI's inhibition on the thalamus (Blandini et al., 2000). The thalamus can no longer send signals to the motor cortex to produce movement and hallmark motor symptoms of PD are produced (Figure 2).

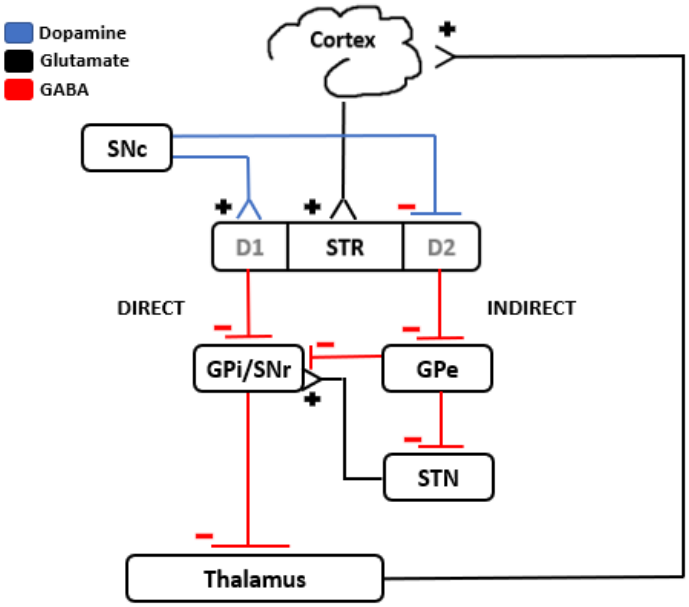

Figure 1. Healthy basal ganglia circuitry. Both direct and indirect pathways are depicted in the figure with blue indicating dopaminergic input, black indicating excitatory glutamatergic projections and red representing inhibitory GABAergic projections. Both the direct and indirect pathways are shown in the diagram. The direct pathway on the left uses D1 receptors within the striatum (STR) and the indirect pathway shown on the right uses the D2 receptors in the STR. The following abbreviations are used in the diagram: substantia nigra pars compacts (SNc), substantia nigra pars reticulata (SNr), internal globus

pallidus (Gpi), external globus pallidus (GPe), subthalamic nucleus (STN), dopamine receptor 1 (D1), dopamine receptor 2 (D2).

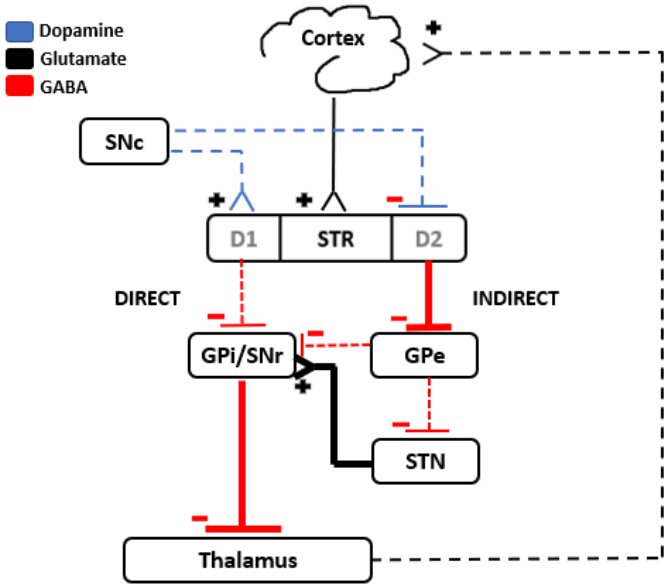

Figure 2. Parkinson's disease basal ganglia circuitry. Both direct and indirect pathways are affected in Parkinson's disease (PD) resulting increased inhibition of the thalamus and the production of hallmark motor symptoms. Dotted lines represent reduced input, while bold lines depict increased activity of that projection within the circuitry. Dopamine neurons in the SN are degenerating in PD and therefore the blue projections from the SNc are dotted. Resulting circuitry is shown using glutamatergic projections in black and GABAergic projections in red. The following abbreviations are used in the diagram: substantia nigra pars compacts (SNc), substantia nigra pars reticulata (SNr), internal globus pallidus (Gpi), external globus pallidus (GPe), subthalamic nucleus (STN), dopamine receptor 1 (D1), dopamine receptor 2 (D2).

1.7 Pathology

1.7.1 Lewy Bodies (LB)

One of the two main hallmark pathologies in PD is Lewy Body (LB) formation. LB's are round, intraneuronal, cytoplasmic inclusions often found in the remaining neurons of the SNc. LB's are mostly composed of the 140 amino acid phosphoprotein α-synuclein, 90% of which is phosphorylated at Ser 129.

Other components of LB's are listed in a review by Wakabayashi et al. and can be divided into several groups: structural elements, α-synuclein binding proteins, synphilim-1 binding proteins, components of the ubiquitin-proteasome system, cellular response proteins, phosphorylation and signal transduction proteins, cytoskeletal proteins, cell cycle proteins and cytosolic proteins (Gai et al., 2000; Voronkov et al., 2018; Wakabayashi et al., 2007). α-synuclein is normally involved in vesicular trafficking by regulating the amount of synaptic vesicles docked at the synapse (Wislet-Gendebien et al., 2006). It has also been suggested to have a role in dopamine synthesis and transport, molecular chaperone activity, and synaptic plasticity (Burré et al., 2018). Physiological functions of α-synuclein require an equilibrium between its monomer and tetramer states, with tetramers destabilizing to become monomers (Bartels et al., 2011). Missense mutations found in familial forms of PD demonstrate that pathological states are produced when there is a decrease in the tetramer:monomer ratio occurs and monomers begin to aggregate forming oligomers (Dettmer et al., 2015). These oligomers are an early toxic species and a pre-fibrillary form of α-synuclein able to disrupt cell membranes and axonal transport (Colla et al., 2012; Rocha et al., 2018; van Rooijen et al., 2008; Laura A. Volpicelli-Daley et al., 2014). Misfolding of monomers also leads to α-synuclein aggregation and restricts surveillance of molecular chaperones furthering misfolding and aggregation (Auluck et al., 2002). Oligomers are intermediates before α-synuclein fibrillation occurs and serve as the base for elongation (Luk et al., 2009; Wood et al., 1999). These fibers recruit more α-synuclein and develop into established LB's (Laura A Volpicelli-Daley et al., 2014; Volpicelli-Daley et al., 2011). This accumulation of α-synuclein is believed to precede the second most well documented pathological hallmark of PD, dopaminergic degeneration (Burré et al., 2018; Raza et al., 2019).

1.7.2 Dopaminergic Degeneration

The other critical pathology of PD is the loss of dopaminergic neurons within the SNc that project to the striatum. Once 60% of the neurons in the SNc are lost, there is approximately an 80% decrease of dopamine within the striatum. The loss of dopamine dysregulates the direct and indirect pathways of the

basal ganglia as described above. Motor symptoms that are required for a diagnosis of PD only develop once this 60% and 80% decrease are reached (Cheng et al., 2010). Dopaminergic neuron degeneration can be visualized in the SNc by a loss of neuromelanin, a protein that creates a dark pigment in dopamine neurons (Vila, 2019). LB formation likely plays a role in dopamine neuron degeneration as their presence slows cellular transport processes and impairs other cellular functions. The pathways leading to dopaminergic degeneration have not been specifically determined, however oxidative stress and endoplasmic reticulum (ER) stress have been separately proposed to lead to this degeneration and explain LB formation.

1.8 Pathophysiology

Although the origins and causes of PD have not been identified, the pathology of PD supports two interconnected processes to likely be at play: oxidative stress and ER stress. Oxidative stress can lead to ER stress through various pathways and increased ER stress furthers oxidative stress thus, the two pathways reinforce each other.

1.8.1 Oxidative stress

Oxidative stress is produced when there is an imbalance between reactive oxygen species (ROS) production and removal by antioxidants that favours the increased presence of ROS. ROS are mostly produced within the mitochondria through the electron transport chain (ETC) (Puspita et al., 2017). The ETC is made of several complexes that are electron acceptors or donors. Through redox reactions electrons are transferred down the chain to create an electrochemical gradient that aids in the synthesis of ATP. Complex I is the first complex in the ETC and is a major site of potential ROS production, specifically superoxides. Reduced complex I significantly increases ROS production and ROS ultimately produces protein damage, DNA and RNA damage and lipid peroxidation. (Hirst et al., 2008).

1.8.2 Oxidative Stress in Parkinson's Disease

Direct evidence for oxidative stress has been found in PD patients. Cerebrospinal fluid collected from PD patients demonstrated increased concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG), an oxidative stress marker known to be a by-product of DNA oxidation. The concentration of 8-OHdG was positively correlated with disease duration (Isobe et al., 2010). A loss of complex I has also been identified in the SNc and reduced in platelets from PD patients (Krige et al., 1992; Schapira et al., 1990). Other evidence for complex I induced oxidative stress has been found in familial cases of PD through mutations in the Parkin gene. Patients with mutations in the gene demonstrate complex I impairment (Müftüoglu et al., 2004). Further evidence for oxidative stress in PD is related to dopamine metabolism. Metabolism of this neurotransmitter can produce radicals and may be why dopaminergic neurons are selectively damaged at earlier stages of the disease. When dopamine is released into the synapse excess dopamine is either metabolised by monoamine oxidase (MAO) or by auto-oxidation. This dopamine auto-oxidation produces quinone and a superoxide which is a ROS and therefore increases oxidative stress within dopaminergic neurons (Zucca et al., 2014).

1.8.3 Endoplasmic Reticulum Stress and the Unfolded Protein Response

ER stress is produced when there is abundant protein misfolding, protein aggregation, protein production imbalances and reduced calcium stores in the ER (Glembotski et al., 2012; Lindholm and Saarma, 2010; Jyoti D Malhotra and Kaufman, 2007; Rao et al., 2004; Ron and Walter, 2007). Ribosomes on the surface of the rough ER translate proteins which are then folded. Only properly folded proteins then travel to the golgi for transportation throughout the cell. When a neuron is under ER stress the cell initiates the unfolded protein response (UPR). The UPR slows protein translation, degrades misfolded proteins and activates pathways that will increase molecular chaperones to unfold misfolded proteins. There are three known transmembrane proteins that mediate the UPR through separate pathways: inositol-requiring enzyme 1 (IRE1), activating transcription factor-6 (ATF6) and protein kinase RNA-like endoplasmic reticulum kinase (PERK). Each of these three receptors are membrane-bound and remain

inactive under normal conditions by glucose regulated protein 78 (GRP78), also known as binding immunoglobulin protein (BiP). Once ER stress is detected, GRP78 is a molecular chaperone that unbinds from the three UPR proteins and binds to misfolded proteins. This triggers activation of IRE1, ATF6 and PERK. Each of these proteins then mediates the UPR through different pathways with some overlap (Figure 3). The IRE1 pathway involves dimerization of IRE1 and autophosphorylation, which splices the transcription factor X-Box Binding Protein 1 (XBP1). Once in the sliced form, XBP1s is translocated to the nucleus where it increases transcription of chaperones and other proteins needed for maturation and degradation of proteins. After long periods of ER stress XBP1s favours upregulating apoptotic factors (Lee et al., 2003; Urra et al., 2013). ATF6 is another UPR mediating protein. Upon release from GRP78, ATF6 is translocated to the golgi where it is processed and ATF6f is released. This fragment then controls gene expression of endoplasmic reticulum associated degradation (ERAD) and XBP1(Hetz, 2012; Lee et al., 2002). The last pathway involved in the UPR is the PERK pathway. PERK phosphorylates eukaryotic initiation factor 2 (eIF2α) which translates transcription factor ATF4. ATF4 is known to upregulate C/EBP homologous protein (CHOP) a well-documented apoptosis inducer(Galehdar et al., 2010). The UPR is a compensatory mechanism that can overcome acute ER stress. Once ER stress becomes chronic, apoptotic pathways such as those mediated through CHOP are favoured over the UPR. It is the chronic ER stress that has been implicated in neurodegenerative diseases.

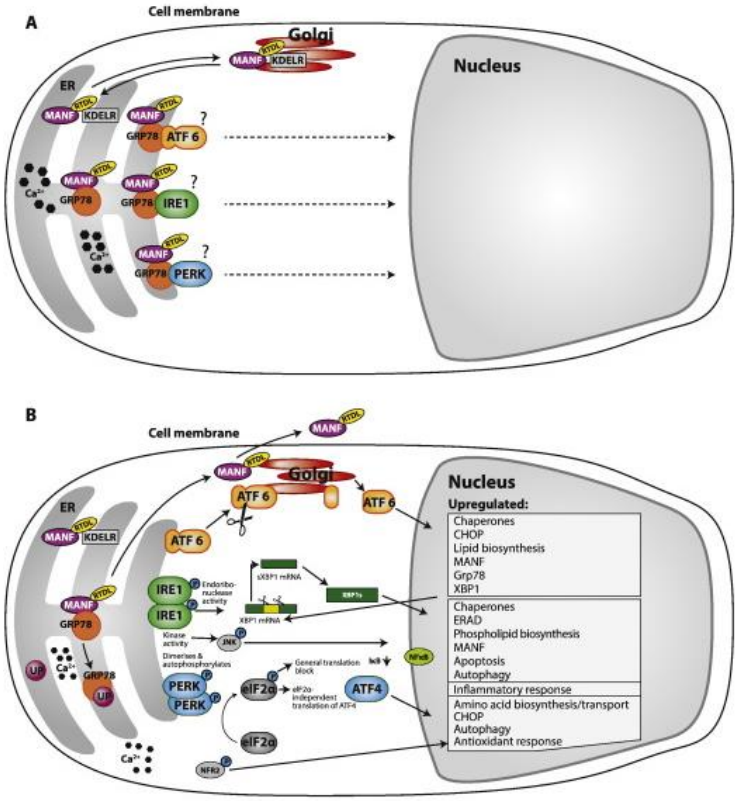

Figure 3. Unfolded protein response pathways initiated during ER stress. A) Without the presence of ER stress, GRP78 remains bound to unfolded protein response (UPR) proteins and keeps them inactive. **B)** Under ER stress GRP78 dissociates from UPR proteins and initiates the UPR to overcome ER stress or eventually lead to apoptosis. (Image from Lindahl et al. 2017 *Neurobiology of Disease*. Reproduction rights obtained and shown in Appendix 1)

1.8.4 Endoplasmic Reticulum Stress and Parkinson's Disease

Evidence for the presence of acute ER stress and chronic ER stress in PD is found in animal models and human patients. In animal studies, GRP78 overexpression demonstrates dopaminergic neuron protection and decreased α-synuclein aggregation(Gorbatyuk et al., 2012). GRP78 is required to regulate the activation of the three UPR pathways and is an important chaperone to aid in recovery from misfolded proteins. There is an age-dependent decrease in GRP78 found in rats examined at 2 months and 24 months with significantly less GRP78 found in the aged animals that coincided with increased α-synuclein

toxicity. The same study reported overexpression of GRP78 could then protect nigral dopamine neurons in the α-synuclein model of PD (Salganik et al., 2015). Age-related loss of GRP78 will not be able to keep the UPR tightly regulated which is confirmed at all levels of the three UPR branches. XBP1s is the functional transcription factor of the IER1 branch and increases chaperone translation. Knockdown studies of XBP1 have exacerbated SNc dopaminergic degeneration and signs of chronic ER stress are shown through increased CHOP expression. AAV-XBP1s administration also demonstrates neuroprotective properties (Valdés et al., 2014). Both XBP1s and ATF6f (the transcription factor of the ATF6 pathway) mediate transcription of GRP78 to produce a neuroprotective effect (Sado et al., 2009). Studies of the protective ATF6 branch of the UPR show that knockout of this UPR protein leads to increased degeneration of dopamine neurons (Egawa et al., 2011; Hashida et al., 2012). Chronic ER stress in PD has also been suggested to arise from this branch as overexpression of α-synuclein blocks the ER to golgi vesicular trafficking of ATF6 (Credle et al., 2015). The final branch of the UPR, the PERK pathway, has been implicated in PD through post-mortem studies. Phosphorylated PERK and eIF2α was found colocalized with α-synuclein in LB (Hoozemans et al., 2007). PERK activation was also found in PD brain tissue by Mercardo et al. as well as genetic and toxin models of PD. This group further demonstrated that PERK inhibition can reduce dopaminergic degeneration (Mercado et al., 2018). Chronic PERK activation is known to influence activating transcription factor 4 (ATF4) which is known to increase CHOP expression and induce apoptosis. CHOP deletion in 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models show reduced degeneration, supporting CHOP is a significant pathway used in dopaminergic neuronal degeneration (Silva et al., 2005). CHOP also depletes glutathione and exacerbates ROS production (McCullough et al., 2001). Together, evidence for chronic ER stress is prominent and will induce pathways to degrade overly stressed neurons. Thus, ER stress is likely responsible for some of the dopaminergic neuron degeneration evident in PD pathology.

1.8.5 Oxidative Stress and ER Stress: Multi-hit hypothesis

The multi-hit hypothesis arises from the drastic evidence for both oxidative stress and ER stress mechanisms in dopaminergic degeneration and α-synuclein aggregation. Oxidative stress produces protein specific damage leading to misfolding of proteins, one specifically is α-synuclein damage. α-synuclein inhibits complex I of the electron transport chain and therefore produces more ROS (Loeb et al., 2010). ROS then damage proteins and the ER is required to these fix misfolded and damaged proteins. With too many proteins damaged the ER becomes overloaded and ER stress begins to occur. Folding and refolding proteins in the ER is a high energy consuming process using adenosine triphosphate (ATP). This forces mitochondrial to work harder to increase ATP production and in turn this manufactures more ROS (Jyoti D Malhotra and Kaufman, 2007). Due to the high energy requirement of protein folding, the mitochondria and ER have a close relationship. There are several direct contacts made between the two organelles that allow for direct metabolite exchange. α-synuclein co-localizes with these contact sites between mitochondria and the ER (Guardia-Laguarta et al., 2014). Overexpression of α-synuclein decreases the number of contact sites and thus changes calcium transfer. Under oxidative stress conditions mitochondrial membrane potential is altered and causes ER fragmentation. Fragmentation and ER stress both increase the leakage of calcium into the cytosol. Mitochondria act to buffer the calcium concentration through reuptake, however increased calcium promotes ATP synthesis within the mitochondria which increases respiratory chain electron leakage and furthers ROS production (Brookes et al., 2004; Görlach et al., 2015). Within the ER, oxidative protein folding takes place and is another source of ROS production. Proteins are folded through the formation of disulphide bonds, whose formation is depended on the redox status within the ER. The ER lumen maintains an oxidizing environment that promotes disulfide bond formation, while the cytosol has a reducing environment. There are two proposed mechanisms for how disulfide bonds generate ROS. One suggests that ROS are a by-product of the transfer of electrons from thiol groups to oxygen. The other theory proposes ROS are formed due to glutathione depletion. Glutathione is a well known antioxidant and the ratio of oxidized glutathione to

reduced glutathione is often used as a marker for redox states (Hwang et al., 1992; Jyoti D. Malhotra and Kaufman, 2007). Unfolded protein accumulation also increases calcium leak into cytosol which then needs to be buffered by the mitochondria and furhter creates more ROS. The increased ROS will exacerbate ER stress which will then intensify ROS production and the two cycles reinforce each other (Figure 4). Both pathways can induce apoptosis and therefore PD pathology is likely due to a multi-hit phenomenon.

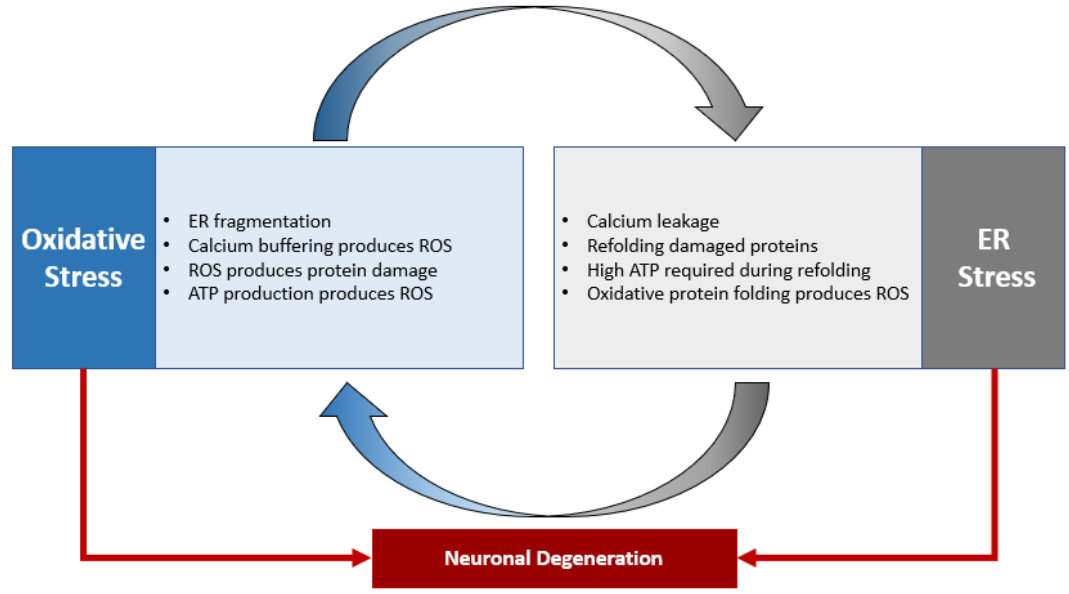

Figure 4. Proposed multi-hit hypothesis of neurodegeneration. Oxidative stress leads to endoplasmic reticulum (ER) stress and ER stress leads to oxidative stress. The multi-hit hypothesis proposes that both stress responses, while reinforcing each other, lead to neuronal degeneration found in Parkinson's disease.

1.9 Animals Models

Preclinical models are a vital resource for therapeutic development and disease mechanism research. Both *in vitro* and *in vivo* models are used in the preclinical stage of drug development. Preclinical models of PD most commonly used are toxin models and genetic models. Both *in vitro* and *in vivo* models utilize these types of models however, this section will focus on animal models.

1.9.1 Toxin Models

When PD was discovered to be a disorder involving dopamine depletion, neurotoxins that targeted dopaminergic neurons became of significant use in PD research. Toxin models include both neurotoxins and pesticides that affect the dopamine system. One of the first toxin models utilized and is today considered the classic model of PD is the 6-hydroxydopamine (6-OHDA) model. This model was first discovered by Ungerstedt in 1968 (Ungerstedt, 1968) and now uses 6-OHDA administration directly into either the striatum or substantia nigra to induce dopaminergic degeneration. 6-OHDA is often administered unilaterally and induces a lesion at the site of administration. Once 6-OHDA reaches the SN by direct administration to the SN or through retrograde transport if injected into the striatum, 6-OHDA uses a dopamine transporter to enter dopamine neurons and auto-oxidizes. This produces increased oxidative stress that leads to apoptosis in upwards of 60% of the dopamine neurons in the SN. The substantial loss of neurons recapitulates motor impairments found in PD and this model has proved useful in motor symptom treatment development. The other most commonly utilized toxin model of PD is the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model. MPTP can be administered systemically and produces selective degeneration of neurons in the substantia nigra. MPTP itself is not toxic, however once in the brain MPTP is converted to the toxic metabolite 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B (MAO-B) (Langston et al., 1984). This metabolite is taken up through dopamine transporters where it becomes concentrated in the mitochondria. MPP+ inhibits complex I and induces oxidative stress that eventually leads to apoptosis of many dopamine neurons in the substantia nigra (Langston, 2017). The MPTP model has been significantly helpful in identifying molecular mechanisms specifically the mitochondrial impairments that underlie PD pathology. Both 6-OHDA and MPTP models only recapitulate the dopaminergic neuronal loss characteristic of the disorder and do not present with any LB formation. Other toxin models have been explored that are produced using pesticides, most commonly paraquat and rotenone. Paraquat is an herbicide that structurally resembles MPP+ and

therefore is thought to act through similar mechanisms. Paraquat enters the brain using neutral amino acid transporters and is believed to produce oxidative stress though redox cycling and activation of ROS generating enzymes such as NADPH oxidases (Bové and Perier, 2012). Paraquat models also have the benefit of upregulating α-synuclein and increasing aggregation (Manning-Bog et al., 2002). However, inconsistent data has been found for paraquat inducing detectable behavioural deficits in mice (Bové and Perier, 2012). Rotenone is another pesticide that is used as both an herbicide and an insecticide. This pesticide can also produce a model of PD through systemic administration. Rotenone is able to freely cross cellular membranes and impairs oxidative phosphorylation in the mitochondria causing systemic complex I inhibition and severe oxidative stress that is not specific to dopamine neurons. This model does demonstrate behavioural impairments such as reduced locomotor activity as well as deposits of α-synuclein, suggesting it may also present LB-like pathology (Bové and Perier, 2012; Carriere et al., 2017; Yuan et al., 2015). Toxin models have been very useful in understanding mechanisms of PD, and in the battle of developing neuroprotective treatments as these models consistently demonstrate dopamine degeneration.

1.9.2 Genetic Models

The other avenue of PD models used in preclinical research are genetic models that are created with known mutations that lead to familial PD and genetic related sporadic PD. These models take advantage of both autosomal dominant and autosomal recessive genes. Autosomal dominant models include α-synuclein models and LRRK2 models, while Parkin, PINK1 and DJ-1 are all autosomal recessive genetic models (Hisahara and Shimohama, 2010). Of the autosomal dominant genes, a routinely used genetic model of PD is the α-synuclein A53T model. These transgenic rodents express the human A53T mutation and develop progressively severe motor impairments that are similar to human manifestations. This model also shows α-synuclein aggregations throughout the central nervous system. Other human mutations of the SNCA gene are also used as genetic models including the E46K mutation in α-synuclein.

These transgenic rodents show age dependent motor impairments and have more α-synuclein that aggregates forming Lewy-body like structures(Emmer et al., 2011). The other autosomal dominant genetic model of PD is the LRRK2 model. The LRRK2 model with the human R1441G mutation shows age-dependent motor impairments that respond to L-dopa and axonal injury (Li et al., 2009). The LRRK2 model has also supported that LRRK2 and α-synuclein likely use similar mechanism to induce PD pathologies. Overexpression of LRRK2 has shown to increase α-synuclein toxicity while inhibition reduced this toxicity (Lin et al., 2009). Autosomal recessive models have also been explored often using knockout models. Parkin knockout models have provided insight into mitochondrial dysfunction and demonstrate reduced locomotor activity (Dawson et al., 2010; Itier et al., 2003). Genetic models have helped identify that another PD associated gene, PINK1, is involved in the same pathway as Parkin. Parkin can rescue PINK1 phenotypes but PINK1 cannot rescue Parkin induced deficits (Park et al., 2006). This finding supports that PINK1 functions up stream from Parkin and knockout models of PINK1 have also implicated PINK1 in mitochondria fission and trafficking (Poole et al., 2008; Vives-Bauza et al., 2010). The final autosomal recessive genetic model used is the DJ-1 model. This model is largely a model of mitochondrial dysfunction in the disease as inconsistent results show dopamine degeneration. There are often no significant changes to the number of dopamine neurons or level of dopamine in the striatum found in knockout animals however there is evidence of abnormal dopamine transmission and mitochondrial dysfunction (Andres-Mateos et al., 2007; Dawson et al., 2010). Like any disease no animal models recapitulates all aspects of PD. Having models that echo specific characteristics of the disease give insight into potential mechanisms and drives forward efforts in treatment development.

1.10 Treatment

1.10.1 Pharmacological Intervention

There is currently no cure or permanent treatment for PD, only symptomatic therapy. Therapy regiments often begin with pharmacological intervention, with or without physiotherapy, and can progress to invasive surgical interventions. The current "gold-standard" therapy for PD is levodopa (also known as L-dopa or L-3,4-dihydroxyphenylalanine), first used as a monotherapy in the 1960's (Birkmayer and Hornykiewicz, 1961). L-dopa is a dopamine precursor and unlike dopamine, is capable of crossing the blood brain barrier (BBB). L-dopa then becomes decarboxylated by amino acid decarboxylase in remaining dopamine neurons and acts as a substitution for the lost dopamine within the striatum (Jost, 2017; Juorio et al., 1993; Stansley and Yamamoto, 2013). To date L-dopa provides robust relief of akinesia and rigor in early disease states and therefore response to L-dopa remains an important part of the diagnostic criteria for PD. L-dopa however, has very low bioavailability and given alone, 70% is decarboxylated peripherally (Jost, 2017; Nutt and Fellman, 1984). To overcome this, L-dopa is currently not prescribed without a decarboxylase inhibitor such as carbidopa or benserazide, which are not able to cross the BBB. Despite the benefits of L-dopa therapy, response to L-dopa diminishes as the disease progresses. This gives rise to the term "honeymoon period" of the disorder and what is known as "on" and "off" periods. The "honeymoon period" of the disease refers to a period when response to L-dopa and other therapeutics can almost completely reverse the motor symptoms (Rascol et al., 2003). The disease eventually progresses to a point when L-dopa loses its efficacy and despite administration provides no relief of symptoms. This is known as an "off" period. These "off" periods become more common as the disease progresses and are intermittent between periods when L-dopa does mitigate motor symptoms, known as the "on" periods. To extend the time before "off" periods begin, a common therapeutic strategy tried before L-dopa in patients with mild symptoms is the use of MAO-B inhibitors. MAO-B inhibitors provide therapeutic benefits by blocking the action of the MAO-B enzyme that normally acts to breakdown dopamine and other neurotransmitters. MAO-B inhibitors are approved for use as monotherapy or a combination therapy. As the disease progresses, L-dopa is added to treatment regiments and the MAO-B

inhibitor can help to slow the breakdown of L-dopa and dopamine, reducing dose requirements and lengthening the efficacy window for L-dopa. Other combination treatments given with L-dopa are catechol-o-methyl-transferase (COMT) inhibitors. COMT inhibitors block the degradation of catecholamines such as dopamine by COMT and can prolong the effect of L-dopa. COMT inhibitors reduce the amount of L-dopa required to elicit symptom relief and increase L-dopa bioavailability (Jost, 2017). An alternative to dopamine replacement therapy using L-dopa is through direct stimulation of post-synaptic dopamine receptors in the striatum by dopamine agonists. Dopamine agonists have been approved for use as a monotherapy or a combination therapy and can be used at both early or late stages of PD. Dopamine agonists range in their selectivity for different dopamine receptors but regardless, provide symptom relief of akinesia, rigor and tremor. Several dopamine agonists also demonstrate antidepressant affects, however that can also produce problems related to impulse control. Patients treated with dopamine agonists also report hallucinatory side effects which can be treated with clozapine (Connolly and Lang, 2014; Jost, 2017). Overall the most effective monotherapy discovered so far is L-dopa. The problem currently faced in PD is that the disease progresses to a point where this pharmacological intervention does not provide enough relief to improve quality of life.

1.10.2 Surgical Intervention

Once pharmacological therapy options become ineffective or are no longer feasible, a surgical option for treatment is deep brain stimulation (DBS). DBS entails the implantation of electrodes into either the subthalamic nucleus (STN) or the globus pallidus internus (GPi), which is then stimulated using an implanted pulse generator by the clavicle (Jakobs et al., 2019). DBS first emerged in the 1990's and the Food and Drug Administration (FDA) approved the procedure targeting the STN in 2002. The following year, the FDA approved the GPi as another viable target for DBS (Aum and Tierney, 2018). The STN is more commonly targeted, however, there is evidence to support both targets as efficacious with mixed support for one target over the other (Almeida et al., 2017). Studies find that DBS at the STN requires lower doses

of L-dopa years later versus DBS at the Gpi (Moro et al., 2010; Odekerken et al., 2016). Patients can receive unilateral DBS or bi-lateral electrode implantation depending on disease progression and severity. DBS has been shown to improve motor symptoms significantly and has had some success with treating non-motor symptoms of depression and cognitive decline (Combs et al., 2015).

1.10.3 Future Treatment Needs

Of the current pharmacological and surgical interventions available, none provide adequate neuroprotection. As PD is a neurodegenerative disease, identifying a neuroprotective therapy would have a significant impact on the course of the disease and the quality of life for those living with PD. The significant need of neuroprotective and more sustainable treatments for PD has been acknowledged by Health Canada. The organization has included PD as a disorder eligible for their "fast-track" drug submission program. This program is called the Priority Review of Drug Submissions Process and has a target of 180 days for new drug submission review. One goal of this policy is to give priority review to new therapies, preventatives and diagnostic agents to help life-threatening or severely debilitating diseases. In order to develop therapeutics with neuroprotective potential, molecular targets must be identified that are involved in the proposed two pathways involved in PD pathology, ER and oxidative stress.

1.11 Overall Thesis Aims and Hypotheses:

Aim 1: To support the multi-hit hypothesis for neurodegeneration and identify a potential neuroprotective target related to ER stress (Chapter 2).

Hypothesis: An ER stress related neurodegeneration target, and support for this hypothesis can be identified via the target altering ER homeostasis in the substantia nigra to increase ER stress producing neurodegeneration.

Aim 2: In support of the other half of the multi-hit hypothesis an oxidative stress-related molecular target will be evaluated and proposed as a neuroprotective target (Chapter 3).

Hypothesis: Oxidative stress induces pro-apoptotic pathways that can be targeted for neuroprotective purposes.

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Chapter 2: MANF as an ER stress-related molecular target for neuroprotection in Parkinson's disease

Preface

Endoplasmic reticulum (ER) stress is a major hypothesis involved in the pathophysiology of Parkinson's disease (PD). ER stress as discussed in the previous chapter leads to activation of the unfolded protein response (UPR) that functions to overcome acute levels of misfolded proteins, protein aggregation or other causes of ER stress. During chronic periods of ER stress that the UPR cannot overcome there is a shift in pathways from self-preservation to self-degradation, and apoptotic pathways begin to be favoured. ER stress is one of the hypotheses behind neurodegeneration and thus is important in neuroprotective research. To support this half of the multi-hit hypothesis, and show ER stress is responsible for neurodegeneration, mesencephalic astrocyte neurotrophic factor (MANF) will be evaluated (Aim 1 – Chapter 1). MANF is a recently discovered neurotrophic factor that may regulate ER stress in dopaminergic neurons and therefore was investigated as a potential neuroprotective therapeutic target for PD.

2.0 Background

2.1 MANF Astrocyte-Derived Neurotrophic Factor

MANF is a neurotrophic factor that is expressed in neuronal and non-neuronal tissues such as the brain, pancreas and salivary glands. The largest quantities of MANF are found within the brain. MANF is widespread and has been specifically identified in the olfactory bulb, cortex layers II-VI, hippocampus, thalamus, striatum, and substantia nigra (Lindholm et al., 2008). During early development, MANF levels are highest during postnatal day 3-6, after which levels gradually decrease as up to investigated postnatal day 30 (Wang et al., 2014). This relatively recently discovered neurotrophic factor is believed to maintain ER homeostasis and has significant implications in injury and neurodegenerative disorders.

2.1.1 MANF Structure

MANF is a small, soluble protein encoded for in humans by a gene on chromosomal band 3p21.2. MANF is evolutionarily conserved among invertebrates and vertebrates and contains several important functional domains. The N-terminus of the protein directs MANF to the ER where cleavage of the signal peptide gives rise to the mature MANF peptide that can be secreted (Mizobuchi et al., 2007). The mature protein has 5 alpha-helices at the N-terminus, 3 alpha-helices at the C-terminus and a linker region (residues 96-103) between the two domains (Hellman et al., 2011). The N-terminus is a saposin-like domain that can interact with lipid membranes. Two patches of positively charged lysine and arginine residues allow for the interaction with negatively charged phospholipids (Lindahl and Lindholm, 2017; Parkash et al., 2009). The C-terminus is a SAP-like domain which is thought to be involved in the chromosomal organization (Aravind and Koonin, 2000). This domain interacts with apoptotic inducing BCL2 associated X (BAX) protein and keeps it inactive, thereby preventing apoptosis (Hellman et al., 2011). At the extreme end of the C-terminus is the RTDL sequence responsible for ER retention of MANF. This sequence binds to KDEL receptors and cycles MANF from the golgi to the ER (Mark J. Henderson et al., 2013). Removal of this sequence has demonstrated increased secretion of MANF and increases relocalization from the ER to the golgi (Christopher C Glembotski et al., 2012; Mark J. Henderson et al., 2013; Oh-hashi et al., 2012). Regulation of MANF levels can be controlled by ER stress levels in the cell. During ER stress there is increased expression and increased regulation of MANF. The promoter region of MANF contains an ER stress response element II (ERSE II) that is recognized by UPR associated proteins, ATF6 and XBP1 (Lee et al., 2003; Mizobuchi et al., 2007; Oh-Hashi et al., 2013). MANF's promoter region also contains ERSE I recognized by XBP1s (D. Wang et al., 2018). During ER stress that induces the UPR, these downstream proteins can then modulate the expression of MANF and increase its expression. Within the promoter region of MANF, there is also an activator protein 1 (AP-1) binding site. AP-1 is a transcription factor that is activated upon inflammation and results in enhanced MANF expression (C.

Wang et al., 2018). Secretion of MANF is also increased during times of ER stress however, the functionality of secreted MANF has yet to be determined.

2.1.2 MANF Function

The exact functions and mechanisms of MANF have yet to be elucidated however evidence continues to support MANF in ER stress homeostasis, dopaminergic development, and dopaminergic protection. Under normal conditions, MANF is localized within the ER and bound to GPR78. MANF inhibits ADP release and ATP binding from GRP78 to inhibit GRP78 client release. This stabilizes the complex GRP78 has with UPR regulatory proteins and keeps the UPR process inactivated (Yan et al., 2019). MANF association with GRP78 is a calcium-dependent interaction. Lower amounts of calcium in the ER lumen reduced GRP78/MANF association. Reduced calcium also increases MANF secretion from the ER, although the role of secreted MANF is still unknown partly because cell membrane receptors for MANF have not been identified (Christopher C. Glembotski et al., 2012; Yang et al., 2018). Upon MANF release from GRP78, the complex with UPR proteins is less stable and this initiates UPR processes. This evidence suggests MANF plays a regulatory role in the UPR process through GRP78 (Yan et al., 2019). Consistent evidence also shows MANF expression is increased during ER stress (Christopher C. Glembotski et al., 2012; Mark J Henderson et al., 2013). This is likely produced through UPR mediated XBP1 and ATF6 association with ERSE I and II in the promoter region of MANF as described above. MANF's role in maintaining ER homeostasis extends into its protective and maintenance functions of dopaminergic neurons. MANF has been shown to selectively protect dopamine neurons over GABA or serotonin neurons (Petrova et al., 2003). Dopaminergic specific functions are conserved across several species. *Drosophila* knockout models have impaired dopaminergic neuronal development, altered dopamine uptake, increased UPR activation, altered neurite extension and altered neuronal migration that can be rescued with MANF administration (Palgi et al., 2012, 2009). *C. elegans* without *manf-1* develop age-dependent dopaminergic degeneration (Richman et al., 2018) and zebrafish with a MANF knockdown have fewer

dopamine neurons and reduced dopamine concentrations (Chen et al., 2012). MANF's role in neuronal development has been explored using mice knockout models revealing normally high expression in neural stem cells of the subventricular zone. Animals without MANF do not show altered stem cell proliferation however, display delayed migration to the cortex and significantly decreased neurite extensions (Tseng et al., 2017). Another protective mechanism potentially implemented by MANF is a role in immune modulation. MANF can promote phenotype switch of macrophages from a pro-inflammatory state to an anti-inflammatory function through alternative activation of macrophages (Neves et al., 2016). Little is known about this mechanism and therefore future research should investigate MANF in inflammatory functions. Much of the current focus for MANF research has been investigating its therapeutic potential in models of neurodegenerative diseases.

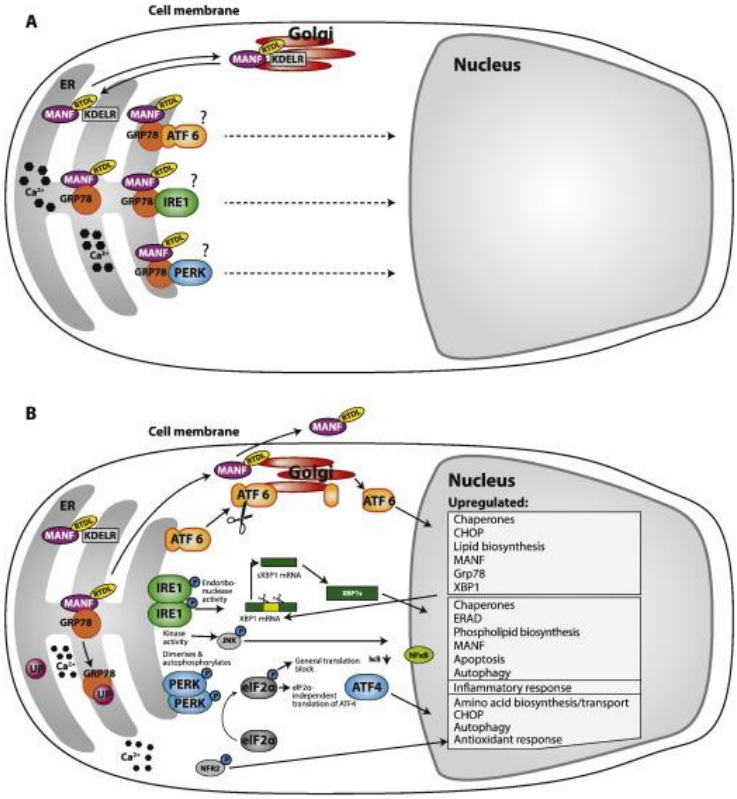

Figure 2i. Hypothesized role of MANF in the unfolded protein response. A) MANF stabilizes the association between GRP78 and UPR proteins keeping them inactive when ER stress is not present in the

cell. **B)** Under ER stress MANF no longer associates with GRP78 in a calcium dependent manner and GRP78 dissociates with the three UPR proteins. The downstream effects of each UPR protein are shown with the ultimate goal of overcoming ER stress or under chronic ER stress conditions, inducing apoptotic effects. (Image from Lindahl et al. 2017 *Neurobiology of Disease*. Reproduction rights obtained and shown in Appendix 1).

2.1.3 MANF in Parkinson's Disease Models

The dopaminergic selective protection exhibited by MANF opened the door to the investigation of MANF as a neuroprotective therapy for PD. Studies have been conducted using several models of PD in various species to demonstrate the protective capabilities of the neurotrophic factor. 6-hydroxydopamine (6-OHDA) models of PD with intrastriatal administration of MANF show dopamine neuron protection and there is evidence for MANF to have restorative potential (Hao et al., 2017; Voutilainen et al., 2009). MANF further protected against developing rotarod motor impairments in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD and protected dopamine and tyrosine hydroxylase levels. Using MPTP as a cellular model, cell viability was increased with MANF administration and mitochondrial membrane potential loss was reversed (Liu et al., 2018). This supports that MANF can reverse oxidative stress-inducing processes in addition to its ER stress reversing capabilities mediated through ER homeostasis, lending that MANF can affect both factors in the multi-hit hypothesis of PD.

2.2 Study Significance

Therapeutic potential for MANF has been preclinically demonstrated to be neuroprotective however investigation of this neurotrophic factor in the pathophysiology justifying it as an ER stress-related therapeutic target has not. Theoretically, with low levels of MANF regulating GRP78 and the UPR, ER stress pathways will be chronically activated favouring apoptotic pathways and lead to dopaminergic degeneration. This study, therefore, tested this hypothesis using lentiviral mediated shRNA to knockdown

MANF chronically and investigate phenotypic manifestations of motor impairments to evidence therapeutic target potential of MANF. Additionally, the effect of chronic MANF reductions allowed for the investigation of ER stress vs apoptosis pathway mediators.

2.3 Specific Study Aims and Hypotheses

Aim 1: Determine if knockdown of MANF in the substantia nigra using shRNA lentiviral mediated technology can produce motor impairments similar to PD.

Hypothesis: Without MANF, chronic ER stress will lead to neurodegeneration in the substantia nigra and produce PD-like motor impairments.

Aim 2: Investigate MANF in the context of ER stress and apoptosis pathways.

Hypothesis: Reduced MANF will have a chronic ER stress response that favours apoptosis and show degeneration in the substantia nigra.

2.4 Author Contributions

Mishra R. designed the study with Bernardo A. and Shawaf O. Bernardo A. and Shawaf O. performed surgeries and conducted the behavioural experiments. Nawar K. performed RT-qPCR biochemical analysis and Bernardo A conducted immunohistochemistry. Statistics were completed by Bernardo A., Shawaf O. and Nawar K. Bernardo, A. wrote the manuscript with assistance from Shawaf, O, and Nawar. K. All authors reviewed and approved the final manuscript.

2.5 Statement of Paper Status: This study is currently being submitted to journals.

Title: Reduced expression of MANF affects ER stress pathways and motor function: MANF's role in Parkinson's disease pathophysiology

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Declarations of interest: None

Abstract:

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a critical protein that promotes the survival of a variety of neuronal and other cell types, namely midbrain dopaminergic neurons. MANF has been implicated in the pathophysiology of Parkinson's disease (PD) and plays a key role in ameliorating endoplasmic reticulum (ER) stress through its activation of the unfolded protein response (UPR). Chronic ER stress that the UPR cannot overcome leads to apoptosis and is hypothesized to be one of the factors involved in dopaminergic neuronal loss in PD. Using lentiviral mediated shRNA technology, this study produced a localized knockdown of MANF in the substantia nigra of rats and assessed motor functions by examining balance, gait, and coordination over a period of several months. Immunohistochemistry for tyrosine hydroxylase and RT-qPCR quantification of ER stress markers and apoptotic factors was also determined. Motor deficits were identified using the narrow beam transversal and fixed-speed rotarod tests, while local asymmetry was displayed using the cylinder test and amphetamine-induced rotations. Deficits were also accompanied with reduced tyrosine hydroxylase levels in the hemisphere with reduced MANF. RT-qPCR quantification of glucose-regulated protein 78 (GRP78) revealed ER stress present at the site of localized MANF knockdown and increased levels of apoptotic marker C/EBP homologous protein (CHOP). In conclusion, knockdown of MANF in the substantia nigra recapitulates key motor features of parkinsonism and affects ER stress pathways. This supports chronic ER stress in the pathophysiology of PD and advocates for further investigation of MANF's therapeutic potential to combat ER stress in PD pathophysiology.

Key Words: Mesencephalic astrocyte-derived neurotrophic factor (MANF), Motor symptoms, Glucose regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), Tyrosine hydroxylase (TH), Parkinson's disease (PD)

Abbreviations: Activating transcription factor 6 (ATF6), Bovine serum albumin (BSA), C/EBP homologous protein (CHOP), Endoplasmic reticulum (ER), Fixed speed rotarod (FSRR), Glucose regulated protein 78 (GRP78), Inositol-requiring enzyme 1 (IRE1), Intraperitoneal (IP), Mesencephalic astrocyte-derived neurotrophic factor (MANF), Neurotrophic factor (NTF), Parkinson's disease (PD), Phosphate buffered saline (PBS), Protein kinase RNA-like endoplasmic reticulum kinase (PERK), Substantia nigra (SN), Tyrosine hydroxylase (TH), Unfolded protein response (UPR)

Introduction

Neurotrophic factors (NTFs) are closely related biomolecules critical for the growth, survival, and differentiation of neurons and other cell types within the central and peripheral nervous systems(Lindahl and Lindholm, 2017; Skaper, 2012). Current literature suggests that NTFs such as mesencephalic astrocyte-derived neurotrophic factor (MANF), also known as Arginine-rich mutated early tumors (ARMET), are involved in regulating endoplasmic reticulum (ER) stress(Hakonen et al., 2018; Richman et al., 2018; Zhang et al., 2018). MANF is a recently discovered and evolutionary conserved NTF identified in both invertebrate and vertebrate species that is mainly localized within the ER due to a KDEL ER retention signal at the C-terminus(Petrova et al., 2003).

The ER is an organelle involved in the proper folding and processing of translated proteins directed for secretion. ER stress can be caused by several factors including protein misfolding, protein aggregation, protein production imbalance, reduced ER calcium stores, errors in protein glycosylation and viral infections(Glembotski et al., 2012; Lindholm and Saarma, 2010; Malhotra and Kaufman, 2007; Rao et al., 2004; Ron and Walter, 2007). Accumulation of proteins in the ER lumen leads to ER stress, and if prolonged causes apoptosis(Kadowaki and Nishitoh, 2019; Lindholm and Saarma, 2010; Rashid et al., 2015). To combat prolonged ER stress-induced apoptosis, the unfolded protein response (UPR) counteracts ER stress through three distinctive pathways: mRNA degradation and termination of protein translation;

regulating protein refolding by ER chaperones; and activation of ER-associated degradation of unfolded proteins(Voutilainen et al., 2015). The UPR is mediated through three ER membrane receptors: protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1)(Hughes and Mallucci, 2019; Lindholm and Saarma, 2010). All three receptors are bound to ER chaperone glucose-regulated protein 78 (GRP78), maintaining their inactive state(Glembotski et al., 2012; Lindholm and Saarma, 2010). MANF is thought to primarily function within the ER to maintain ER homeostasis and reduce ER stress through the UPR of dopaminergic neurons. MANF interacts with GRP78 in a calcium-dependent manner, facilitating MANF secretion in pathological states of decreased calcium due to the accumulation of misfolded proteins(Glembotski et al., 2012; Voutilainen et al., 2015).

The accumulation of misfolded proteins within the ER leads to dissociation of GRP78 from ATF6, IRE1, and PERK, thereby activating the UPR(Bertolotti et al., 2000; Dudek et al., 2009; Zhou et al., 2006). During prolonged ER stress, PERK mediates phosphorylation of eIF2α, leading to the activation of ATF4, which subsequently upregulates CHOP, a(Y. Li et al., 2014; Nishitoh, 2012)(Y. Li et al., 2014; Nishitoh, 2012) transcription factor present at higher levels during prolonged ER stress and used as a marker for chronic ER stress and cell death(Y. Li et al., 2014; Nishitoh, 2012).

Although MANF's mechanism has not been fully elucidated, it is implicated in survival and protection of dopaminergic neurons and pertinent in the pathology of neurodegenerative disorders, such as Parkinson's disease (PD)(Lindahl and Lindholm, 2017; Petrova et al., 2003). PD is a progressive disorder characterized by rigidity, akinesia, bradykinesia and postural instability(Brakedal et al., 2014). A hallmark of PD is the loss of dopamine neurons in the substantia nigra (SN), affecting the basal ganglia's ability to coordinate excitatory and inhibitory motor signals. Increasing evidence from tissue samples of PD patients and rodent models of PD indicate ER stress is also associated with the pathophysiology of PD(Colla et al.,

2012; Hughes and Mallucci, 2019). Markers of chronic ER stress have been identified in MANF mutant *drosophila*(Palgi et al., 2009), and MANF deficient mice report chronically increased UPR with poor dopaminergic neuronal development(Lindahl et al., 2014; Lindholm and Saarma, 2010). Administration of MANF as a therapeutic agent observed in models of PD and traumatic brain injury show protection against dopaminergic neuron death(Airavaara et al., 2009; Hao et al., 2017; Liu et al., 2018). Compelling evidence links MANF to some level of neuroprotection and has indirectly linked MANF to PD pathology. MANF has not been specifically investigated regarding motor function and ER stress relating to PD pathology.

MANF's role in regulating ER homeostasis, the increasing evidence for ER stress-related dopaminergic neuron loss in the pathophysiology of PD coupled with the success of MANF administration as a neuroprotectant and regulator of ER stress necessitates the further investigation of MANF's role in PD. The objective of this study was twofold: the study aimed to investigate whether knockdown of MANF in the substantia nigra using shRNA technology was capable of producing PD-like motor impairments and to investigate how reduced MANF would affect ER stress and apoptotic markers.

Methods

Animals

Male Sprague-Dawley rats (N=14) were obtained from Charles River Laboratories (St. Constant, Canada), and individually housed at the McMaster University Central Animal Facility. All work was completed in accordance with the Canadian Council on Animal Care. Animals were on a reverse 12:12 light/ dark cycle and food-restricted to 90% of their free-feeding body weight with unlimited access to water. Animals were trained and baseline values were obtained. Animals were randomly assigned to

groups prior to shRNA induction. No significant differences identified between groups during baseline testing. Experiment timeline is summarized in Figure 1.

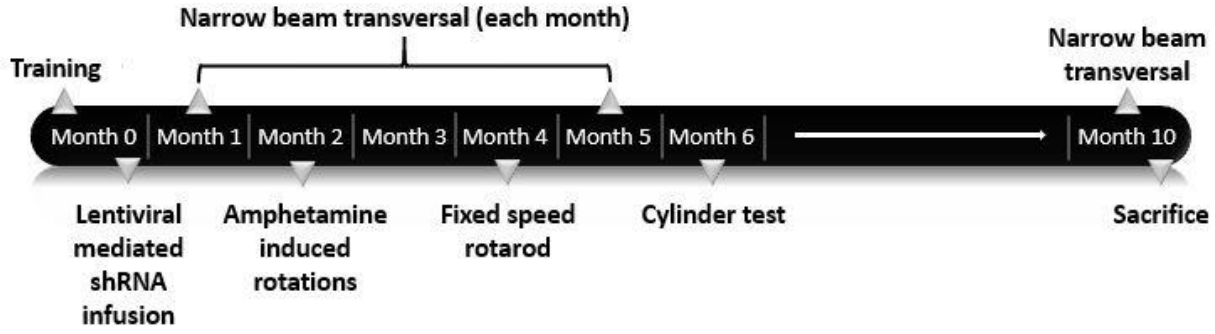

Figure 1: Experimental Timeline. The timeline depicts the sequence of events and behavioural testing the animals underwent from zero to ten months. Grey triangles represent behavioural testing time points. All animals underwent behavioural testing and were sacrificed 10 months post-shRNA lentiviral mediated particle infusion for nucleic acid quantification analysis.

Lentiviral mediated shRNA particles

Lentiviral mediated particles were obtained from Origene Technologies (Rockville, USA). 0.5µL of four unique 29mer MANF (ARMET) specific lentiviral particles with >106TU/mL were combined to make a cocktail (shMANF). This was administered via unilateral stereotaxic injections into the SN, described below. Control animals were infused with scrambled control (shCtrl) sequences also obtained from Origene Technologies. Non-specific effects of the intervention such as tissue damage are controlled for by using the scrambled control sequence. A scrambled control incorporated the viral component of the treatment and therefore differences seen between the groups is a result of reduced MANF induced by shMANF and not non-specific effects of the viral intervention.

Surgeries

Animals were anesthetized and maintained using isoflurane. Animals were mounted on a stereotax and given fluids, analgesics, and local anesthetics. Bregma was located and recorded. The SN was located (A/P: -5.3mm; M/L: ±2.3mm) in reference to bregma using the stereotax and a hole was drilled in the skull above the SN. A syringe containing either shCtrl or shMANF cocktail was lowered into the SN (D/V: -7.8) and 2µL of the solution was infused unilaterally at 0.5µL/min using the UMP3 UltraMicroPump. After infusion, the syringe was left at the site for 8 minutes to ensure complete diffusion into brain tissue. Animals were monitored for 7 days in a biosafety level 2 room then returned to specific pathogen free level. Two weeks was allotted post-surgery before behavioural testing.

Narrow Beam Traversal

Narrow beam transversal tests motor coordination in rodents and specifically identifies changes in coordination and balance(Carter et al., 2001). Animals were trained to transverse a 100cm long and 9cm wide beam elevated 60cm between two platforms. The starting platform was a flat surface and a 20cm x 20cm x 20cm enclosure was on the ending platform. During training, the width of the beam was gradually decreased to 1.5cm over the course of three weeks. Latency to initiate beam transversal and time to transverse the beam was recorded. Rats performed three consecutive trials before returning to their home cage and narrow beam traversal was repeated each month to detect consistency in motor dysfunction.

Amphetamine Induced Rotations

Amphetamine causes increased dopamine release from presynaptic vesicles. When amphetamine interacts with the dopamine transporter it reverses the transport of dopamine, which results in a surge of dopamine in the synaptic cleft(Sulzer, 2011). After amphetamine administration, rotational behaviour is a robust test that phenotypically demonstrates dopaminergic neuron loss and is normally used in the unilateral 6-OHDA lesion model. Unilateral loss of dopaminergic neurons results in an imbalance of

dopamine between the left and right hemispheres after amphetamine administration producing rotational behaviour. The direction can change based on the degree of dopaminergic neuron loss(Mintz et al., 1986; Robinet and Bardo, 2001). Animals received an intraperitoneal (IP) injection of 2.5mg/kg amphetamine and were placed in a clear cylinder (31cm in height and 30cm in diameter). Animals were filmed for 30 minutes from above and their number of rotations was analyzed between the 20-30-minute interval.

Fixed Speed Rotarod

Fixed speed rotarod (FSRR) employs a motor-driven revolving rod (7cm diameter) to measure fore-and hindlimb motor coordination and balance. Rats were placed on the rod and were required to remain on the rotating rod as long as possible. A switch on a landing platform below recorded their endurance upon contact with the falling rat. During testing, each rat was placed on the rotarod for a maximum of 60 seconds for three consecutive trials at 10 rotations per minute (rpm). Latency to fall off the rotarod for each trial was recorded and used in subsequent analysis.

Cylinder Test

Cylinder test exploits natural exploratory behaviour of rodents placed in an unfamiliar environment through quantifying rodent rearing, as demonstrated by pressing their forelimbs against the walls of the cylinder for truncal stability while they survey their surroundings(Schallert et al., 2000). Asymmetry in rodents' forelimb use provided a measure for forelimb akinesia after the rodent is placed in a transparent cylinder 31cm in height and 30cm in diameter(Kriks et al., 2011). The independent use of the left or right forelimb to contact the cylinder wall during a rear was scored for a total of 20 independent forelimb contacts. The initial limb contacting the cylinder wall was recorded and the rodent had to remove

both forepaws from the wall before another independent forepaw wall contact was scored. Data are expressed as the number of ipsilateral forelimb wall contacts per 20 wall contacts(Kriks et al., 2011).

Immunohistochemistry

Investigation of dopamine levels was indirectly measured in the SN using immunohistochemistry for tyrosine hydroxylase (TH) to validate that the MANF knockdown had a deleterious effect on dopaminergic neurons. A 75mg/kg ketamine and 10gm/kg xylazine mixture was used as an anesthetic. The animal was then transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) and the brain was removed and stored in 4% PFA overnight. The brain was then immersed in 10% sucrose for 24 hours followed by 30% sucrose until no longer floating. Each brain was then flash frozen using 2-methyl butane and stored at -80°C. Chromium-gelatin-coated were prepared and 18µm thick serial, coronal sections were obtained of the SN using a cryostat and slides were stored at -80°C. Immunohistochemistry protocol used began by washing the slides 3 times in PBS, then post fixing the slides with 4% PFA for 10 minutes. Another 3 washes with PBS was completed and slides were blocked using bovine serum albumin (BSA), normal goat serum, 0.1% Triton X and PBS before incubation with anti-TH antibody (dilution 1:500) (Cedarlane Cat.# CL8876AP, Lot#167621) overnight at 4°C. Specimens were then washed in PBS and incubated for one hour at room temperature using Texas red goat anti-rabbit antibody (1:1000) (Invitrogen, Cat #T2767 Lot# 1905916). Coverslips were adhered using PureLong Gold mounting medium containing DAPI (Life technologies Cat. #P36935, Lot # 1789705). Sections were then imaged using a Leica DM6 B microscope and the Leica Application Suite X (LAS X) software. Immunofluorescence was then analyzed in each hemisphere (control hemisphere with no treatment and shMANF hemisphere with shMANF administration) by ImageJ software. Integrated density was used to quantify differences as this method controls for a discrepancy in size and allows for ease of comparison between multiple sections. This aspect of the paper is merely demonstrating that dopamine has been

affected by reduced MANF while cell death is demonstrated through RT-qPCR analysis. Images were converted to 8-bit greyscale images and background was subtracted. Integrated density was calculated by the number of pixels that are above the threshold and the mean grey value of those pixels, specifically the product of the number of pixels above threshold and the mean grey value pixels.

RNA extraction, cDNA synthesis, and RT-qPCR

Rats were anesthetized and sacrificed immediately through decapitation. Brain regions collected for analysis included the substantia nigra, striatum, and cortex, which were flash-frozen and stored at -80°C. Total RNA was extracted via the manufacturer's instructions using TRIzol reagent (ThermoFisher Scientific, Catalog#15596018). Genomic DNA was removed using the DNAse I enzyme (ThermoFisher Scientific, Catalog#EN0521). RNA concentration and purity were determined using the NanoDrop 2000 Spectrophotometer. Finally, cDNA was made using qScript cDNA SuperMix (QuantaBio, Catalog#95048-100). To determine Real Time-quantitative PCR (RT-qPCR) specificity, negative reverse transcription controls were made for random samples without the addition of the reverse transcriptase enzyme. Rat MANF, GAPDH, CHOP, and GRP78 primers were used for qPCR analysis (MANF forward 5'-CGGTTGTGCTACTACATTGGA-3' and reverse 5'-CTGGCTGTCTTCCTTCTTGACC-3') (GAPDH forward 5'-CAACTCCCTCAAGATTGTCAGCAA-3' and reverse 5'-GGCATGGACTGTGGTCATGA-3'), (CHOP forward 5'-GTCTCTGCCTTTCGCCTTTG-3' and reverse 5'-CTACCCTCAGTCCCCTCCTC-3') (GRP78 forward 5'-CTGGGTACATTTGATCTGACTGG-3' and reverse 5'-GCATCCTGGTGGCTTTCCAGCCATTC-3'). The final concentration of all primers used was 1µM. RT-qPCRs were completed using manufacturer's specifications of SYBR Green PCR kit (Qiagen CAT # 204056). No template control of nuclease-free water was used for every plate. Triplicates of each sample were analyzed, and Ct values were limited to the variability of ±0.5. Relative gene expression was compared using the delta-delta Ct method as previously described(Joshi et

al., 2018) using the equation ΔΔCt=2-((Ct-Gene of interest–Ct-Housekeeping)–(Ct-Avg Ctrl Gene of interest–Ct-Avg Ctrl Housekeeping). GAPDH was used as the housekeeping gene.

Statistics

Statistical analyses were carried out using GraphPad Prism 6.0 software (GraphPad Software, USA). Before analyses, outlier detection was performed using the GraphPad Outlier Tool. Amphetamine induced rotations, fixed speed rotarod, cylinder, and RT-qPCR results were analyzed using an unpaired Student's t-test. The narrow beam transversal test was analysed using subject matching and a repeated measures two-way ANOVA for each parameter (latency to transverse and transversal time) separately. The relative standard curve method and the mRNA copy numbers were used to confirm no differences in the levels of the housekeeping gene GAPDH. In all statistical tests, significance was defined as *$p<0.05$, **$p<0.01$ and ***$p<0.001$. Results are presented as ± standard error of the mean (SEM).

Results

shMANF administration produced a localized reduction of MANF mRNA in the substantia nigra (SN)

Following 10 months of behavioural testing, rats were sacrificed, and cDNA was prepared. RT-qPCR analysis was performed to confirm reduced MANF expression at the site of shMANF administration in the SN (Figure 2A). Results verified that there was an effective MANF knockdown in the SN as shown by decreased MANF expression in the shMANF group compared to shCtrls ($t(7)=3.170$, $p<0.05$). In order to confirm the localization of the knockdown within the SN and demonstrate no migration of the shMANF lentiviral particles to unintended brain regions after 10 months, the cortex and striatum were analyzed. Regions did not exhibit significant changes in MANF expression compared to shCtrl animals (Figure 2B and 2C). We, therefore, concluded that shMANF produced a localized knockdown of MANF in the SN and the changes in the SN did not significantly affect MANF levels in the striatum or the cortex.

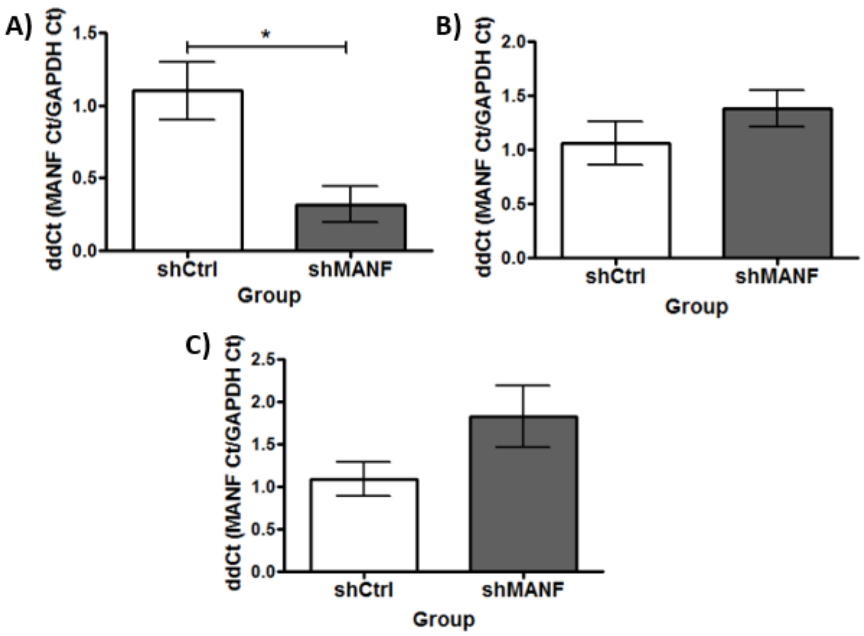

Figure 2. shMANF administration significantly reduced MANF in the SN without affecting other brain regions. (2A) Confirmed knockdown of MANF mRNA levels was shown to be significantly reduced in the SN of animals that received shMANF lentiviral mediated particles compared to those that received shCtrl ($t(7)=3.170$, $p<0.05$). **(2B)** No significant changes to MANF levels were noted in the striatum **(2C)** or the cortex using RT-qPCR and delta-delta CT method of analysis ($\Delta\Delta Ct=2^{-((Ct\text{-Gene of interest}-Ct\text{-Housekeeping})-(Ct\text{-Avg Ctrl Gene of interest}-Ct\text{-Avg Ctrl Housekeeping})}$.

Tyrosine hydroxylase is reduced in the SN of shMANF treated hemisphere

Due to the impact, MANF has on dopamine neurons and dopamine's established role in PD we confirmed that changes in dopamine were present using tyrosine hydroxylase (TH). TH is an enzyme imperative for dopamine production and is often used as an indicator of dopamine neurons in PD related studies. Therefore, tyrosine hydroxylase (TH) was investigated as a marker for dopamine neurons to demonstrate that shMANF influenced dopamine levels in the SN. Using immunohistochemistry, we evaluated TH immunofluorescence of a representative sample, allowing us to maintain a higher sample size for RT-qPCR analysis. Above it was demonstrated that shMANF did not migrate from the site of

injection and therefore we demonstrated the difference in TH levels between the SN in the untreated hemisphere of the brain (used as an internal biological control) and the SN of the shMANF treated hemisphere. The integrated density of TH immunofluorescence (n=3 per hemisphere), was found to be significantly reduced in the shMANF hemisphere compared to the control untreated hemisphere ($t(4)=2.8059$, $p<0.05$) (Figure 3). This confirmed the deleterious effect on the dopaminergic neurons in the SN produced by reduced MANF by shMANF.

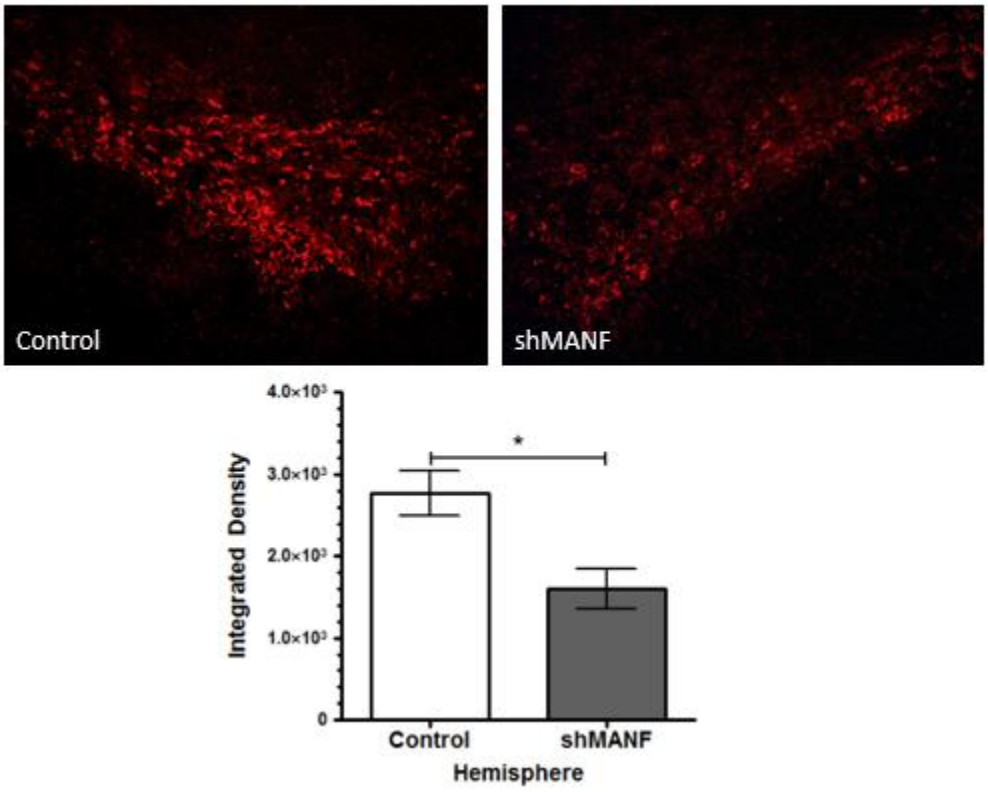

Figure 3. Tyrosine hydroxylase is reduced in the shMANF treated hemisphere. Immunohistochemistry images for tyrosine hydroxylase (TH) in the control hemisphere of the brain and the shMANF treated hemisphere of the brain. Immunofluorescence for TH demonstrated that the integrated density of the shMANF treated hemisphere was significantly less than that found in the untreated control hemisphere ($t(4)=2.8059$, $p<0.05$).

Persistent motor impairments over 10 months using narrow beam traversal

To establish whether reduced expression of MANF resulted in motor impairments, we performed the narrow beam traversal test. This test reliably measures basal ganglia disturbances in genetic models of PD with minimal nigrostriatal damage(Brooks and Dunnett, 2009). It has been shown to detect changes in dopamine function in older animals and consistently implicates nigrostriatal dopamine loss by measuring skilled walking(Meredith and Kang, 2006). Therefore, we utilized the narrow beam transversal test repeatedly over 5 months to establish if the MANF knockdown demonstrated impairments in movement initiation, balance and coordination impairments. The test was then repeated at month 10 to determine impairment persistence 10 months post shMANF administration. The first parameter analyzed was latency to cross the beam. This depicts the time it takes an animal to initiate movement, a major component of gait difficulties present in PD patients(Smulders et al., 2016). Using a repeated measures two-way ANOVA, treatment had a significant effect on latency time $F(1,10)=3.841$, $p<0.01$). shMANF animals demonstrated significantly longer durations to initiate transversal across the narrow beam and Bonferroni post hoc testing revealed significant differences between shCtrl and shMANF groups at months 2, 3, 4, and 5 (Figure 4A). Time was also a significant factor ($F(1,5)=8.575$, $p=<0.0001$), however, time did not affect treatment and treatment did not affect time as the interaction between these two variables was not significant ($F(1,5)=0.6820$, $p=0.6392$). The second parameter investigated using the narrow beam test was transversal time. This parameter focused on the animals' gait and coordination. Repeated measures two-way ANOVA revealed significantly longer traversal durations in the shMANF group when compared to shCtrl animals ($F(1,10)=1.713$, $p<0.005$) with significant Bonferroni post hoc difference found at month 10 (Figure 4B).

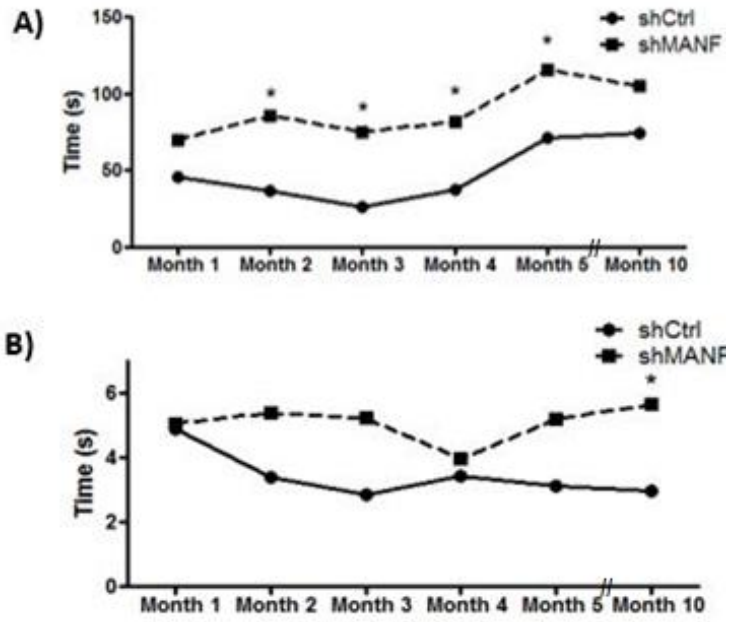

Figure 4. MANF Knockdown affects latency and traversal duration 1-10 months post-surgical infusion using the narrow beam test. Rats were tested on the narrow beam test and measurements were recorded as the mean of three consecutive trials for each parameter. **(4A)** Depicts latency time to initiate beam transversal. Repeated measures two-way ANOVA found shMANF animals took significantly longer to initiate narrow beam transversal $F(1,10)=3.841$, $p<0.01$). Bonferroni post hoc testing revealed significant differences ($p<0.05$). **(4B)** Transversal duration is depicted showing the time it took animals to transverse the beam in seconds.shMANF animals took significantly longer to transverse the beam than shCtrl animals ($F(1,10)=1.713$, $p<0.01$). All values in both graphs are represented as the mean ± SEM values.

Amphetamine induced a contralateral rotation after MANF knockdown

Amphetamine-induced rotation is commonly used in the preclinical 6-OHDA model of PD to evaluate the reduced dopaminergic activity after unilateral lesion. The administration of amphetamine induces ipsilateral or contralateral rotation dependent on the degree of dopaminergic neuron loss(Mintz et al., 1986; Robinet and Bardo, 2001). Upon establishing that behavioural impairments remained present two months post-shRNA infusion, we investigated amphetamine-induced rotations to determine

phenotypically if changes in dopamine levels may be occurring. Therefore, we conducted this test to phenotypically determine if a reduction of MANF would affect this dopamine-related behavioural test and potentially be responsible for the motor impairments observed following the narrow beam transversal test. Results from this test revealed two months after shMANF administration, shMANF animals rotated significantly more in the contralateral direction compared to shCtrl animals ($t(12)=3.401$, $p<0.01$) as determined using a t-test (Figure 5A).

Fixed Speed Rotarod exhibits gait and coordination impairment after MANF knockdown

Fixed speed rotarod (FSRR) test was performed four months after shMANF administration. This test was performed to further evaluate motor function impairments in the shMANF group, specifically through investigation of their gait. FSRR is designed to automate the measurement of neurological deficits in rodents and is one of the most used and sensitive tasks assessing motor function in this population(Brooks and Dunnett, 2009). This test was therefore used to corroborate the narrow beam transversal test results and provide a more sensitive measure to detect gait abnormalities. This test demonstrated marked gait impairments as evidenced by shCtrl animals remaining on the rotating rod for significantly longer durations than shMANF animals at 10rpm ($t(12)=2.380$, $p<0.05$) (Figure 5B).

Forelimb asymmetry found in shMANF animals

The cylinder test was created to detect forelimb impairments in rodents with unilateral 6-OHDA lesions and has proved to be a simple and efficient test for the evaluation of unilateral deficits and asymmetry in voluntary forelimb use(Cenci and Lundblad, 2007). Utilizing an animal's natural exploratory behaviour in new environments, the cylinder test was run six months after shMANF infusion. The cylinder test revealed significant ipsilateral forelimb preference in shMANF animals compared to shCtrl animals ($t(11)=3.303$, $p<0.01$). shCtrl rats did not show favouring, using each forelimb approximately 50% of the time and indicating no impairment to either limb (Figure 5C).

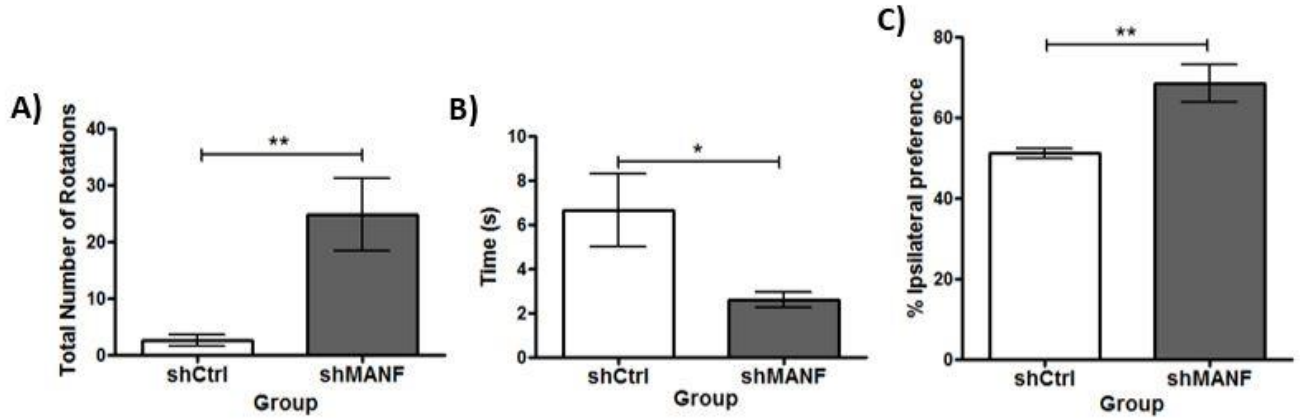

Figure 5. Several behavioural changes are identified in shMANF animals 5A) shMANF rats rotate contralateral to the infusion site after amphetamine administration. Rats were injected IP with 2.5mg/kg of amphetamine. Animals were placed in a clear cylinder and the number of contralateral rotations was measured over 10 minutes in reference to the infusion site. shMANF animals made significantly more contralateral rotations compared to shCtrl animals ($t(12)=3.401$, $p<0.01$). Data are represented as the mean ± SEM values. **5B) The effect of shMANF on the latency to fall at 10 rpm 4 months post-infusion.** Measurements were the mean of three consecutive trials for each parameter and the latency to fall was measured in seconds. An unpaired t-test revealed that treatment did have a statistically significant effect at 10rpm compared to shCtrl rats ($t(12)=2.380$, $p<0.05$). Data are represented as the mean ± SEM values. **5C) The effect of shMANF on ipsilateral forelimb preference 6 months post-surgery.** The data represents the percent of ipsilateral forelimb wall contacts per 20 wall contacts. An unpaired t-test revealed that treatment with shMANF lentiviral mediated particles significantly increased the preference for ipsilateral forelimb wall touches in shMANF animals compared to shCtrl rats ($t(11)=3.303$, $p<0.01$). Data are represented as the mean ± SEM values.

Reduced GRP78 in the substantia nigra

The effects of reduced MANF in the SN on ER stress were investigated through analysis of GRP78. We determined mRNA levels of GRP78 were significantly reduced in the shMANF group ($t(7)=6.060$, p-value<0.001) (Figure 6A). Reduced GRP78 is indicative of the presence of chronic ER stress in the SN of shMANF animals, whereby the UPR is no longer regulated and thus, proapoptotic pathways are unopposed.

Increased mRNA expression of apoptotic marker CHOP

Reduced GRP78 levels found 10 months after shMANF administration suggests prolonged ER stress results in the UPR activating pathways that lead to cell death. Therefore, investigation of the proapoptotic factor CHOP was conducted to investigate the prolonged effects of ER stress in the SN. RT-qPCR analysis of CHOP revealed significantly increased expression of CHOP mRNA in the SN of shMANF compared to shCtrl using an unpaired t-test ($t(8)=2.405$, p-value<0.05) (Figure 6B).

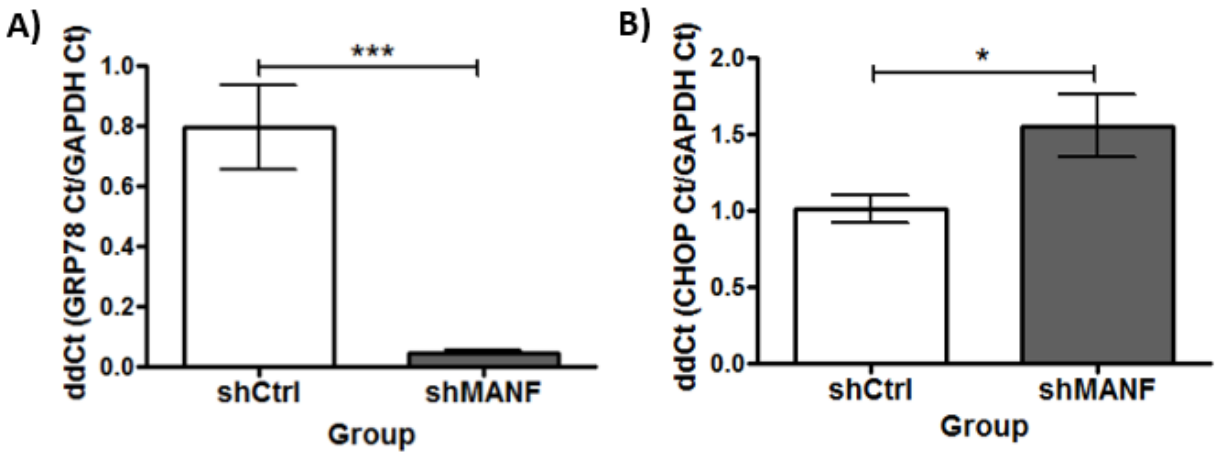

Figure 6. RT-qPCR analysis identified ER stress and apoptotic related changes after MANF Knockdown. qPCR analysis, using the delta-delta Ct method of analysis ($\Delta\Delta Ct=2^{-((Ct\text{-Gene of interest}-Ct\text{-Housekeeping})-(Ct\text{-Avg Ctrl Gene of interest}-Ct\text{-Avg Ctrl Housekeeping})}$), following lentiviral mediated knockdown of MANF in the SN produced **(6A)** a reduced GRP78 mRNA expression in shMANF animals compared to shCtrls ($t(7)=6.060$, p-value<0.001) and **(6B)** shMANF animals demonstrated a significant increase in CHOP mRNA expression compared to shCtrl animals ($t(8)=2.405$, p-value<0.05).

Discussion

The role of NTFs in ER stress and their potential neuroprotective and neurorestorative abilities for PD patients continues to be investigated(Cordero-Llana et al., 2015). In clinical trials, NTFs show some safety however, more research is needed regarding their therapeutic potential(Bartus et al., 2013). Results from this study highlight the role of MANF in ER stress and motor repercussions of ER stress reminiscent of PD-like motor impairments. Localized knockdown of MANF in the SN produced key motor phenotypes of parkinsonism, including deficits in movement initiation, balance, gait and motor coordination. These PD-like motor impairments were chronically maintained 10 months post shMANF administration, as shown by the narrow beam traversal test. Biochemical analysis in this study demonstrates MANF is required to maintain GRP78 levels, further linking MANF to the ER stress pathway. This study demonstrates MANF's essential role in the survival and maintenance of neurons, as supported by the increased presence of C/EBP homologous protein (CHOP) after MANF knockdown.

Despite the mechanism of MANF not being fully understood, evidence continues to support MANF as an ER-resident protein involved in protecting cells intracellularly against and ameliorating ER stress(Apostolou et al., 2008; Glembotski et al., 2012; Henderson et al., 2013; Tadimalla et al., 2008). Proposed mechanisms currently suggest MANF binds with KDEL receptors and GRP78 in a calcium-dependent manner(Glembotski et al., 2012; Henderson et al., 2013). Without the presence of ER stress, GRP78 is bound to UPR receptors IRE1, PERK and ATF6(Bertolotti et al., 2000; Lindahl and Lindholm, 2017; Shen et al., 2002). Under ER stress conditions, GRP78 dissociates from the UPR receptors, initiating the unfolded protein response through binding to misfolded proteins(Kozutsumi et al., 1988; Lindahl and Lindholm, 2017). The purpose of the interaction between MANF and GRP78 has not been definitively identified however, our results support that MANF may act to mediate GRP78's dissociation from UPR

receptors. MANF's interaction with GRP78 is calcium-dependent, whereby calcium imbalance initiates MANF's dissociation from GRP78 to trigger the UPR. The calcium-dependent interaction not only initiates MANF's dissociation from GRP78 but also increases MANF secretion than can have paracrine and autocrine protective functions(Glembotski et al., 2012). Increased MANF levels also upregulate GRP78(Huang et al., 2016) and as demonstrated in this study, reduced MANF levels resulted in reduced GRP78. Decreased GRP78 impedes neurons from triggering the UPR response forcing it into apoptosis. A study downregulating GRP78 found an upregulation of CHOP, consistent with findings in this study(H. Li et al., 2014). Coupled together, the decrease in GRP78 and increase in CHOP expression found indicates that without MANF, the UPR response is unmounted to compensate for ER stress, thus favouring apoptosis. Increased CHOP expression has also been identified in 6-OHDA and MPTP toxin models of PD(Holtz and O'Malley, 2003; Silva et al., 2005). Overexpression of CHOP and subsequent apoptosis has significant implications in many neurodegenerative disorders(Y. Li et al., 2014; Silva et al., 2005).

Behavioural results from this study support a diminished UPR response leading to ER stress-induced apoptosis capable of producing PD-like motor impairments. Amphetamine induced rotations are commonly used in the 6-OHDA model of PD to establish the imbalance of dopamine between the two hemispheres of the brain. This is due to receptor supersensitivity after 6-OHDA destroys >98% of the dopamine terminals producing an ipsilateral rotation. The ipsilateral rotation occurs because amphetamine blocks the reuptake of dopamine, however in the lesioned side of the brain there is insufficient dopamine release, thus producing hallmark ipsilateral rotation. Prior to >98% of dopamine depletion studies have commonly found animals rotate in the contralateral direction, similar to findings from this study(Mintz et al., 1986; Robinet and Bardo, 2001). Extrapolating from the amphetamine-induced rotation results, some dopamine loss is likely present, as animals rotated significantly in a contralateral direction. Previous studies have attributed this to the supersensitivity of receptors produced

after significant dopamine depletion. If depletion is between 80-97% the remaining residual dopamine neurons release a small amount of dopamine capable of super stimulating the postsynaptic dopamine receptors to produce a contralateral rotation(Carey, 1992). In the present study, the significant contralateral rotational behaviour is likely due to a dopamine depletion of less than 97% and postsynaptic supersensitivity was stimulated by residual dopamine neurons. Asymmetrical motor function was also identified without pharmacological challenge in this study as depicted by the cylinder test. Rats favoured their ipsilateral forelimb indicative of contralateral impairment to some degree. Immunohistochemical analysis of TH also further demonstrates that changes to the dopaminergic system were produced by shMANF. These changes are likely responsible for motor impairments identified as TH was reduced, indicative of dopamine neuronal loss, in the shMANF treated hemisphere.

PD patients have motor disabilities including bradykinesia, rigidity, gait abnormalities, poor postural balance, and akinesia associated with movement initiation. The narrow beam traversal task used two parameters to assess different motor impairments seen in clinical populations. Latency duration is a marker for akinesia, while traversal duration evaluates bradykinesia and balance(Allbutt and Henderson, 2007; Brooks and Dunnett, 2009; Glajch et al., 2012; Pothakos et al., 2009). FSRR established gait, balance and coordination abnormalities to provide a well-rounded investigation of several motor disabilities characterized in PD**.** Deficits identified in this study suggest MANF is required to maintain proper motor control likely due to increased ER stress and subsequent apoptosis arriving after failed maintenance and protection by MANF. MANF assists in protein folding and ameliorating ER stress, thereby preventing neuronal apoptosis and degeneration in PD and suggesting it as a potential therapeutic in the maintenance of midbrain dopaminergic neurons(Lindholm and Saarma, 2010; Parkash et al., 2009). *Drosophila* and *c. elegans* studies identified MANF mutants to have increased dopaminergic degeneration, potentially through the activation of the UPR leading to cell death under irreversible ER damage(Palgi et

al., 2012, 2009; Richman et al., 2018). MANF has also been used as a therapeutic for neuroprotection in toxin models and alpha-synuclein models of PD(Liu et al., 2018; Voutilainen et al., 2009; Zhang et al., 2017). Together, these findings demonstrate the importance of MANF's presence in the SN for maintenance and neuroprotection from ER stress-related damage in neuronal populations.

Despite our best efforts, behavioural tests such as cylinder, FSRR, and narrow beam traversal share the inevitability of practice effects, whereby animals' performance may improve due to progressive familiarity with the task during each testing session(Schallert et al., 2000). To overcome this limitation, we investigated several motor tests only once. Narrow beam test, however, was used continuously to establish the long-term presence of behavioural impairments after MANF knockdown. To reduce practise effects, animals were tested repeatedly for 5 months and then testing was stopped between months 6 through 9 before testing was reinitiated at month 10. Rodent models for the study of neurodegenerative disorders present challenges when attempting to translate preclinical findings to clinical correlates. Animal models aim to recapitulate the disease in three different ways, including: phenotypically (referred to as face validity); pathophysiologically (known as construct validity); and the ability to predict pharmacological translatability to humans (referred to as predictive validity). This study has shown reduced MANF produced both face and construct validity, evidenced by motor impairments and increased ER indicators respectively. Future studies should investigate non-motor feature subtypes of PD(Marras and Chaudhuri, 2016) in a MANF knockdown model and investigate predictive validity using PD treatments such as L-DOPA and carbidopa and exogenous MANF. Furthermore, MANF levels can be specifically investigated in clinical populations of PD through post-mortem analysis.

In conclusion, this study demonstrated MANF's role in ER stress pathways. Moreover, we demonstrated the major implication of reduced MANF within neurodegeneration, therefore, acknowledging its significance as a neuroprotective agent. The etiology of PD remains to be elucidated

and this is the first study demonstrating that MANF knockdown recapitulates key motor features of parkinsonism. Thus, these findings coupled with previous literature that supports MANF overexpression in the SN protecting dopaminergic neurons, suggests the vital role of MANF in both the pathophysiology of and potential treatment for PD.

Acknowledgments

Authors thank William Brett McIntyre, Dima Malkawi, Sumitha Sivagnanasundram and Vidhi Patel for their assistance in behavioural work and all members of the Mishra laboratory for their help and support throughout the project.

Funding:

This work was supported by the Canadian Institutes of Health Research [grant no. 126004].

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3.0 Chapter 3 – CDK5/p25 as an Oxidative stress-related molecular target for neuroprotection in Parkinson's disease

Preface

As stated in Chapter 1: Introduction, oxidative stress is one of the prominent theories for dopaminergic degeneration in Parkinson's Disease (PD) and other neurodegenerative disorders. The increased presence of reactive oxygen species (ROS) during oxidative stress can lead to DNA damage, RNA damage and eventually overwhelms a cell's natural defense systems, which initiates programmed cell death. Oxidative stress induces pro-apoptotic pathways such as those through cyclin-dependent kinase 5 (CDK5)/p25 hyperactivity that can be targeted for neuroprotective purposes. The neurotoxin 6-hydroxydopamine (6-OHDA) is known to induce apoptosis through oxidative stress (Konnova and Swanberg, 2018), therefore CDK5/p25 hyperactivation was investigated in this model as a molecular target for neuroprotection related to oxidative stress.

3.0 Background

3.1 Cyclin dependent kinase 5 (CDK5)

Cyclin dependent kinases are a family of proteins that are primarily involved in the regulation of the cell cycle. Identified, so far, are cyclin dependent kinase (CDK) 1-13 and all phosphorylate their substrates are either a Ser-pro or Thr-Pro site. Cyclin dependent kinase 5 (CDK5) is an atypical CDK as it is not involved in the regulation of the cell cycle but rather, axonal guidance, lamination, and migration during development, differentiation and neuronal survival. It is also functional in post-mitotic cells which is not common among other CDKs (Camins et al., 2006). CDK5's role in survival functions, unique regulation compared to other CDKs and CDK5 hyperactivation in the oxidative stress theory of PD make CDK5 a kinase of interest in neurodegenerative disorders.

3.1.1 Localization and Structure

CDK5 is expressed in neurons, cardiomyocytes, and podocytes. Within each cell type, regulation is hypothesized to occur through distinct activators (Hagmann et al., 2015). In neurons, the activators responsible for CDK5's regulation are p35, p39, p10, and p25. The two most common being p35 and p25. These activators are specific to CDK5 regulation in neurons and are not expressed in other tissues of the body (Lew et al., 1994; Shah and Lahiri, 2014; Tsai et al., 1994). Localization of CDK5 within a neuron is based on these regulators as they retain CDK5 in different subcellular locations but evidence has not shown that these activators are responsible for shuttling the kinase to different regions (Hagmann et al., 2015). Association with membrane-bound regulators p35, p39 or p10 will retain CDK5 within the perinuclear region or plasma membrane (Asada et al., 2012). When bound to p25, CDK5 becomes redistributed within the nucleus and cytoplasm as this regulator does not retain a membrane binding domain (Shah and Lahiri, 2014). Structurally, CDK5 is a kinase made up of a small N-terminal lobe and a C-terminal helical lobe. Between these is an activation loop also known as the T loop of approximately 20 amino acids. When inactive the T loop acts as a steric blockade of the activation site. When active, this activation loop is extended and assists with substrate specificity and stabilization (Tarricone et al., 2001). As stated above association with p35 and p25 determines CDK5 activity as well as localization. These activators bind to PSAALRE in an αC helix of CDK5 (Mapelli and Musacchio, 2003; Tarricone et al., 2001). Binding with p35 and p25, as well as the other less understood regulators p39 and p10, regulate CDK5 activity as discussed below.

3.1.2 CDK5 Regulators

CDK5 is activated by its binding to specific activators, p35, p39 and cleaved forms of these (p10, p25, and p39) (Shah and Lahiri, 2014). The first activator to be discovered was p35, a pro-survival activator that when bound to CDK5 forms a complex that phosphorylates targets in a physiological manner. The N-

terminal of p35 contains a myristoylated region that keeps this activator bound to the plasma membrane or perinuclear membrane which further regulates its phosphorylation targets. p35 is cleaved by the calcium-dependent enzyme calpain to create an N-terminal component p10 and a C-terminal component p25. Once cleaved p10 retains the myristoylated region resulting in membrane targeting. p25 is not membrane bound and free to move around the cell and often found in the cytoplasm or the nucleus. The N-terminal p10 is also created by cleavage of p39 into p10 and p29 however very little is known about p39, p29 or even p10. Under neuronal insult or pathological conditions, such as excess calcium release from the ER during ER stress, there is an increase in intracellular calcium which requires mitochondria to buffer extra calcium and produces excess ROS. The extra calcium then hyperactivates calpain which cleaves p35 into p10 and p25 (Kai-Hui Sun et al., 2008; Lee et al., 2000). p25 is the truncated form of p35 known to be present during oxidative stress and leads to neurotoxic effects in neurons through hyperactivated CDK5. Both p35 and p10 are easily degraded through ubiquitination pathways giving them very short half-lives, however, p25 is not able to be degraded by ubiquitination, and stabilizes the active conformation of the T loop. This results in a 5-fold longer half-life of p25 than both p35 and p10 and producing hyperactivated CDK5 (Patrick et al., 1999; Shah and Lahiri, 2014). Due to the lack of myristoylated region in p25, the hyperactivated form of CDK5 travels to the cytoplasm and nucleus where it hyperphosphorylates physiological targets and phosphorylates non-physiological targets leading to apoptotic cascade activation. Cleavage of p35 into p25, therefore, changes both half-life and substrate specificity of CDK5.

3.1.2 CDK5 Activation

CDK5 activation by the regulators discussed above is unique as these are CDK5 specific. Other CDK's require a two-step activation process which entails binding of a cyclin and phosphorylation of the T loop. CDK5 is atypical in this regard as it has a single step activation process. Binding of any of the above

activators (p35, p25, p10, p39, p29) is enough to activate the kinase. CDK5, therefore, does not require phosphorylation of the T loop to become activated. Instead binding of activators forms several interactions with CDK5 in a way that bypasses the requirement for phosphorylation to be activated (Tarricone et al., 2001). CDK5 has over 20 substrates, some of which include the dynein associated protein Nudel, synapsin I, Tau and DARPP32 a striatum specific protein that regulates dopamine signaling (Patrick et al., 1999; Tarricone et al., 2001). CDK5 substrates depend on which complex was formed, CDK5/p35 or CDK5/p25.

3.1.3.1 CDK5/p35

CDK5 functions as a serine-threonine kinase that is proline-directed. When CDK5 forms a complex with p35, pro-survival, development-oriented processes are favoured (Figure 3i). CDK5/p35 is critical for development as knockout produced an adult lethal phenotype (Chae et al., 1997). CDK5/p35 is also critical for proper cortical organization and lamination (Chae et al., 1997; Ohshima et al., 1996). Other functions of this complex include aiding in axonal migration, process outgrowth and neuronal signalling (Paglini et al., 2001; Rashid et al., 2001). CDK5 has been proposed to fine-tune synaptic vesicle ratios between the resting and recycling pool of presynaptic neurons through phosphorylation of synapsin I (Matsubara et al., 1996).

3.1.3.2 CDK5/p25

The CDK5/p25 complex has neurotoxic effects within a neuron and eventually leads to apoptosis. Several mechanisms support this observation such as hyperphosphorylation of Tau by CDK5/p25 which leads to aggregation then neurofibrillary tangles and golgi fragmentation and ultimately oxidative stress (Shah and Lahiri, 2014; K.-H. Sun et al., 2008). Lamins are another substrate of the CDK5/p25 complex which disrupts the nuclear envelope inevitably leading to cell death (Chang et al., 2011). Through these pathways and

evidence from PD models and post-mortem samples, CDK5/p25 has been proposed to contribute to neuronal degeneration (Figure 3i).

3.1.4 CDK5 Hyperactivation in Parkinson's Disease

Evidence for hyperactive CDK5/p25 in PD is supported by preclinical studies and post-mortem analysis at multiple points in the pathway leading to hyperactivation. Calpain is required to convert p35 into p25, therefore, without calpain there is reduced p25. Inhibition of calpain has shown preventative effects on neuronal degeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model, suggesting it is the CDK5/p25 complex that is detrimental to the neurons (Crocker et al., 2003). The preclinical MPTP model also increases CDK5/p25 activity and increases the degradation of p35 (Endo et al., 2009). Further MPTP studies show CDK5 specifically is the mediator of dopamine neuron loss, which is partly mediated by phosphorylation of myocyte enhancer factor 2 (MEF2). Phosphorylation of MEF2 inactivates this survival factor and leads to cell death (Smith et al., 2006, 2003). CDK5/p25 supports the oxidative stress theory of PD pathology as CDK5/p25 phosphorylates Prx2 to downregulate its peroxidase activity and stops DNA repair enzyme apurinic endonuclease I by decreasing its activity through phosphorylation, both leading to oxidative stress and cell death (Huang et al., 2010; Qu et al., 2007a). CDK5/p25 activation also supports the ER stress hypothesis of PD as activation of this kinase furthers cell death in ER stress neurons, and overexpression of alpha-synuclein producing ER stress induces calpain overactivation which hyperactivates the CDK5/p25 complex (Czapski et al., 2013; Saito et al., 2007). In human studies, evidence for increased activation of the calpain/p25 pathway was identified in PD post mortem samples and CDK5 is colocalized within LBs found in PD brains (Alvira et al., 2008; Brion and Couck, 1995; Nakamura et al., 1997). Parkinson's associated genes further implicate CDK5/p25 hyperactivation as CDK5/p25 also phosphorylates Parkin triggering Parkin aggregation and inactivation which consequently decreases ubiquitin ligase activity (Avraham et al., 2007; Rubio de la Torre et al., 2009). Areas identified with Parkin

over phosphorylation are also correlated with areas of p25 reinforcing that it is the CDK5/p25 complex responsible for hyperphosphorylation effects (Rubio de la Torre et al., 2009). Involvement of CDK5/p25 hyperactivation in PD pathophysiology is growing and therefore warrants investigation of its potential as a therapeutic target.

3.2 Truncated Peptide 5 (TP5)

Truncated peptide 5 (TP5) is a 24 amino acid truncated peptide that was derived based on the structure of p35 (Binukumar et al., 2015). Originally, a 126 amino acid fragment of p35 was found to specifically inhibit CDK5/p25 activity without affecting CDK5/p35 (Zheng et al., 2002). Serial truncation of CIP then led to the identification of P5, a 24 amino acid peptide with similar CDK5/p25 specific inhibitory properties. A modification was made to allow this peptide to cross the blood brain barrier (BBB) and was then termed truncated peptide 5 (TP5) (Binukumar and Pant, 2016). TP5 has the novel and unique ability to inhibit the CDK5/p25 complex activity without interfering with endogenous CDK5/p35 activity (Zheng et al., 2010, 2002). This specific CDK5/p25 inhibition is critical as CDK5 inhibitors show some protective properties, however, they also inhibit the beneficial, physiological effects such as neural signalling produced by the CDK5/p35 complex. Specific inhibition of CDK5/p25 hyperactivation allows regular CDK5/p35 activities to continue while apoptotic pathways induced by CDK5/p25 can be inhibited. The exact molecular mechanism of selectivity has not been fully elucidated however a study by Cardone et al. supports that TP5's inhibitory properties are through selective competitive inhibition of p25 (Cardone et al., 2016) (Figure 3i). Potential therapeutic benefits of TP5 have begun to be investigated in pre-clinical models of PD. *In vitro* studies of dopamine neurons show TP5 is protective against cell death and inhibits inflammation induced by MPTP. *In vivo* studies of the MPTP model were then conducted and found TP5 was again capable of protecting neurons from cell death and decreasing inflammation in addition to suppression of astroglial and microglial activation induced by MPTP (Binukumar and Pant, 2016;

Binukumar et al., 2015). Toxic effects of this peptide have not been identified and further investigation of TP5 in other preclinical models of PD and on behavioural functions is warranted.

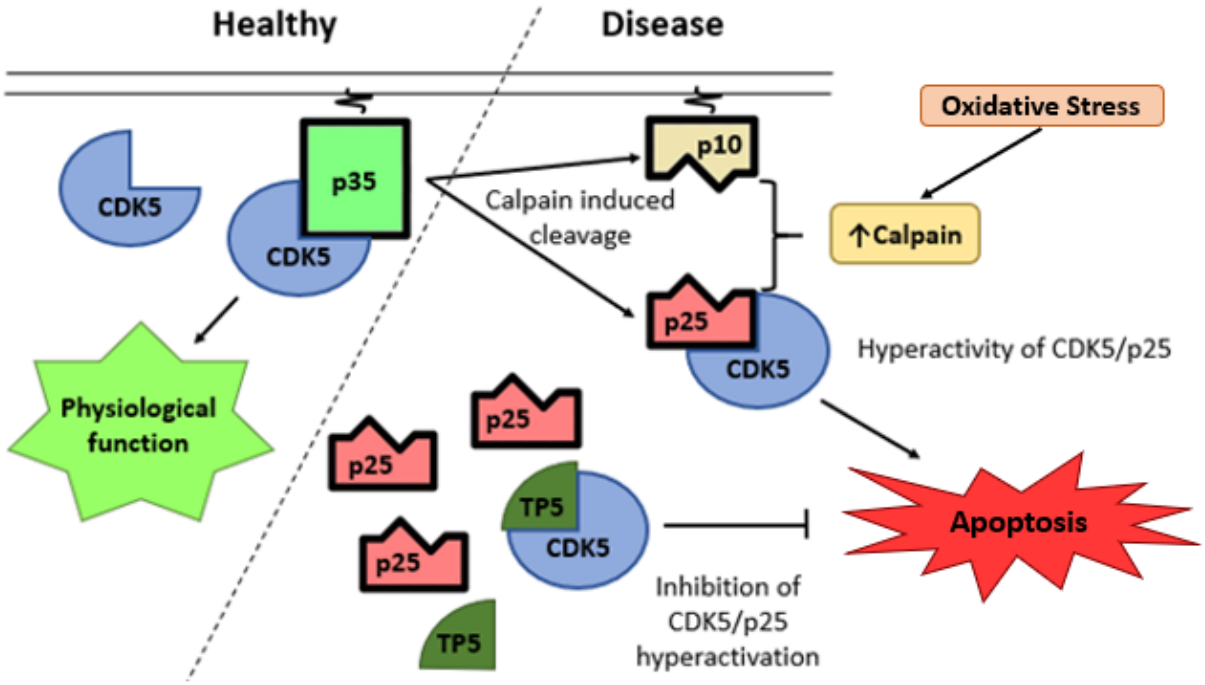

Figure 3i. Diagram of CDK5 regulation via its different activators. CDK5 forms a complex with p35 during physiological states to produce pro-survival functions such as proper migration and neuronal organization. In disease states and in the presence of oxidative stress, calcium-dependent enzyme calpain activity is increased and cleaves p35 into p10 and p25. p25 is no longer membrane-bound and has a longer half-life and a higher affinity for CDK5. The CDK5/p25 complex is hyperactive and leads to apoptosis. TP5 is a novel CDK5/p25 inhibitor and inhibits p25 from binding to CDK5 thus inhibiting apoptosis while not affecting CDK5/p35 functions.

3.3 Study Significance

Oxidative stress is proposed to contribute to neurodegeneration and CDK5/p25 hyperactivation can both promote oxidative stress and be a result of oxidative stress that increases calcium (Görlach et al., 2015; Guo et al., 2018; K. H. Sun et al., 2008). The current study specifically investigates CDK5/p25

hyperactivation in the degenerative pathology of PD through increased CDK5/p25 activity induced by the neurotoxin 6-OHDA. Additionally, the study investigates a novel potential therapeutic peptide that targets CDK5/p25 hyperactivation to support CDK5/p25 as a beneficial target for neuroprotection which is currently a highly unmet need in PD treatment.

3.4 Specific Study Aims and Hypotheses

Aim 1: Identify CDK5/p25 hyperactivity in the 6-OHDA model.

Hypothesis: CDK5/p25 activity will be increased in animals treated with 6-OHDA compared to aCSF controls.

Aim 2: Demonstrate neuroprotective properties of TP5 through behavioural and biochemical data supporting CDK5/p25 as a potential molecular target.

Hypothesis: Animals pretreated with TP5 prior to 6-OHDA will perform better on motor tasks and display less degeneration within the brain.

3.5 Author Contributions

Mishra R. and Bernardo A. designed the study with the assistance of Pant H. Bernardo. A performed all surgeries and infusions as well as conducted all behavioural and immunohistochemistry experiments and analyses. Amin N. conducted CDK5 immunoprecipitation and assay and data were analyzed by Bernardo. A. Bernardo A. wrote the manuscript. All authors edited and reviewed the final manuscript.

3.6 Statement of Paper Status: This paper has been submitted to Experimental Neurology and is currently awaiting review.

Title

Inhibition of hyperactive cyclin dependent kinase 5/p25 is protective in the 6-hydroxydopamine model of Parkinson's disease

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Abstract

Background: Cyclin-dependent kinase 5 (CDK5) is a multifunctional enzyme involved in neuronal development, maturation and survival functions. CDK5 activity is tightly regulated by association with regulatory proteins p35 and p39. Upon neuronal insults, increased intracellular calcium activates calpain. This enzyme cleaves p35 to p25, which has a higher affinity for CDK5. Association with p25 hyperactivates CDK5, initiating apoptotic cascades that lead to significant dopaminergic loss and plays a major role in neurodegenerative disorders, such as Parkinson's disease (PD).

Objective: This study investigates the selective inhibition of CDK5/p25 by a 24-amino acid peptide derived from p25/p35 called truncated peptide 5 (TP5) and its neuroprotective properties.

Methods: Neuroprotective capabilities of TP5 were tested in the 6-hydroxydopamine (6-OHDA) model of PD. Motor assessments included locomotor activity, beam transversal, fixed speed rotarod, and amphetamine-induced rotations. Immunohistochemistry investigated tyrosine hydroxylase levels and immunoprecipitation and assay investigated CDK5 activity.

Results: Pre-administration of TP5 maintained locomotor activity, preserved beam transversal scores, protected motor coordination on the fixed speed rotarod and attenuated amphetamine-induced rotations in 6-OHDA lesioned rats, all indicative of neuroprotection by TP5. CDK5 activity in TP5+6-OHDA treated animals was not found to be significantly different from aCSF treated sham surgery controls. Immunohistochemistry revealed a significant increase in tyrosine hydroxylase within the substantia nigra in animals pretreated with TP5.

Conclusions: Hyperactive CDK5/p25 inhibition in the 6-OHDA model has neuroprotective effects capable of protecting against the development of PD-like motor phenotypes and pathology. This supports CDK5/p25 specific inhibition as a target for further neuroprotective therapeutic development.

Keywords: CDK5/p25, TP5, 6-hydroxydopamine, Parkinson's disease, tyrosine hydroxylase

Highlights:

- Novel CDK5/p25 specific inhibitor, TP5, protects against PD like behavioural phenotype development in 6-OHDA SN lesion model
- Dopaminergic neuroprotection is demonstrated by TP5
- CDK5/p25 inhibition has potential neuroprotective therapeutic effects for neurodegenerative disease

Introduction

Parkinson's disease (PD) is a debilitating neurodegenerative disorder characterized by degeneration of dopamine neurons within the substantia nigra and nigrostriatal circuitry(Radhakrishnan and Goyal, 2018). Resulting motor symptoms include tremors, bradykinesia, and muscle rigidity while non-motor symptoms include memory loss, speech impairments, depression and anxiety(Galvan and Wichmann, 2008; Magrinelli et al., 2016; Schapira et al., 2017). The "gold standard" treatment is dopamine replacement therapy levodopa (L-dopa) however, with disease progression L-dopa loses its efficiency and coincides with other motor and non-motor side effects(Rascol et al., 2003). L-dopa is also metabolized by gut microbiota reducing the availability of the drug(van Kessel et al., 2019). Progressive dopaminergic degeneration diminishes treatment response and therapeutics have a limited capacity to provide neuroprotection. A significant theory for dopaminergic depletion in PD is increased oxidative stress, endoplasmic reticulum stress and subsequent hyperactivation cyclin-dependent kinase 5 (CDK5)(Blesa et al., 2015; Cruz et al., 2003; Qu et al., 2007b; Schapira, 2008).

CDK5 is a neuron-associated serine/threonine kinase involved in neuronal migration, differentiation, synapse development and synapse function(McLinden et al., 2012). Regulation by

different activators allow CDK5 to mediate anti-apoptotic effects or induce apoptotic effects. Specific binding of CDK5 to activators p35, p25 (a c-terminus fragment of p35) or p39 determine the anti-apoptotic or pro-apoptotic downstream effects of CDK5 activity(Hisanaga and Endo, 2010; McLinden et al., 2012). The role of p35 in neuronal survival is well established by inhibiting c-Jun N-terminal kinase 3 (JNK3) and activating the neuregulin/ phosphatidylinositol 3-kinase (PI3K)/Akt pathway(Li et al., 2003, 2002). During physiological stress, p35 is cleaved by the calcium-dependent enzyme calpain into p25. Cleavage converts pro-survival p35 into the shorter, pro-apoptotic p25 fragment(Kusakawa et al., 2000). CDK5 bound to p25 phosphorylates nuclear proteins inducing pro-apoptotic events, damages DNA and arrests the cell cycle. In disease states such as PD, increased presence and longer half-life of p25 hyperactivates CDK5. The CDK5/p25 complex increases neuronal degradation by inhibiting paired-related homeodomain protein 2 (Prx2), a reactive oxygen species (ROS) scavenger, resulting in the accumulation of ROS and oxidative stress(Qu et al., 2007b). Treatment strategies attempting to inhibit elevated CDK5 activity have proven ineffective because they also inhibit normal pro-survival p35-bound CDK5 activities(Binukumar et al., 2015; Siklos et al., 2015).

A novel peptide, truncated peptide 5 (TP5), is a 24-amino acid peptide designed based on the structure of p35. TP5 inhibits CDK5/p25 specifically while having minimal effects on the endogenous CDK5/p35 complex(Zheng et al., 2010, 2005). TP5 has been investigated for neuroprotective properties in models of neurodegenerative disorders such as Alzheimer's disease (AD) and ischemic stroke and has decreased neurofibrillary tangles, inflammation and amyloid plaques(Ji et al., 2017; Shukla et al., 2013). Neuroprotective properties have also been documented using TP5 in 1–methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP/MPP(+)) animal and cell culture models of PD(Binukumar et al., 2014a, 2015). Functional neuroprotection related to PD is important to evaluate during the investigation of novel potential therapeutics. This study considered a battery of motor function and motor coordination tasks to investigate if neuroprotection exhibited by TP5 extends to protecting against the development of PD-

like motor impairments in the 6-hydroxydopamine (6-OHDA) model of PD. We further demonstrate the neuroprotective properties of TP5 through investigation of CDK5 levels and tyrosine hydroxylase (TH).

Methods

Animals

Sprague-Dawley rats, 8 weeks old, weighing 250-300g were obtained from Charles River Laboratories (Quebec, Canada). Animals were individually housed at McMaster University Central Animal Facility at 21^{o}C, on a 12-hour dark/light cycle and on a food-restricted diet to maintain 95% of their free-feeding weight. Rats received water *ad libitum*, and all experimental procedures were completed in accordance with the Animal Research Ethics Board and the Canadian Council on Animal Care.

Surgery

Animals were anesthetized using Isoflurane and oxygen. Animals were mounted on a stereotax and an incision was made along the skull. Bregma was located and stereotaxic coordinates were documented. At stereotaxic coordinates A/P: -5.3mm and M/L: ±2.3mm in reference to bregma, from the Paxinos and Charles Watson's rat brain atlas (2nd edition), a hole was drilled above the substantia nigra (SN). A second hole was drilled close to the first hole and a jeweller's screw was secured to act as an anchor for the permanent cannula. The permanent cannula (HRS Scientific, Quebec) was lowered into the substantia nigra (D/V: -7.3mm) using the stereotax. Dental cement was used to attach the cannula to the jeweller's screw. Once secured, a dust cap was inserted into the cannula to avoid clogging and prevent infection. The incision was closed, and animals were given 5 days to recover before infusions began.

Treatments

TP5 and Scrambled peptide (ScP) were obtained from Dr. Pant (NIH, USA). 1mg of TP5 was dissolved in 150µL of sterile water. This dilution optimized the largest quantity of TP5 sufficiently dissolved in the

smallest amount of aqueous solution allowing for the most TP5 able to be administered into the SN. ScP was prepared identically to TP5.

6-OHDA was obtained from Sigma-Aldrich (Oakville, Ontario) and dissolved in 0.9% saline containing 0.2% ascorbic acid. All animals received 8µg 6-OHDA in 3µL of solution. 6-OHDA was kept on ice and away from light.

Infusions

Infusions commenced 5 days after surgical placement of cannulas. The infusion cannula was connected to a Hamilton syringe placed in a Harvard Apparatus 2000 syringe pump via a short tube. The infusion cannula was checked for blockages, inserted into the permanent cannula (D/V: 7.8mm) and attached using the connector assembly (HRS scientific, Quebec). All animals received 3 infusions under anesthesia at an infusion rate of 1uL/min. Infusion one occurred 10 hours prior to 6-OHDA ensuring time for solution uptake by cells. Depending on treatment group animals received 4µL of aCSF, ScP or TP5 as infusion one. Infusion two (2µL of the same treatment previously received) occurred 1.5 hours prior to 6-OHDA insult. Infusion two increased the amount of treatment at the site of 6-OHDA administration to account for any degradation by peptidases. The third infusion was 8µg 6-OHDA in 3µL administered to all treatment groups other than the control used in biochemical assessment, which received aCSF.

Behavioural testing

Animals were habituated, trained and baseline tested for motor tasks prior to surgery. No differences were found between groups prior to surgery. Behavioural testing was conducted 7 days after infusions. Experimental timeline is depicted in Figure 1.

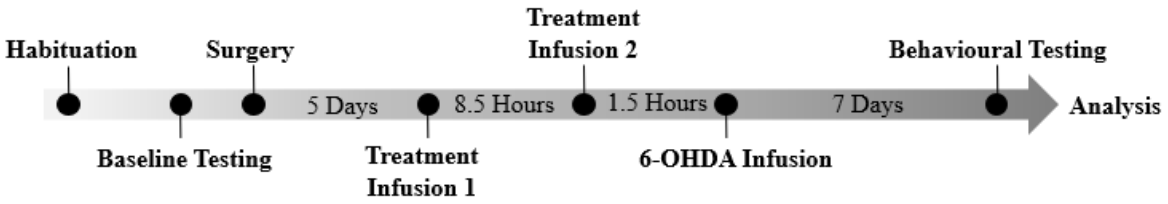

Figure 1. Experimental Timeline. Visual representation of the experimental treatment timeline and behavioural work. Important temporal elements depicting the amount of time between two events that are shown as black circles. Treatments given at infusion 1 and 2 were dependent on treatment group: Control received aCSF at both infusions, aCSF+6-OHDA group received aCSF for both infusions, ScP+6-OHDA animals received ScP for both infusions and the TP5+6-OHDA group received TP5 at both infusion time points.

Locomotor activity

Animals were placed in AccuScan computerized chambers (Accuscan Instruments, Columbus, OH). Chambers measured animals' locomotor activity in the x and y planes using infrared beams evenly distributed front to back and left to right. When the animal travels, specific beams are broken, and VersaMax Analyser software identified the total distance travelled by the animal. Total distance travelled was recorded for all animals over a 2-hour period.

Beam walk

Beam walk measured motor coordination and balance(Allbutt and Henderson, 2007). A flat surface starting platform and 20cmx20cmx20cm enclosed finishing platform were 60cm above the table and 1 meter apart. Animals were trained until they could easily transverse a 1.5cm wide beam using a Kellogg's Froot Loop as an incentive at the finishing platform. The following published scoring system evaluated performance: 0= transverses with no difficulty, 1=transverses with little difficulty, 2=walking disability but

can transverse, 3=considerable time to transverse the beam, 4=unable to transverse beam, 5=unable to remain on starting platform(Urakawa et al., 2007).

Fixed speed rotarod

The fixed speed rotarod (FSRR) evaluates balance and sensorimotor coordination(Monville et al., 2006). The FSRR apparatus has a revolving 7cm in diameter, motor-driven rod with an automated switch below. Animals underwent three trials at 10rpm. The latency to fall off the rod in each trial was recorded by the switch below and averaged for analysis.

Amphetamine induced rotations

Amphetamine induced rotation is a phenomenon present in unilateral 6-OHDA lesion models of PD. Receptor supersensitivity in one hemisphere of the brain produces rotation after 6-OHDA induced damage and phenotypically evaluates dopamine loss(Björklund and Dunnett, 2019; Mishra et al., 1980, 1974; Ungerstedt and Arbuthnott, 1970). 2.5mg/kg of amphetamine was administered intraperitoneally, and animals were placed in a clear cylinder 31cm tall and 30cm in diameter. Animals were recorded over a 30-minute period. The total number of rotations was analyzed from the last ten minutes of the video by a blind researcher.

Tissue collection for CDK5 assay

Animals were anesthetized using isoflurane and subsequently decapitated. The whole brain was removed, the SN was dissected while on ice and flash-frozen before storage at -80°C.

Tissue collection for immunohistochemistry

Animals were anesthetized using a ketamine (75mg/kg) and xylazine (10mg/kg) mixture and perfused with PBS then 4% paraformaldehyde (PFA). The brain was extracted and stored in 4% PFA overnight. The following day the brain was immersed in 10% sucrose for 24 hours followed by 30% sucrose. Each brain

was flash-frozen using 2-methyl butane and stored at -80°C. Chromium-gelatin-coated slides were prepared and 16μm thick serial, coronal sections were obtained of using a cryostat. Slides were stored at -80°C.

CDK5 immunoprecipitation and assay

Kinase assays were performed as described previously, with modification(Binukumar et al., 2014a). Briefly, CDK5 was immunoprecipitated with polyclonal C8 antibody for 2 h at 4°C and protein A-Sepharose beads were used to isolate immunoglobulin. Immunoprecipitates were washed three times with lysis buffer and once with 1X kinase buffer. 1X kinase buffer contained 5 mM MOPS, pH 7.4, 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, and 5 mM $MgCl_2$. Samples were added to the reaction mix containing kinase buffer, 50 μM ATP, 20 μg of histone H1, and 0.1mCi of [γ^{32}P]ATP containing 0.1mm DTT and 1X Halt protease and phosphatase inhibitor (Thermo Fisher) and incubated at 30°C for 1 h. Reactions were stopped by adding Laemmli sample loading buffer, and samples were electrophoresed on 12% SDS–PAGE gels. Histone bands were visualized by Coomassie blue staining and gels were autoradiographed and were scanned on a PhosphorImager. Radioactive band density was analyzed using ImageJ software, and statistical analysis was performed.

Immunohistochemistry

Slides were washed 3 times in PBS, postfixed with 4% PFA for 10 minutes and washed 3 times with PBS. Slides were blocked using BSA, normal goat serum, 0.1% Triton X and PBS before incubation with anti-TH antibody (dilution 1:500) (Cedarlane, Ontario Cat.# CL8876AP, Lot#167621) overnight at 4°C. Specimens were washed in PBS and incubated for one hour at room temperature using Texas red goat anti-rabbit antibody (1:1000) (Invitrogen, Ontario). Coverslips were adhered using PureLong Gold mounting medium containing DAPI (Life Technologies). Sections were imaged using a Leica DM6 B microscope and Leica Application Suite X (LAS X) software. Immunofluorescence was analyzed by ImageJ software and reported

as integrated density. Integrated density was obtained after conversion to 8-bit images and background was subtracted.

Statistical analysis

Behavioural and biochemical data were analysed using one-way Analysis of Variance (ANOVA) with Tukey's post hoc tests using GraphPad Prism software apart from beam walk. The beam walk test was analysed using a repeated measures one-way ANOVA with Bonferroni post hoc testing as this test was run twice. Treatment was always considered the independent variable and significance were considered as *p<0.5, **p<0.01 and ***p<0.001. Outliers were removed using a Grubbs outlier test calculator where indicated. All error bars are represented as standard error of the mean (SEM).

Results

TP5 attenuates reduced locomotor activity in 6-OHDA lesioned rats

Locomotor activity was assessed through total distance travelled to distinguish the rodent equivalent phenotypic manifestation of rigidity, akinesia, and bradykinesia. Accuscan technology found TP5+6-OHDA animals travelled significantly more than those receiving aCSF+6-OHDA ($p<0.05$).This was determined using a one-way ANOVA with a Tukey's post hoc test ($F(2,10) = 4.720$, $p=0.0360$). Post hoc testing did not reveal any significant differences between aCSF+6-OHDA (n=5)and ScP+6-OHDA (n=4) as well as no differences between ScP+6-OHDA and TP5+6-OHDA (n=4) treatment groups (Figure 2A). One outlier was detected and removed from the aCSF+6-OHDA group during analysis. The significantly higher total distance travelled found in TP5+6-OHDA treated animals compared to aCSF+6-OHDA animals (*$p<0.05$), suggests pre-treatment with TP5 was able to protect the development of reduced motor activity inflicted by 6-OHDA.

TP5 improves beam walk in 6-OHDA lesioned rats

Beam walk measured balance and coordination. Using a predetermined scale described in methods, beam walk considers animals' ability to initiate movement onto the beam, speed of transversal and balance abnormalities through hindlimb slips off the beam. Animals pre-treated with TP5 before 6-OHDA achieved a score indicative of little difficulty crossing the beam. A significant effect of treatment was found using a repeated measures one-way ANOVA ($F(2,3)=121.0$, $p=0.0082$). Bonferroni post hoc testing revealed significantly higher scores in the aCSF+6-OHDA (n=6)(*$p<0.05$) and ScP+6-OHDA (n=4) treatment groups (*$p<0.05$) compared to the TP5+6-OHDA (n=4) treatment group (Figure 2B). Groups not receiving TP5 demonstrated considerable difficulty traversing or complete inability to transverse the narrow beam supporting TP5 protection against balance impairments from 6-OHDA.

TP5 pre-treated animals do not display significantly reduced endurance using fixed speed rotarod

Fixed speed rotarod (FSRR) highlights gait and coordination impairments. At 10 rotations per minute (rpm), FSRR confirmed significant differences in gait between treatment groups ($F(2, 11)=21.39$, $p=0.0002$) using one-way ANOVA. Tukey's post hoc test revealed a significant difference between aCSF+6-OHDA (n=6) animals compared to TP5+6-OHDA (n=4) animals (***$p<0.001$). ScP+6-OHDA (n=4) treated rats did not perform well and had significantly reduced endurance compared to TP5+6-OHDA treated animals (***$p<0.001$) (Figure 2C). TP5 prior to 6-OHDA insult was able to protect rats from developing gait and coordination impairments, as seen through their ability to remain on the rotating rod compared to groups that did not receive TP5 prior to 6-OHDA.

TP5 inhibits amphetamine-induced rotations

To phenotypically assess if TP5 protects dopaminergic neurons from 6-OHDA, amphetamine induced rotations were evaluated. One-way ANOVA found treatment produced a significant effect ($F(2, 11)=6.904$, $p=0.0114$) and Tukey's post hoc indicated significantly more rotations completed in the aCSF+6-OHDA (n=6) (*$p<0.05$) and ScP+6-OHDA (n=4) (*$p<0.05$) groups compared to the TP5+6-OHDA (n=4) group over

a ten minute period (Figure 2D). The data phenotypically reveals TP5's ability to prevent the loss of dopamine neurons acting as a protective agent. This data was reaffirmed using biochemical analysis.

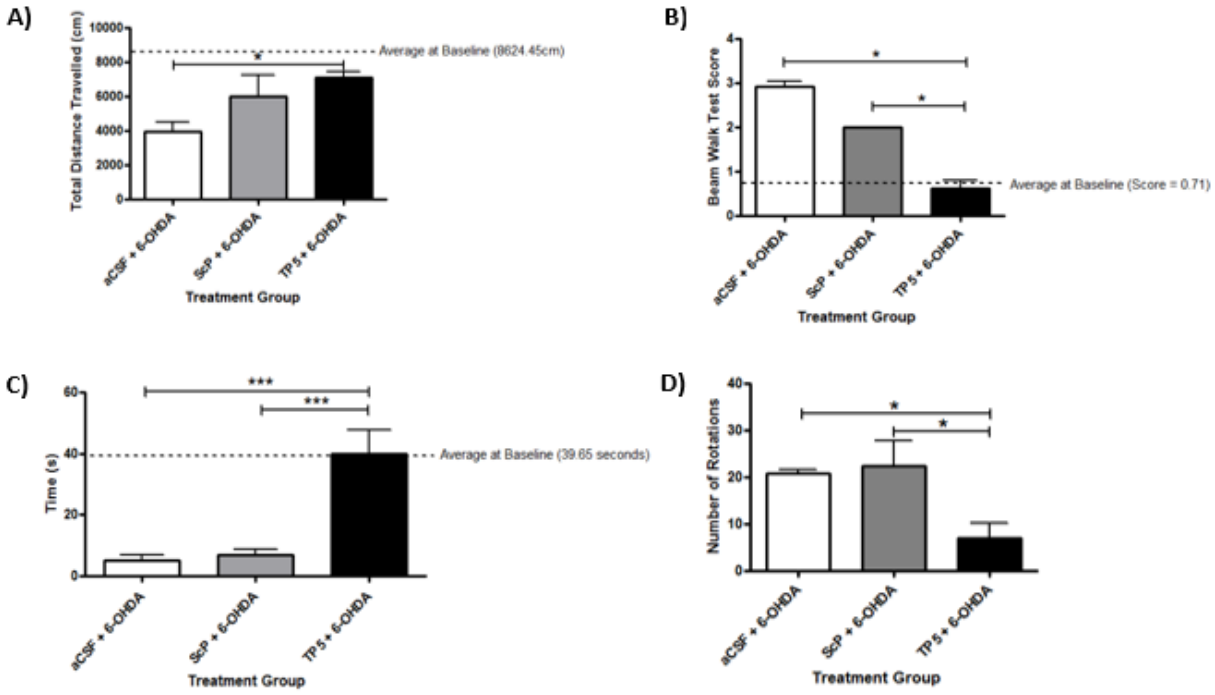

Figure 2. Motor function behavioural assessments A) Locomotor activity is higher in animals treated with TP5 prior to 6-OHDA. Baseline data averaged for all animals shown as a dotted line (8624.45cm) for a visual representation of "normal" for this cohort. A significant decrease in total distance travelled by aCSF+6-OHDA compared to TP5+6-OHDA (*p<0.05) was found using one-way ANOVA with Tukey's post hoc test. No significant difference in distance travelled was found between animals in ScP+6-OHDA and TP5+6-OHDA. **2B) Beam walk scores are lower in TP5 pretreated rats.** Higher scores represent increased difficulty in completing the beam test. Collective baseline average is shown on the graph by a dotted line for reference to a "normal" score (0.71). Repeated measures one-way ANOVA with Bonferroni post hoc test show aCSF prior to 6-OHDA have increased scores, therefore, increased difficulty performing the beam test compared to TP5 pre-treated animals (*p<0.05). ScP prior to 6-ODHA had no protective effect

showing significantly higher scores than TP5 pre-treated animals (*p<0.05). **2C) Pre-treatment with TP5 has a significant effect on fixed speed rotarod endurance.** The dotted line represents average endurance times of all animals at baseline (39.65 seconds). TP5+6-OHDA animals remained on the rod at 10 rotations per minute, significantly longer than both aCSF+6-OHDA (***p<0.001) and ScP+6-OHDA (***p<0.001) groups. **2D) TP5 before 6-OHDA reduced amphetamine induced rotations.** TP5 pretreated animals made significantly fewer rotations than both the aCSF (*p<0.05) and ScP (*p<0.05) pre-treated animals.

Neuroprotection identified through tyrosine hydroxylase in TP5+6-OHDA treated animals

Motor testing revealed significant impairments in the aCSF+6-OHDA and ScP+6-OHDA groups, while TP5+6-OHDA animals maintained motor function performing close to average baseline values. Thus, dopaminergic protection was investigated indirectly through analysis of tyrosine hydroxylase (TH) using immunohistochemistry and integrated density values. TH was used to identify dopamine neurons as this enzyme is a rate-limiting step in dopamine synthesis. Immunohistochemical results validated motor impairments found using n=3 per group with the exception of a significant outlier identified in the aCSF+6-OHDA. One-way ANOVA confirmed an effect of treatment ($F(3,7)= 147.8$, $p<0.0001$) and Tukey's post hoc test revealed significantly less TH in the aCSF+6-OHDA (**p<0.01) and ScP+6-OHDA (***p<0.001) groups compared to control group that had no intervention and the TP5+6-OHDA treatment group (Figure 3). Increased TH found in TP5 treated animals depicts dopaminergic protection by TP5 and no significant difference was found between control and TP5+6-OHDA treatment groups.

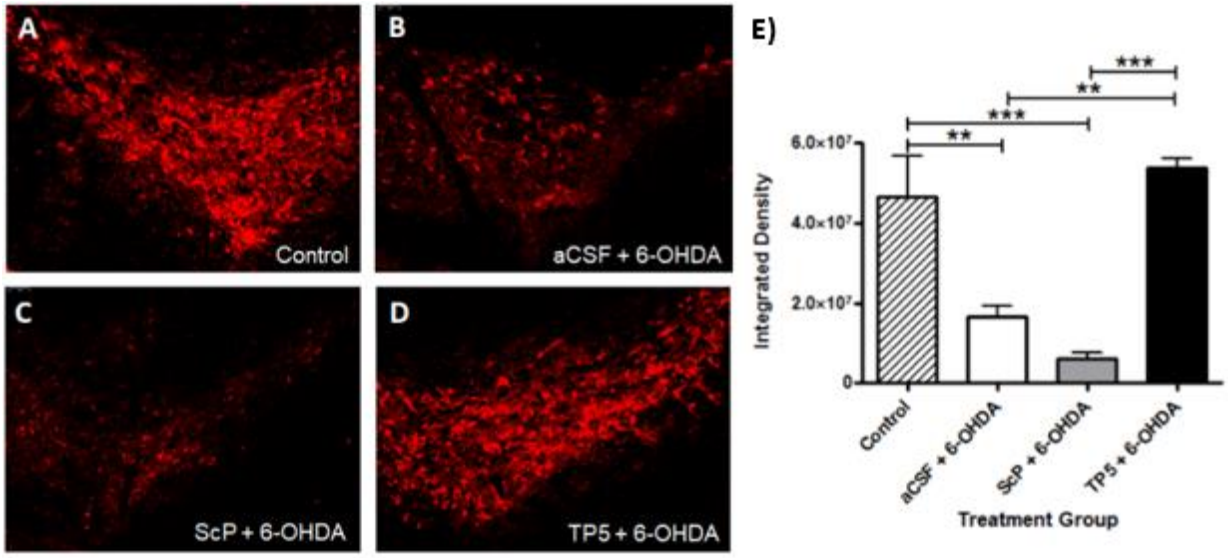

Figure 3. **Pre-treatment with TP5 provides protection for dopamine neurons as shown through tyrosine hydroxylase (TH).** TH in the SN was detected using immunohistochemistry. Images shown correspond to the following treatment groups **A)** Health control with no treatment **B)** aCSF+6-OHDA **C)** ScP+6-OHDA **D)** TP5+6-OHDA. **E)** Integrated density quantified the presence of TH. Significantly higher integrated density was found in control samples than aCSF+6-OHDA (**p<0.01) and ScP+6-OHDA (***p<0.001). No difference was found between control and TP5+6-OHDA. A significant difference was found between TP5+6-OHDA and aCSF+6-OHDA and TP5+6-OHDA and ScP+6-OHDA.

TP5 inhibits hyperactivated CDK5 activity in 6-OHDA lesioned rats After establishing neuroprotective effects of TP5, the mechanism for neuroprotection was considered. TP5 is known to specifically inhibit CDK5/p25 to reduce CDK5 hyperactivity in pathological states. CDK5 activation in the SN (site of injection) was investigated. All values are expressed as % of control, the control was a sham surgery group receiving only aCSF in place of treatment and 6-OHDA. This controlled for volumes administered and potential changes in CDK5 activity produced by surgical intervention. Figure 4 demonstrates a significant effect of treatment using a one-way ANOVA ($F(3,13)=16.11$, $p=0.0001$). One outlier was identified in the aCSF+6-OHDA group and removed. Both aCSF+6-OHDA (n=5) (**p<0.01) and ScP+6-OHDA (n=4) (***p<0.001) groups had significantly increased CDK5 activity compared to aCSF only treated controls (n=4). This

specifically shows 6-OHDA does hyperactivate CDK5/p25. TP5+6-OHDA % of control values did not significantly differ from aCSF treated animals ($p>0.05$). TP5 treatment prior to 6-OHDA (n=4) reduced CDK5 hyperactivation and suggests a mechanism by which TP5 was able to provide neuroprotection from 6-OHDA induced dopaminergic degeneration.

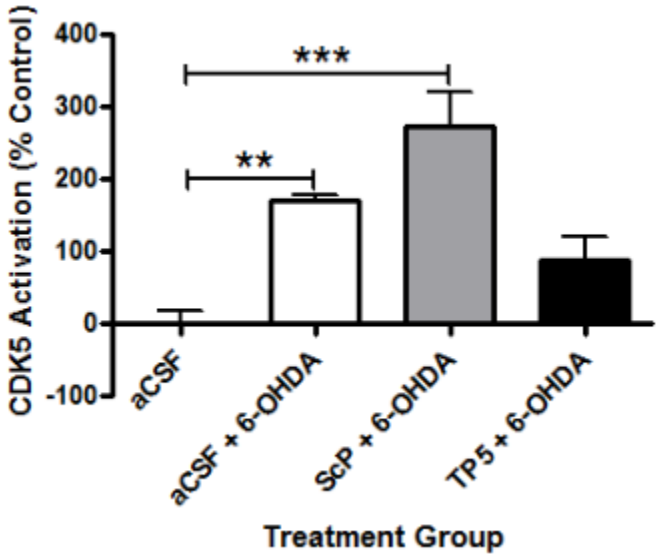

Figure 4. CDK5 activity reduced by TP5. The graph depicts CDK5 activation based on the percent of aCSF sham surgery controls. Using one-way ANOVA, a significant effect of treatment was found ($F(3,13)=16.11$, $p=0.0001$). Tukey's post hoc test revealed increased CDK5 activation in aCSF+6-OHDA (**$p<0.01$) and ScP+6-OHDA groups (***$p<0.001$), while TP5+6-OHDA treatment was not significantly different from aCSF sham surgery animals ($p>0.05$).

Discussion

The underlying mechanisms for dopaminergic neuronal loss of PD are not fully understood. Current intervention supplements dopamine for symptomatic treatment but remaining neurons degenerate eliciting the need for neuroprotective agents. The 6-OHDA model produces significant dopaminergic loss and manifests motor abnormalities similar to PD patients, thus was used in the current study to further evidence the therapeutic potential of TP5 in PD(Binukumar et al., 2014a, 2015). Through motor and biochemical evaluation, this study successfully demonstrated treatment with TP5 prior to 6-

OHDA significantly performed as a protective intervention. Biochemical analysis proved CDK5/p25 hyperactivity was reduced and dopamine neurons were protected based on increased immunostaining of TH in the SN. This study demonstrates inhibition of CDK5/p25 is an important therapeutic target for PD and shows behavioural and biochemical neuroprotective properties of TP5. These findings are consistent with previously reported observations in the MPTP model of PD(Binukumar et al., 2014a, 2015)

Oxidative stress is a current hypothesis for dopaminergic degeneration. The neurotoxin 6-OHDA induces oxidative stress(Smith and Cass, 2007) and this study demonstrated oxidative stress produced by 6-OHDA then increased CDK5/p25 activity. Oxidative stress increases the influx of calcium and produces many pathological changes including the hyperactivation of CDK5/p25, further increasing oxidative stress(K. H. Sun et al., 2008). Calcium-dependent enzyme calpain cleaves p35 (pro-survival activator) to p25 (pro-apoptotic activator) and favours CDK5/p25 complex. CDK5/p25 complex facilitates apoptotic processes through phosphorylation of p53(Zhang et al., 2002). Hyperactivation of CDK5/p25 can induce more oxidative stress and apoptotic processes leading to neurodegeneration. Specifically, dopaminergic apoptosis was demonstrated through decreased TH immunofluorescence in aCSF+6-OHDA treated animals. Hyperactivation of the CDK5/p25 complex is an important therapeutic target identified in neurodegenerative disorders as oxidative stress plays a role in many diseases.

TP5 is an innovative peptide that takes advantage of activator specific regulation of CDK5 by selectively inhibiting the CDK5/p25 complex. Current therapeutics aiming to reduce CDK5 activity also inhibit the beneficial effects of CDK5/p35 and lack therapeutic efficacy(Shah and Lahiri, 2014; Siklos et al., 2015). This study supports CDK5/p25 hyperactivity as a viable treatment target and provides evidence for TP5 as a potential therapeutic through the protective effects of TP5 at a behavioural and cellular level. Behavioural tests investigating motor skills provide phenotypic results and are targeted at evaluating several aspects of PD related motor function allowing for translatability. The motor tests chosen in this

study reflect rigidity, bradykinesia, akinesia, gait abnormalities, and postural imbalances(Taylor et al., 2010). Protective attributes of TP5 were seen in both motor activity and motor function tests as TP5+6-OHDA treated animals performed close to baseline levels. Protective properties were demonstrated by the preservation of the dopaminergic neurons in the SN shown by TH.

Proof of concept studies evaluating hallmark dopaminergic degeneration is imperative for identifying neuroprotective qualities of potential PD therapeutics. This study exemplified protection towards dopaminergic neurons by TP5 supporting further investigation of this peptide and conformationally constrained analogs that easily penetrate blood brain barrier. Future studies should investigate CDK5/p25 inhibition using TP5 in the alpha-synuclein model of PD to investigate the Lewy body component of PD pathology. In post mortem studies CDK5 is co-localized with Lewy bodies suggesting CDK5/p25 plays a role in Lewy body formation(Brion and Couck, 1995; Nakamura et al., 1997; Takahashi et al., 2000). Non-motor features of PD should also be considered in drug discovery and design. Non-motor symptoms often go untreated due to the involvement of a neurotransmitter system other than dopamine(Schapira, 2009; Schapira et al., 2017). Therefore, TP5's ability to cause changes to non-dopaminergic systems should also be considered.

The use of neurotoxins such as 6-OHDA includes some risk by producing a large amount of toxicity in the aCSF+6-OHDA and ScP+6-OHDA groups. Two of the animals in the study were euthanized due to the endpoint from these groups. No animals from the TP5+6-OHDA group reached an endpoint.

Given the progressive nature of PD, the need for protective treatments is high and remains to be a significant therapeutic challenge. This study demonstrated CDK5/p25 specific inhibition by TP5 produces neuroprotective effects when used in the 6-OHDA lesion model of PD at phenotypic and molecular levels. This study also further supports the involvement of CDK5/p25 hyperactivity as a possible mechanism in the pathophysiology of PD as its inhibition can reduce dopaminergic degeneration and protect against

motor symptom development. Overall this study supports further investigation of CDK5/p25 hyperactivity's involvement in PD pathology and investigation of TP5 as a potential protective therapeutic for neurodegenerative disorders.

Acknowledgments

The authors would like to acknowledge Aurore Latragna for assistance with behavioural tests. Authors also appreciate the support from all Mishra laboratory members in the completion of this work.

Authors Roles

A. Bernardo designed the experimental design with H. Pant and R. Mishra. A. Bernardo conducted surgeries, treatments, behavioural work, and immunohistochemistry. N. Amin performed CDK5 immunoreactivity. A. Bernardo completed all statistical analyses and wrote the manuscript. All authors were involved in reviewing and editing the manuscript.

Conflict of Interests and Financial Disclosures

The authors declare no conflict of interest. This work was supported by grants from NSERC, CIHR and the IDRF grant from McMaster University. This research was also supported by the Intramural Research Programs of the National Institute of Neurological Disorders and Stroke, National Institutes of Health.

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Zheng, Y.-L., Kesavapany, S., Gravell, M., Hamilton, R.S., Schubert, M., Amin, N., Albers, W., Grant, P., Pant, H.C., 2005. A Cdk5 inhibitory peptide reduces tau hyperphosphorylation and apoptosis in neurons. EMBO J. 24, 209–220. https://doi.org/10.1038/sj.emboj.7600441

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3.8 Additional Support for CDK5/p25: Preliminary Investigation of TP5 Fragment Peptide A

3.8.1 Peptide A

In collaboration with Dr. Gunning at the University of Toronto, TP5 is being further optimized for several properties including potency, ability to cross the BBB and stability. Low concentrations of TP5 did not have significant effects in the MPTP model, however, higher concentrations did demonstrate protective effects. Increasing the potency of TP5 may allow lower concentrations to be administered (Binukumar et al., 2015). Modifications made to TP5 added a TAT sequence enabling TP5 to cross the BBB. Another way TP5 is being modified to assist with brain bioavailability is a reduction in the size of the peptide. Dr. Gunning's group was able to create a conformationally constrained 8 amino acid long peptide of TP5 that is currently being investigated with the aim of further reducing the sequence to a target of 4 amino acids. Finally, modifications to functional groups can provide increased stability within cellular conditions to increase the half-life of the peptide by protecting it from peptidase activity that may break it down. This is an ongoing effort and therefore preliminary behavioural studies were performed using Peptide A to investigate its potential to provide neurorestorative effects on behaviour after 6-OHDA lesions. Preliminary evidence for Peptide A is shown below to further evidence that investigation of these shorter peptides is warranted.

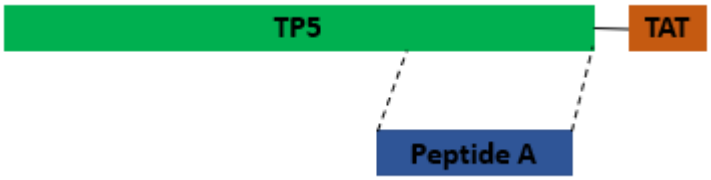

Figure 3ii. Visual representation of Peptide A. Peptide A is a fragment of TP5 that does not have the TAT tag modification. The image shows a visual representation of Peptide A in reference to TP5.

3.8.2 Aims and Hypothesis

Aims: Use pilot studies to determine if a short fragment of TP5 can demonstrate similar properties to TP5.

Hypothesis: If the active portion of TP5 is retained in Peptide A, similar effects of Peptide A and TP5 will be detected.

3.8.3 Methods

Catalepsy Behavioural Testing

Drug Preparation

Haloperidol was prepared in 0.2% acetic acid and then the pH was increased to 5.0 using NaOH. 1 mg/kg of Haloperidol was then administered IP and all animals performed the catalepsy test at 30, 60 and 90 minutes post-injection.

Peptide A was investigated using the haloperidol-induced catalepsy test (described below). For this behavioural test, Peptide A was administered using an intraperitoneal injection (IP). Peptide A was prepared in saline to administer a dose of 10mg/kg 45 minutes prior to haloperidol challenge.

Catalepsy Behaviour Paradigm

Catalepsy is a symptom of PD characterised by muscle rigidity and is also a known side effect of Haloperidol. Haloperidol was administered IP and all animals performed the catalepsy test at 30, 60 and 90 minutes post-injection. The two front paws of the rat were placed on a metal bar 10cm high and the amount of time taken to correct this unusual posture (removal of paws from the bar) was recorded. This was repeated three times with a 10 second delay between trials. If 70 seconds was reached the animals were removed from the bar until the next trial. Times were then scored on the following rating scale. 1= 1-20 seconds, 2= 21-40 seconds, 3= 41-60 seconds, 4= >60 seconds.

Amphetamine induced rotations in the 6-OHDA lesioned model of Parkinson's

Surgery and 6-OHDA Lesions

Surgery and 6-OHDA induced lesions were performed identically to those described in the above paper however cannulas were implanted into the striatum at coordinates A/P +1.0mm, M/L ±3.0mm in reference to bregma, with a cannula lowered D/V-4.5mm and 15ug of 6-OHDA in 3uL of solution was used to induce a lesion in the striatum.

Drug preparations

6-OHDA was dissolved in 0.9% saline containing 0.2% ascorbic acid as described in the above paper. Peptide A was infused using the same methodology as described in the above paper with the modification of 40ug in 6uL being infused to the striatum as a neurorestorative treatment, therefore after a 6-OHDA lesion is created. Amphetamine was prepared by dissolving it in sterile saline. 2.5mg/kg of amphetamine was administered IP to each animal prior to completing the amphetamine-induced challenge.

Amphetamine Induced Rotations Paradigm

Amphetamine induced rotations were also performed and methodology was identical to that described in the above paper included in this chapter. Peptide A was infused 2 days after 6-OHDA lesions and animals were tested 45 minutes after Peptide A infusion.

Protein kinase assay

This assay was performed similarly to methods described in the above paper in this chapter and in Binukumar et al. (Binukumar et al., 2014).

3.8.5 Results

Peptide A decreased cataleptic behaviour induced by Haloperidol

To behaviourally determine if an IP route of administration is effective for Peptide A as it is smaller than TP5, the catalepsy test was conducted in healthy animals. This test also determined if Peptide A can counteract haloperidol's dopaminergic antagonism, suggesting a role in dopamine signalling. An IP injection of Peptide A was administered 45 minutes prior to an IP injection of haloperidol. Two-way ANOVA found Peptide A significantly reduced catalepsy scores ($F(1,2)=52.01$) with Bonferroni post hoc testing identifying significant differences at 30 minutes ($*p<0.05$), 60 minutes ($**p<0.01$)and 90 minutes ($*p<0.05$). This suggests that an active portion of TP5 is likely retained in Peptide A and IP administered is capable of ameliorating haloperidol-induced catalepsy. Further, this mildly evidences that Peptide A is having some effect on dopaminergic signalling however specific changes can not be determined using only these results (Figure 3iii).

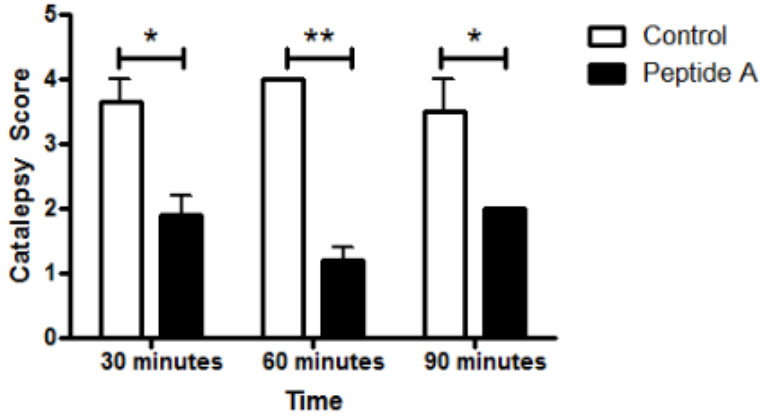

Figure 3iii. Peptide A reduced catalepsy induced by Haloperidol. At all time points following Haloperidol administration, animals that received an IP injection of Peptide A had reduced catalepsy scores compared to those that did not ($F(1,2)=52.01$). Significant Tukey's post hoc testing is depicted as * = $p<0.05$ and **= $p<0.01$.

Amphetamine induced rotations are reduced after Peptide A administration

Preliminary Peptide A results indicated that infusion of Peptide A after 6-OHDA induced lesion significantly reduced amphetamine induced rotational behaviour similar to TP5 results shown by TP5.

One-way ANOVA found a significant effect of treatment ($F(3,17)= 6.222$, $p=0.0048$). Tukey's post hoc testing also found significant differences between the 6-OHDA group and the group treated with TP5 after the lesion ($*p<0.05$) and Peptide A ($p>0.05$). This comparison is shown to depict that Peptide A has similar capabilities on behaviour compared to TP5 (Figure 3iv).

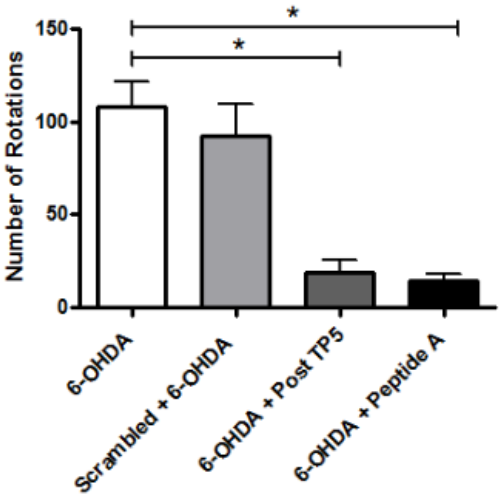

Figure 3iv. Reduced amphetamine induced rotations in TP5 and Peptide A treated animals. The number of rotations made by both TP5 and Peptide A infused animals was significantly lower than those without ($F(3,17)= 6.222$, $p=0.0048$. Both infusions occurred after 6-OHDA induced lesions were created and TP5 and Peptide A showed similar results. The * indicates $p<0.05$ level of significance found by Tukey's post hoc testing.

CDK5 immunoprecipitation assay

Preliminary evidence for Peptide A's ability to inhibit CDK5/p25 was investigated through a kinase assay. Evidence demonstrates that and 2.5µM of either TP5 or Peptide A is not enough to reduce CDK5/p25 activity. However, increasing the concentration to 10µM both TP5 and Peptide A can reduce kinase activity in a similar way. Both TP5 and Peptide A were analyzed here to show a direct comparison of the two peptides (Figure 3v).

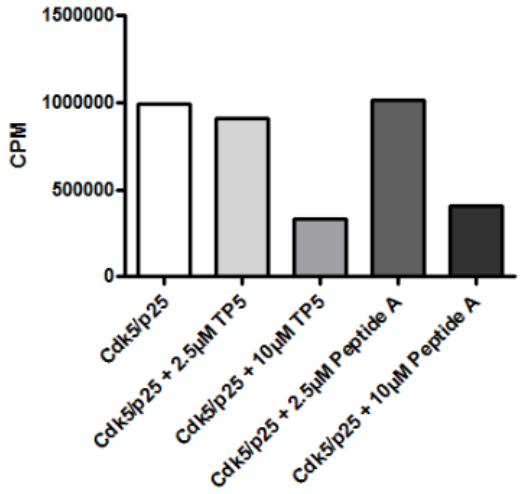

Figure 3v. Peptide A lowers CDK5/p25 kinase activity. Low concentrations of both TP5 and Peptide A were unable to reduce CDK5/p25 activity. Higher concentrations of both TP5 and Peptide A lowered the counts per minute (cpm) detected in a similar manner.

3.8.5 Discussion

Together these preliminary results suggest Peptide A may contain an active portion of TP5 as it demonstrates similar behavioural and kinetic inhibition properties to TP5. This data is very preliminary and small sample sizes were used however, through these small experiments Peptide A may prove to have therapeutic potential similar to TP5. Behavioural results in this study support that these peptides might modulate dopamine signalling as Haloperidol induced catalepsy was mitigated or overcome and amphetamine induced rotations were also altered. How these changes are occurring at a molecular level can not be determined from these results and therefore future studies should include larger studies replicating these results and analysis of CDK5/p25 inhibition on dopamine receptors and their signalling.

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Chapter 4: Synapsin II and Schizophrenia

Preface

Chapter 4 is a miscellaneous chapter that includes evidence for synapsin II in the pathophysiology of negative and cognitive symptoms of schizophrenia (SZ) suggesting synapsin II as potential target for future therapeutic design. This chapter will begin with a brief overview of SZ and the gap in current treatment regimens followed by an introduction to synapsin II, the proposed molecular target that is focused on in this chapter. This chapter focuses on a third Aim for this thesis: **Aim 3 (Miscellaneous):** Investigate a molecular target involved in neurotransmission regulation and its role in the pathology of SZ to propose a molecular target for negative and cognitive symptoms that elude current treatments, specifically synapsin II. **Hypothesis:** Synapsin II knockdown will recapitulate schizophrenia-like symptoms and lend support to the hyper vs hypofrontality debate in schizophrenia. Overall synapsin II knockdown will implicate this protein in the pathophysiology of schizophrenia and its importance as a molecular target for negative and cognitive symptom relief.

4.0 Background

4.1 Schizophrenia (SZ)

SZ is a complex brain disorder that lacks a comprehensive understanding of the underlying pathophysiology. Affecting approximately 1% of the global population, SZ contributes to unemployment rates, suicide rates and financial burdens to both society and individuals (Owen et al., 2016). 6.3% of the homeless shelter population in the United States are patients diagnosed with schizophrenia (Cloutier et al., 2016). Social stigma, fear from society and psychiatric challenges influence the overwhelming 80-90% unemployment rate that can lead to homelessness of schizophrenia patients. Unemployment also negatively impacts their recovery, propagating a vicious cycle (Marwaha and Johnson, 2004). The financial implications associated with SZ are substantial. A study conducted using data from 2013 covering the

economic burden of SZ in the United States determined an estimated $155.7 billion dollar economic burden for the 2013 schizophrenia population. This value was attributed to direct health care costs such as medications, hospitalizations, etc., direct non-health care costs including law enforcement, police protection, and judicial services, and indirect costs included unemployment and caregiving (Cloutier et al., 2016). In 2004 England spent $11.8 billion to cover medical and living expenses for patients, while in Canada $6.8 billion was associated with healthcare, non-healthcare costs and productivity loss due to SZ (Goeree et al., 2005; Owen et al., 2016). This financial strain is aggravated by the heterogeneous and chronic nature of symptoms that usually present between the ages of 15-24.

4.1.1 Symptomology

Symptom profiles can vary from patient to patient, as well as range in the severity of each individual behavioural abnormality. Diagnostically, symptoms can be categorized into three major classifications including, positive, negative and cognitive symptoms. Positive symptoms include the addition of a behaviour that is not present in a healthy functioning individual. In SZ these positive symptoms frequently manifest as delusions and/or hallucinations. These are often in the form of verbal auditory stimulation, such as hearing or participating in a conversation with voices (Owen et al., 2016). Negative symptoms relate to the loss or reduction in thoughts and/or behaviours that are present in healthy individuals. In schizophrenia patients, some negative symptoms specifically include diminished motivation, anhedonia (the inability to feel pleasure), and reduced social interaction (Wójciak and Rybakowski, 2018). The third category of symptomology corresponds to the cognitive symptoms, which are described as mental processing impairments that hinder an individual's functioning within society. Common cognitive symptoms present in SZ include, but are not limited to, deficits in working memory, attention, perception, problem solving, general intelligence as determined by lower Intelligence Quotient scores and executive functioning (Bowie and Harvey, 2006).

4.1.3 Pathophysiology

At the root of this varying symptom profile is the underlying pathophysiology that has yet to be understood in detail. Researchers have hypothesized various theories to explain different aspects of the symptomology, none of which can encapsulate all aspects of the disorder (Hasan et al., 2014). The commonly agreed-upon pathophysiology involves a biochemical imbalance of neurotransmitters within the brain. The neurotransmitter system involved, however, is still under examination.

4.1.2.1 Dopamine Hypothesis

One of the most prevailing hypotheses in SZ is the dopamine hypothesis. There are four major dopamine pathways within the brain, mesolimbic, mesocortical, tuberoinfundibular and nigrostriatal. Each of these pathways participates in various functions such as reward, cognitive function, prolactin secretion, and movement respectively (Beaulieu and Gainetdinov, 2011). The dopamine hypothesis of SZ states that there is hyperdopaminergic function in the mesolimbic structures of the responsible for the onset of positive symptoms, while in the mesocortical regions of the brain exists hypodopaminergic function (Bennett, 1998). This has been supported by research demonstrating that dopamine agonists are able to aggravate the positive symptoms, such as auditory hallucinations, and induce stereotypic behaviours in animal models (Randrup and Munkvad, 1967). Additionally, the ability of dopamine antagonists illustrating remedial effects on the psychotic symptoms of SZ provides further validation for this theory (Baandrup et al., 2016; Bruijnzeel et al., 2015).

4.1.2.2 Glutamate Hypothesis

The dopamine hypothesis encompasses many hallmarks of SZ however, it is unable to provide an explanation for treatment-resistant SZ and fails to address why SZ presents within adolescence to young adulthood. Glutamate, the most common excitatory neurotransmitter in the central nervous system, has been proposed to participate in the biochemical imbalance involved in SZ. Evidence for glutamatergic

dysfunctions within SZ stems from glutamate receptor antagonists, such as phencyclidine (PCP) and ketamine. These can both induce all three symptom domains of SZ (Hasan et al., 2014; Itil et al., 1967). Post-mortem samples of patients with SZ have also reinforced the glutamate hypothesis by demonstrating disrupted glutamatergic signalling plays a role in structural abnormalities, such as decreased dendritic spine densities in the dorsolateral prefrontal cortex (Hasan et al., 2014). The hypoglutamatergic state within the cortex is largely associated with cognitive and negative symptom presentation and further exerts neurochemical signals on the major inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), system.

4.1.2.3 GABA Hypothesis

Glutamate and GABA work in a complimentary fashion therefore, alterations to the glutamatergic system will have repercussions on the GABAergic system and vice versa. There has been considerable evidence to support a hypofunctional state of GABAergic neurotransmission in SZ. Reduced cortical GABA has been found in patients with SZ (Charych et al., 2009). Additionally, the enzyme responsible for GABA synthesis, glutamic acid decarboxylase (GAD), has been reported to show decreased levels within temporal and prefrontal cortices of patients with SZ (Lang et al., 2007). The implications of an imbalance between glutamate and GABA have been postulated to result in inappropriate synaptic pruning and ultimately develop altered connectivity within the SZ brain (Hasan et al., 2014).

4.1.3 Treatments

The lack of understanding of the diverse presentation and array of symptoms limit treatment options for patients with SZ. Current interventions include antipsychotic drugs (APDs) in combination with family intervention and community treatment (Baandrup et al., 2016). APDs are most effective in ameliorating positive symptoms of SZ. However, they inadequately provide relief of the negative and cognitive symptoms. Multiple generations of APDs have been developed in the aim of ameliorating all

symptom domains of SZ but none are capable of covering all areas so far. Typical APDs or first generation APDs, such as Haloperidol, utilize the dopamine hypothesis to manage positive symptoms of SZ via antagonism of the dopamine D2 receptor (D2R) (Seeman, 1987). Increased dopamine within the mesolimbic regions of the brain has been implicated in producing psychosis in SZ, and is a prevailing theory for the underlying pathophysiology of positive symptoms of SZ. First generation APDs display a high affinity for the D2R and therefore antagonism of these receptors can lead to decreased dopamine levels within mesolimbic and mesocortical regions brain. Specifically, decreased levels of dopamine in the mesocortical pathway are thought to contribute to behaviours such as decreased social interaction and decreased cognitive function. Dopamine hypofrontality can be further aggravated by antipsychotics via D2R antagonism, effectively further decreasing dopamine levels within the cortex (Mailman and Murthy, 2010; Miyamoto et al., 2005). Another limitation to first generation APDs are the extrapyramidal side effects they induce. These include abnormal involuntary movements, parkinsonian symptoms (rigidity, bradykinesia, and tremors) and tardive dyskinesia which is a chronic movement disorder described as involuntary, repetitive movements of the lips, tongue, jawbone, trunk, and limbs (Seigneurie et al., 2016).

The second generation, or atypical antipsychotics, are a more favourable treatment option as they demonstrate a drastic reduction in extrapyramidal adverse effects. First generation APDs are associated with three times greater risk of tardive dyskinesia in elderly patients than those medicated with second generation APDs (O'Brien, 2015). Second generation APDs have a distinct affinity profile that includes a higher affinity for serotonin receptors (5-HT2A) and then secondly has a lower affinity for the D2Rs allowing increased dissociation (Kusumi 2015). This wider affinity profile is proposed to be why second generation APDs have slightly better efficacy treating negative and cognitive symptoms than first generations. The effectiveness is still not substantial and could be evident because second generation APDs do not exacerbate negative and cognitive symptoms as sometimes seen with first generation drugs.

APDs also have significant metabolic side effects that pre-dispose patients to diabetes and cardiovascular disease (Rummel-Kluge et al., 2010).

Third generation antipsychotics have been proposed and are described to portray functional selectivity, which is a term used to explain different signalling patterns exerted on a single receptor. This can include partial agonistic characteristics within one pathway and partial antagonistic traits within another. The functional selectivity of these APDs, such as aripiprazole, allows them to exert their unique binding and dissociation patterns more specifically to either pre or postsynaptic receptors depending on the signalling environment of the D2R (Mailman and Murthy, 2010). In areas of high extracellular dopamine, aripiprazole can compete with dopamine and produce a partial antagonism effect. While in areas of low dopamine concentrations aripiprazole can bind to additional receptors and produce agonistic effects (Keltner and Johnson, 2002). This flexibility is central to the potential ability of these APDs to ameliorate both positive and negative symptoms of SZ. However, all three generations of APDs struggle to improve the negative and cognitive symptoms of SZ sufficiently. This lack in efficacy stems from the lack of knowledge about the underlying pathophysiology of negative and cognitive symptoms and how it is likely a combination of neurotransmitter systems that are affected.

4.2 Synapsin II (SynII)

Synapsins are a family of phosphoproteins that are evolutionarily conversed across invertebrate and vertebrate species (Kao et al., 1999). Three synapsins have been identified, synapsin I, synapsin II and synapsin III, making up approximately 9% of all vesicle proteins (Rosahl et al., 1995). Synapsin II is the synapsin that has been most associated with Schizophrenia and therefore will be the focus of this chapter. Below is a brief overview of synapsin II. For an in-depth review of synapsin II please read the “Synapsin II” chapter by Bernardo et al. in *The Encyclopedia of Signalling Molecules* (Bernardo et al., 2016).

4.2.1 Synapsin II Structure

Synapsin II can be alternatively spliced into synapsin IIa or synapsin IIb isoforms. Within the two isoforms, the N- terminus is conserved while the C-terminus contains variations (Sudhof et al., 1989). Several domains (A, B and C) are conserved across all synapsins. Following domain C, is a domain unique to synapsin II termed domain G and in this domain in an actin-binding site (De Camilli et al., 1990). Synapsin IIa then contains an H and E domain. The E domain is conversed across all other synapsin "a" isoforms, and is thought to be responsible for reserve pool maintenance via interaction with actin and the cytoskeleton. Synapsin IIb lacks an H or E domain and instead, following the G domain is an I domain with unknown specific functionality (Figure 4i) (Bernardo et al., 2016).

Figure 4i. Domains of synapsin IIa and synapsin IIb. The different domains of the synapsin IIa and synapsin IIb are visually represented. The A,B, and C domains are conserved across all synapsins while the G domain is unique to synapsin II isoforms. The E domain is conserved across all "a" isoforms of synapsins as shown in synapsin IIa.

4.2.2 Localization and Regulation

Synapsin II is vastly present in the presynaptic terminals of both the central nervous system and the peripheral nervous system. The expression seems to differ based on region. Synapsin IIa and IIb have specifically been identified in mossy fibers and hippocampal neurons however, synapsin IIa is not readily found within Purkinje fibers of the cerebellum (Sudhof et al., 1989). This regional distribution often follows a 1:2 ratio of expression for synapsin IIa:synapsin IIb with the exception of the olfactory bulb where synapsin IIa is more prevalent than synapsin IIb (Walaas et al., 1988). This exception is thought to be due to the constant remodelling of the contacts within the olfactory bulb (Bernardo et al., 2016; Walaas et al., 1988). Regulation of synapsin II occurs through 3 different transcription factors, early growth response

factor (EGR-1), activation protein 2-alpha (AP-2α), and polyoma enhancer activator 3 (PEA-3) (Petersohn et al., 1995). Dopamine has been shown to indirectly regulate synapsin II transcription via AP-2α (Skoblenick et al., 2010) as well as methylation via CpG islands within synapsin II promoter region (Cruceanu et al., 2016).

4.2.3 Function

Synapsin II has established functions at several stages of the vesicle cycle including clustering, reserve pool maintenance, delivery to the active zone, and synchronization of neurotransmitter exocytosis (Figure 4ii) (Bernardo et al., 2016). Many of these functions occur through a dynamic balance between the phosphorylation and dephosphorylation of synapsin II (Bykhovskaia, 2011; De Camilli et al., 1990). Synapsin IIa is believed to be the major player in clustering of synaptic vesicle as is it can form cross-linkages between synaptic vesicles and actin filaments. Upon phosphorylation, synapsin II dissociates from actin in a phosphorylation-dependent manner and liberates the vesicle from the reserve pool into a readily releasable pool or recycling pool (Bernardo et al., 2016; Bykhovskaia, 2011). At excitatory and inhibitory synapses, a different specific function of synapsin II has been proposed. In excitatory synapses, synapsin II seems to play a role in reverse pool maintenance function while at inhibitory synapses, synapsin II more regulates the size of the readily releasable pool (Gitler et al., 2004). The next stage that synapsin II is involved in is the shuttling of the vesicle from the readily releasable pool to the active zone and docking of vesicles at the active zone of the presynaptic terminal. This occurs with the cooperation of Rab3 as a complex with synapsin. The complex promotes dissociation from actin and targets active zone machinery (Bykhovskaia, 2011). Finally, synchronization of neurotransmitter release is proposed to occur through synapsin coating vesicles and changing membrane properties in order to promote accelerated fusion in the presence of calcium however this has not been fully elucidated (Bernardo et al., 2016). Synapsin II has also been found to influence synaptogenesis, synapse maintenance, and plasticity. Studies

of synapsin II knockdown reveal flawed cytoskeletal organization, reduced neurite branching and slow axonal outgrowth that affects overall synapse maintenance and function (Brenes et al., 2015).

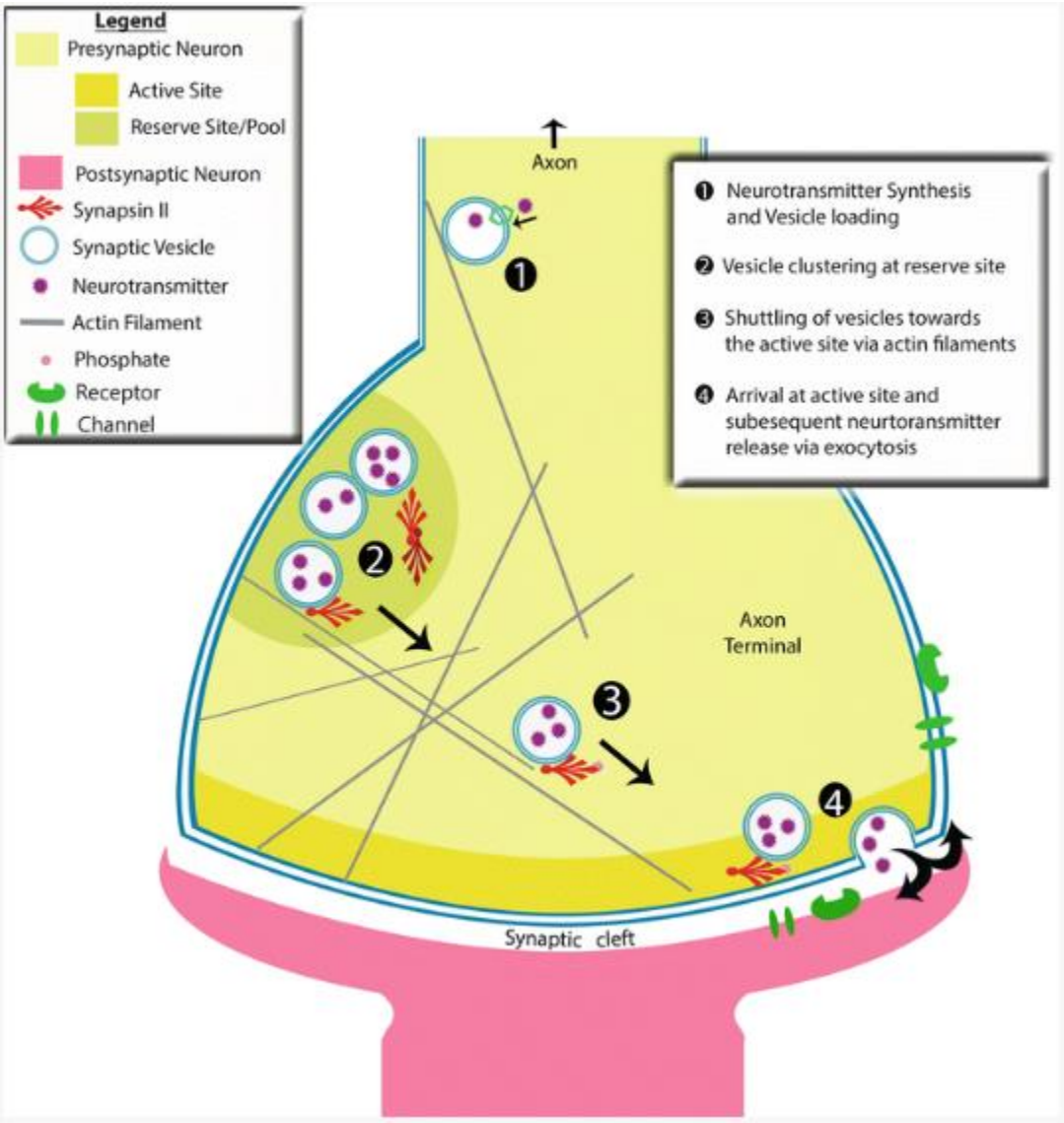

Figure 4ii. Visual representation of synapsin II functions in neurotransmission. Synapsin II is involved in several stages of the vesicle cycle and neurotransmitter release. The involvement of synapsin II at important stages of the vesicle cycle is outlined in the figure however this is a simplified representation. 1) Neurotransmitters are loaded into synaptic vesicles, 2) Vesicles are clustered in the reserve pool and synapsin II tethers vesicles to actin filaments to maintain clustering. 3) Phosphorylation of synapsin II

causes dissociation from actin filaments and vesicles travel to the active zone. 4) Synapsin II helps the docking process of vesicle to the presynaptic membrane. Image by Bernardo et al. 2017, The encyclopedia of signalling molecules. Permission obtained and shown in Appendix 2.

4.3 Synapsin II and Schizophrenia

Out of the three established synapsin genes, synapsin II has been most positively associated with SZ. Accumulating evidence from gene association studies, animal studies and post-mortem human sample analysis has supported the role of synapsin II in SZ. First, the location of the synapsin II gene is within a highly vulnerable region for SZ and within families of Northern European and Chinese descent, synapsin II polymorphisms demonstrated a positive association between synapsin II and SZ (Saviouk et al., 2007; Chen et al., 2004). Further, there have been reports of decreased synapsin II levels within the dorsolateral prefrontal cortex of schizophrenia patients and an association between different APD treatments and their effects on synapsin II mRNA levels (Tan et al., 2014; Chong et al. 2002). Accompanying this clinical evidence, synapsin II knockdown experiments have been able to produce schizophrenic-like behaviours in rats that reflect some of the different symptom domains (Dyck et al. 2011). As a modulator of neurotransmission, synapsin II may be an instrumental player in the pathophysiology of SZ. The interaction synapsin II has with multiple neurotransmitter systems including glutamate, and GABA (Dyck et al. 2011) may be able to provide an explanation for the different hypotheses that demonstrate perturbed neurotransmission of multiple neurotransmitters and specifically be involved in the pathology relating to negative and cognitive symptom domains.

4.4 Study Significance

The significance of this study is two-fold. Using knockdown methods, reduced synapsin II was able to recapitulate all three symptom domains of schizophrenia. Moreover, the negative symptoms such as social withdrawal and cognitive symptoms including those of several cognitive domains (memory,

attention, cognitive flexibility, etc.) were displayed. This is important as these domains currently go unmedicated in patients. This evidences that synapsin II is likely involved in the underlying pathology of these symptoms potentially through disrupted regulation of neurotransmission and suggests this as a potential molecular target for these symptoms. Additionally, functional brain imaging supported that reduced synapsin II also produced a global hyperactivation in the brain which lends support to the hyperfrontality theory in schizophrenia. Overall, the study suggests synapsin II as a molecular target for future therapeutic design in the treatment of schizophrenia.

4.5 Specific Study Aims and Hypotheses

Aim 1: Similar to synapsin II levels found in clinical schizophrenia, induce synapsin II reduction in the mPFC and evaluate several symptom domains of schizophrenia with a particular interest in negative and cognitive symptoms.

Hypothesis: Synapsin II knockdown will lead to hyperlocomotion, reduced social interaction and impaired cognitive function corresponding to positive, negative and cognitive symptoms respectively.

Aim 2: Identify if synapsin II knockdown can alter brain metabolic function in support of the hypo/hyperfrontality debate in existing schizophrenia literature.

Hypothesis: Reduced synapsin II will affect vesicle trafficking, neurotransmission and therefore brain metabolic activity will show either increased or decreased metabolism.

4.6 Author Contributions

Experiments were designed by Mishra R., Bernardo A. and Molinaro L.. Bernardo A. and Molinaro L. completed animal training and habituation. They also performed surgeries and conducted behavioural testing. Thomson S. assisted with surgeries and ran western blotting with assistance from Ho J. and Bernardo A. Imaging analysis and statistical analysis was completed by Bernardo A. and Molinaro L. The

manuscript was written by Bernardo A. with assistance from Molinaro L., Thomson S. and Ho J. All authors will have the opportunity to review and edit the manuscript prior to submission.

4.7 Statement of Paper Status: This manuscript has not been submitted as of yet. All authors will be required to review and edit the manuscript prior to submission.

Title

Reduced Expression of Synapsin II Manifests Behavioural and Brain Metabolic Changes: Implications in the Pathophysiology of Schizophrenia

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Abstract

Synapsin II (SynII) is a neuronal phosphoprotein responsible for regulating synaptic vesicle trafficking of multiple neurotransmitters. SynII has been implicated in schizophrenia (SZ) through genome-wide association studies and reduced SynII mRNA levels in SZ post-mortem studies. SZ is a psychiatric disorder with unmet symptoms alleviation, specifically negative and cognitive symptoms. These domains can manifest as social withdrawal and memory, attention, motivation impairment respectively and are thought to be produced due to irregular neurotransmitter release. Effective treatment of these domains must target underlying pathology of the disease therefore, this study investigated SynII's role in manifesting these behavioural changes to elucidate a potential target for antipsychotic drugs (APDs). The emergence of phenotypic SZ like- positive, negative, cognitive symptoms detected using locomotor activity, social interaction paradigm, 5-choice serial reaction time test, and 8-arm radial maze respectively. Hyper and hypo brain function is highly debated in the literature, therefore, this study further used computerized tomography and positron emission test image fusion for investigation of region-specific glucose metabolism profiles and found global hyperactivation in the brains of SynII knockdown animals. Our findings corroborate reduced SynII in the pathophysiology of SZ and reinforce SynII as a potential therapeutic target to alleviate currently untreated symptoms of SZ.

Keywords: Synapsin II, schizophrenia, positive symptoms, negative symptoms, cognitive symptoms, positron emission tomography

Introduction

Synapsins are a family of neuron-specific phosphoproteins that were the first synaptic vesicle proteins identified (Fdez and Hilfiker, 2007). Constituting one of the most abundant families of synaptic proteins, synapsins comprise roughly 1% of total protein in the brain (De Camilli et al., 1990) and 9% of total vesicle protein (Rosahl et al., 1995). Mammalian synapsins are encoded by three distinct genes, synapsin I, II and III; of which give rise to at least 9 isoforms (Kao et al., 1998; Sudhof et al., 1987). Synapsins I and II are located in the adult presynaptic terminals of both the central and peripheral nervous system, whereas synapsin III is developmentally controlled and is not restricted to presynaptic terminals (Gitler et al., 2004). Through alternative splicing, both synapsins I and II give rise to a- and b- isoforms that have similar, precise or overlapping roles (Sudhof et al., 1987; Waites and Garner, 2011). Synapsin II (SynII) protects against synaptic depression by accelerating synaptic vesicle trafficking during repetitive stimulation (Rosahl et al., 1995), and regulating vesicle trafficking between the reserve pool (RP) and readily releasable pool (RRP) (Molinaro et al., 2015). In its unphosphorylated form, SynII tethers synaptic vesicles in the RP to actin filaments of the cytoskeleton, maintaining RP integrity. Upon phosphorylation, SynII dissociates from actin filaments, releasing vesicles from the RP into the RRP and propagating the synaptic vesicle cycle (Cesca et al., 2010). SynII, therefore, regulates the release of multiple neurotransmitters such as dopamine, GABA, and glutamate (Ferreira and Rapoport, 2002; Gitler et al., 2008; Medrihan et al., 2013; Song and Augustine, 2015; Venton et al., 2006). Given the multiple roles of SynII in neuronal functioning, disruption of these roles could result in increased vulnerability towards the development of mental disorders. Dysregulation of SynII has been strongly implicated in the pathogenesis of schizophrenia (Cesca et al., 2010; Dyck et al., 2011; Molinaro et al., 2015).

Schizophrenia (SZ) is a biochemical, psychiatric brain disorder characterised by a spectrum of symptoms divided into positive, negative and cognitive domains. Patients experiencing positive symptoms report visual or auditory hallucinations while the negative symptom domain consists of social withdrawal,

anhedonia, and apathy(Bowie and Harvey, 2006; Owen et al., 2016; Veerman et al., 2017). Cognitive symptoms typically found among patients with schizophrenia are impaired attention/vigilance, reasoning/problem solving, motivation and working memory (Gold et al., 2018). Current antipsychotic treatments are struggling to alleviate all symptoms, largely negative and cognitive domains. The underlying pathology leading to these symptoms is unknown and therefore elucidation of a molecular target for these will aid in therapeutic design and capability.

Considerable evidence implicates reduced expression of SynII in the pathophysiology of SZ. Genome-wide association studies (GWAS) of different geographic populations indicate a positive association between polymorphisms within the human SynII gene and SZ (Chen et al., 2004a, 2004b; Saviouk et al., 2007). Examination of human post-mortem tissue samples revealed a decrease in SynII mRNA within the dorsolateral prefrontal cortex (DLPFC) in patients with SZ compared to control samples (Karoly et al., 2000; Tan et al., 2014). Pre-clinical SynII knockdown experiments established reduced vesicular transport proteins responsible for the transport of glutamate and GABA into vesicles, implicating SynII in the regulation of glutamatergic and GABA-ergic signalling (Dyck et al., 2011). Decreased vesicular transporter mRNA levels are also seen in human post-mortem samples (Eastwood and Harrison, 2005; Schleimer et al., 2004). Together, this data suggests decreased SynII levels and functional abnormalities may contribute to the underlying pathophysiology of SZ.

Functional imaging studies have found modified metabolic activity in the frontal lobe of patients with SZ, more specifically the DLPFC (Minzenberg et al., 2009; Newberg et al., 2011; Perlstein et al., 2001). Literature debates hypofrontality and hyperfrontality, referring to decreased or increased glucose uptake respectively within the frontal lobe, with hypofrontality being reported more often in the DLPFC (Chen et al., 2004a, 2016; Glahn et al., 2005; Karlsgodt et al., 2009; Minzenberg et al., 2009; Shinto et al., 2014; Wolf et al., 2011). Therefore, this study aimed to investigate SynII in regards to this debate and identify if knockdown of the phosphoprotein could play a role in amounting metabolic changes.

The objective of this study is to strengthen the existing literature evidencing decreased SynII in the pathophysiology of schizophrenia. Specifically, we aimed to confirm schizophrenic-like phenotypes of positive, negative and cognitive symptom domains by investigating several domains of cognition that have never been evaluated previously and administered a second-generation antipsychotic to determine if negative symptoms can be reversed. Further, we aimed to establish either hypofrontality or hyperfrontality in rats subsequent to SynII knockdown using antisense-oligonucleotides ribonucleic acids and short hairpin (shRNA). Using both knockdown technologies this study used an established and novel method of knockdown respectively, to investigate SynII in recapitulating all symptom domains of SZ and lend support to the hypofrontality and hyperfrontality debate through brain metabolic activity imaging.

Methods

Animals

Male 8-week-old Sprague-Dawley rats weighing 250-300g were obtained from Charles River Laboratories (Quebec, Canada) and individually housed in the McMaster University Central Animal Facility at 21^{o}C, on a 12-hour dark/light cycle. All animals were food restricted to 95% of their free-feeding weight (16-18g), to produce an incentive for treat retrieval during the 8-arm radial maze. All animals received water *ad libitum*, and all experimental procedures were completed in accordance with the Animal Research Ethics Board and the Canadian Council on Animal Care.

Antisense oligonucleotides and surgery

Antisense (AS) and mismatched (MM) sequences were determined by via NCBI BLAST tools available online and each of the sequences was capped with phosphorothiates to prevent degradation by nucleases. Oligonucleotides were obtained from Integrated DNA Technologies (IDT) and dissolved in nuclease-free water to a final concentration of 1uM. Two-week Alzet osmotic mini-pumps (HRS Scientific, Montreal, QC) were filled with 5nM of a solution of aCSF as a sham control, MM solution or AS solution

for each respective treatment prior to surgery by following manufacturers instructions. Stereotaxic surgeries were performed similarly to the previously published methodology from our lab (Dyck et al., 2009). In brief, rats were anesthetized with isoflurane and bilateral, stainless-steel cannulas were inserted into the mPFC at coordinates: AP +3.5mm, ML ±2.0mm, and DV -3.5mmμ in reference to bregma. Rats were given 4 days to recover prior to recommencing behavioural testing.

shRNA and surgery

Lentiviral particles were obtained from Origene Technologies (Rockville, MD). 0.5μL of four unique 29mer SynII specific lentiviral particles with >10^6 TU/mL were combined to make a cocktail (shSynII). ShSynII was administered via bilateral stereotaxic injections into the mPFC. Control animals were infused with a scrambled control sequence (shCntrl). During surgery, animals were anesthetized and maintained in a surgical plane using isoflurane with a constant supply of oxygen. Once the animal was mounted on the stereotax, an incision was made on the dorsal surface of the skull. Using a manual drill, two holes were drilled above the mPFC (A/P: +2.7mm, M/L ± 1.5mm) in reference to bregma. A Hamilton syringe was lowered 3.0mm into the mPFC. 2μL of the lentiviral scrambled control particle solution or the shSyII shRNA cocktail solution (>10^6 TU/mL) was infused at a flow rate of 1μL /minute. The syringe was left in place for 10 minutes following the injection to ensure sufficient diffusion within the cortex. The syringe was then removed from the brain and the incision was closed using stainless steel wound clips (Durect Corporation, Cupertino, CA). Animals were monitored for 7 days and given 14 days to recover prior to behavioural testing.

Locomotor Activity

Locomotor testing was conducted in specialized AccuScan chambers (AccuScan Instruments, Columbus, OH). Disruption of infrared beams equally distributed along the x and y axes of each chamber

was detected via Accuscan software which determined the total distance travelled by the animal. Baseline locomotor activity was measured prior to surgery to ensure that there were no subtle underlying basal differences in walking speed and distance travelled between groups.

Pre-pulse Inhibition

The pre-pulse inhibition (PPI) test is conducted utilizing the Startle Response System designed by SR Labs (San Diego Instruments, San Diego, CA). Details of the behavioural paradigm have been previously published (Dyck et al., 2009a, 2011; Thomson et al., 2019). Briefly, PPI sessions included a 5 min habituation to the restraint tube, immediately followed by a 15 min testing portion. Acoustic parameters were set as follows: startle pulse - 110dB for 40ms; prepulse - 71dB for 20ms; no stimulus - white noise. PPI was analyzed using the following percent PPI formula: %PPI = [(average response magnitude to startle pulse only trials—average response magnitude to pre-pulse with startle pulse trials)/average response magnitude to startle pulse only trials] × 100. Baseline pre-pulse inhibition was tested prior to surgery to ensure that all animals are equally sensitive to response to the pulse or pre-pulse presented.

5-Choice Serial Reaction Time Test

Both behavioural testing and training were completed in operant boxes from Med Associates (St. Albans, VT) measuring 33m x 31cm x 29cm. Each chamber consisted of 5 apertures, each made up of a light and a detector, located on one wall of the chamber. On the opposite wall was a single aperture was used for reward retrieval as it was attached to a food pellet hopper containing 45mg Dustless Precision Pellets. A light was also located overhead and illuminated to convey an aversive stimulus. Chambers were connected to a computer and run using Med-PC IV software. To reduce noise, keep lighting consistent and avoid other confounding outside stimuli, each chamber was contained in an individual wooden cabinet.

During habituation (2 days) rats were left to explore the chamber while all aperture lights were illuminated, and food pellets were placed in all aperture opening. Training began after habituation days were completed, beginning at training stage 1 (T1). Each 5-CSRTT session consisted of 100 trials or a maximum of 30 minutes. To pass a training stage the following criteria were required: a minimum of 50 correct responses, the accuracy of at least 80% and an omission rate below 20%. Training continued until rats reached training stage 12 (T12) which took 4-8 weeks with individual variabilities. (Bari et al., 2008; Bhandari et al., 2016).

Each session would be initiated with a single food pellet in the reward compartment and LED light illumination. Once the pellet was retrieved trial one commenced. Each trial consisted of illumination of 1 of the 5 aperture lights for a set stimulus duration (SD). The rat can then respond either during the SD or for a specific time after termed the limited hold (LH). If the rat poked their nose in the correct aperture a single pellet of food was dropped from the hopper into the lit reward aperture to be retrieved. After retrieval, the next trial is initiated with a set inter-trial interval (ITI) between every trial. If an incorrect response (nose poke into an unilluminated aperture) the overhead light became illuminated for 5 seconds. The next trial would be started after this 5 second period. If an omissive response was registered (no response/no nose poke into an aperture) the overhead light also turned on for a 5 second period. A premature response, determined to be a response occurring in the ITI therefore before the presentation of a new stimulus, also caused the overhead light to turn on for 5 seconds. At T1 the specific parameter used were: 30 seconds SD, 2 seconds ITI, and 30 seconds LH. Each subsequent training stage became progressively more difficult until T12 parameters were: 0.5 seconds SD, 5 seconds ITI, and 5 seconds LH (Bari et al., 2008; Bhandari et al., 2016). Stable performance at T12 demonstrated an understanding of the paradigm and animals were able to undergo surgery. Rats then underwent surgery to induce the respective treatments as previously described. After recovery from surgery, all animals were tested using

the 5-CSRTT testing paradigm that had varying SD, ITI, and LH parameters in order to test the effects of treatment of several cognitive parameters. Parameters were the same for all rats at each session.

8-Arm Radial Maze

The 8-arm radial maze (8-ARM), a task that stimulates working and reference memory, was used to initiate activity in the frontal lobe prior to imaging. The maze consists of eight arms (70cm x 25.5cm x 12.5cm) attached to an octagonal platform in the center. Each arm has a location at the distal end where a sugar pellet used as food bait was placed. During habituation, 10 minutes was allotted for the animal to enter each arm and receive all treats. The habituation trial was completed after all treats had been consumed, or 10 minutes had elapsed. During testing, a win-shift format was used where four arms were baited and 4 arms were blocked. The animal was given a total of 15 minutes to consume all 4 treats and was then removed from the maze and given a delay period of 15 minutes. During the delay, the maze was cleaned, and all blocked arms were opened and baited. The animal was then returned to the maze for a free run for a total of 15 minutes. During the free run, each animal was scored based on the number of correct arm choices and incorrect arm choices. A correct choice was considered to be the entry into an arm not previously explored, while an incorrect response was an entry into a previously explored arm. Total number of incorrect responses was calculated as follows: (total number of entries) – (total correct). This task was also used for cognitive stimulation prior to imaging to more accurately translate to human SZ patients' functional imaging results. Evidence suggests that SZ patients exhibit impaired cognitive functioning while trying to complete a cognitive task or while initiating the use of these pathways (Newberg et al., 2011).

Social Interaction

A black 100cm x 100cm x 40cm polyvinyl chloride arena was used for testing social interaction. Animals were habituated to the arena individually 5 times prior to testing, to ensure that novelty of the arena did not affect the animal's behaviour during testing. Two animals from the same treatment group, that had never previously interacted, were placed in the arena and filmed from above. Animals interacted for a total of 10 minutes; the first 5 minutes were allotted for habituation followed by a 5-minute period of scored interaction time. Videos were analyzed by a blinded experimenter and the total time spent in a variety of interactions including: sniffing another rat, climbing over or under another rat, aggression towards another rat and following another rat, was recorded.

Olanzapine Preparation

In order to investigate if olanzapine, a current second generation antipsychotic, was able to attenuate the deficits seen in social interaction a 10mg/kg dose of olanzapine was administered 40 minutes prior to the testing. Olanzapine was prepared in 0.1M HCL and using NaOH the pH was adjusted to 6.0.

Imaging

Glucose uptake is an indication of metabolic function within the brain and literature supports aberrant metabolic function in the brain of SZ patients (Newberg et al., 2011; Perlstein et al., 2001). Imaging was performed by the McMaster Centre for Preclinical and Translational Imaging. Images were obtained using Philips Mosaic Dedicated Animal PET system (Philips Medical Systems, Cleveland, Ohio) and Gamma-Medica Ideas X-SPECT (Gamma-Medica Ideal, North Ridge, California). Animals were fasted for 12 hours and received 500µCi of fludeoxyglucose (FDG) via tail vein injection 30 minutes prior to imaging. Animals were anesthetized using a constant flow of 1.5% isoflurane and oxygen for the duration of a 15-minute static emission positron emission tomography (PET) scan and a 5-minute high-resolution computerized tomography (CT) scan. The collected data was then reconstructed using a 3D iterative

reconstruction algorithm, and a Feldkamp filtered back-projection cone-beam reconstruction algorithm for PET and CT data respectively. The transformed data were then fused using software developed by Dr. Troy Farncombe and standard uptake values were obtained for each brain region of interest as previously described by our lab (Daya et al., 2014).

Western Blotting

The mPFC was homogenized via sonication and mechanical agitation in phosphate-buffered saline (PBS), which also contained the mini-C protease inhibitor cocktail (1 tablet per 10 mL of PBS), to prevent tissue degradation. The concentration of protein for each region was then determined using a Bradford assay. To prepare the samples, 10 µg of protein was mixed with 12.5 µL of 2X sodium dodecyl sulfate (SDS) buffer and the final volume of each sample was brought to 25 µL with distilled water. Prior to loading, the samples to be probed for SynII were boiled for 10 minutes and spun down briefly in a microcentrifuge. The samples were loaded into each well of 4-20% precast gradient gels (Bio-Rad) and run at 200 V for approximately 30 minutes to separate the proteins in each sample. The gels were then activated with UV radiation to permit a covalent reaction between trihalo compounds present in the gels and tryptophan amino acid residues in the protein, which permits the immediate detection of the proteins on the gel or on membranes after transfer. After confirming the quality of separation, semi-dry Trans-Blot Turbo system (Bio-Rad) was used to transfer the separated proteins onto polyvinylidene difluoride (PVDF) membranes. A stain-free total protein image of each membrane was obtained using the Bio-Rad ChemiDoc^{TM} Imaging System. Subsequently, membranes were blocked for 1 hour with gentle agitation in 5% non-fat milk, which was prepared by dissolving powdered milk in TBS-T (0.1% Tween 20 and 1X Tris-buffered saline, pH 8.5). The membranes were then incubated in the appropriate primary antibodies overnight at 4°C while shaking on a nutating mixer. Each membrane was probed with only one primary antibody. The primary antibodies were prepared by diluting stock solutions with TBS-T in the following volume ratios: SynII at 1:2500. The following day, membranes were washed with TBS-T twice for 5 minutes

and once for 15 minutes. Membranes probed with SynII were then incubated in the anti-rabbit secondary antibody, diluted with TBS-T at a volume ratio of 1:5000, for 1.5 hours at room temperature. Finally, membranes were washed with TBS-T twice for 5 minutes and once for 10 minutes. Membranes were incubated in 1.4 mL of ECL (Bio-Rad) for 1 minute and imaged using the Bio-Rad ChemiDoc^{TM} MP Imaging System. The images were analyzed using the Image Lab system (Bio-Rad), which normalized the quantification of protein to the relative amount of total protein in each blot as determined from the stain free total protein images. The Image Lab software also accounts for the background (noise), improving the accuracy of the analytical technique. Normalized values were used to calculate percent expression.

Statistics

This study used a variety of statistics to appropriately evaluate the different parameters investigated. A students t-test was used to evaluate western blotting results. One-way Analysis of Variance (ANOVA) with Tukey's post hoc testing was used to detect significance in locomotor activity, PPI and all 5-CSRTT parameters. Two-way ANOVA with Bonferroni post hoc testing was used to analyze social interaction paradigms, 8-ARM and standard uptake values provided by brain imaging results. Significance was considered as the following: *$p < 0.05$, **$p<0.01$, ***$p<0.001$. Outliers were considered using Grubb's test with a significant level of $\alpha \leq 0.05$ and if detected were excluded in the final analysis. Error bars in figures always show the standard error of the mean (SEM).

Results

Confirmed synapsin II reduction in the mPFC

Confirmation of synapsin IIa (SynIIa) and synapsin IIb (SynIIb) isoform protein levels were evaluated after behavioural testing and imaging through western blotting gel electrophoresis. Significantly reduced protein expression of the SynIIa ($t(11)=2.58, p=0.026$) and SynIIb ($t(12)=3.659$, $p=0.003$) isoforms were confirmed in the mPFC (the site of injection) of animals with a SynII knockdown compared to those in the

control group. Therefore, behavioural and imaging data presented subsequently can be attributed to the reduction in SynIIa and SynIIb.

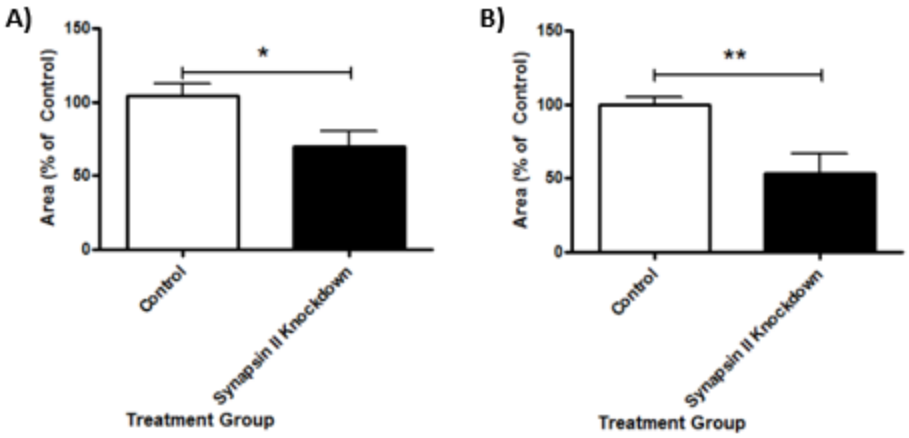

Figure 1. Confirmed reduction of synapsin IIa and synapsin IIb protein levels in mPFC. 1A) Synapsin IIa protein was significantly reduced in the mPFC of synapsin II knockdown animals (* symbolizes $p<0.05$). **1B)** Depicts reduced protein levels of synapsin IIb in the mPFC of synapsin II knockdown animals compared to controls (** indicates $p<0.01$).

Positive Symptoms of SZ are phenotypically displayed

The positive symptoms of SZ are classically considered to be hallucinations resulting from excess dopamine. In rodent models, hallucinations are impossible to quantify therefore the rodent phenotypic equivalent is increased locomotor activity, which is also produced by increased levels of dopamine. In SynII knockdown animals, increased locomotor activity was confirmed ($F(2,21)=4.510$, $p=0.025$) with Tukey's post hoc analysis revealing a difference between aCSF and AS SynII knockdown group (*$p<0.05$). No significant difference was observed between AS and MM groups.

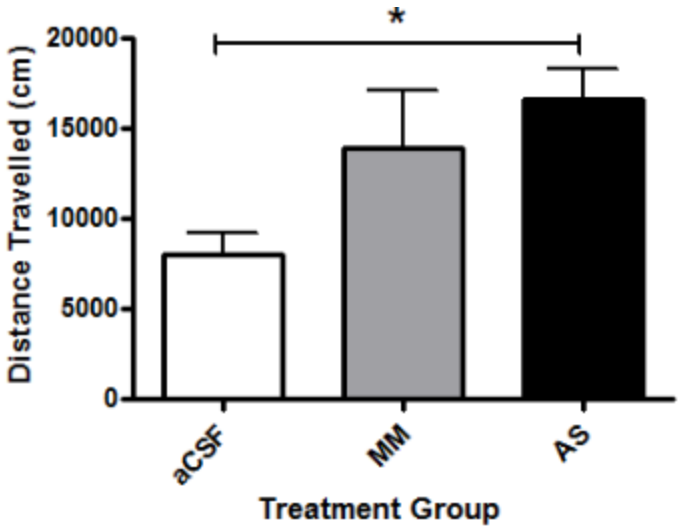

Figure 2. Increased locomotion was found in synapsin II knockdown animals. Total distance travelled in cm was increased in the synapsin II knockdown group (AS) compared to aCSF treated animals. Post hoc testing results are shown using * indicating a significant difference between the two groups of $p<0.05$.

Sensorimotor Gating is disrupted in synapsin II knockdown animals

Deficits in sensorimotor gating is a phenomenon found in clinical SZ. This can be measured using a percent prepulse inhibition (PPI) task that translates efficiently between rodents and humans. In this study SynII knockdown animals also presented with deficits in %PPI. An effect of treatment was found using One-way AVONA with a Tukey's post hoc test, which determined a significant decrease in %PPI in SynII knockdown animals ($F(2,16)=5.956$, $p=0.012$) when comparing the aCSF (**$p<0.01$) and MM (*$p<0.05$).

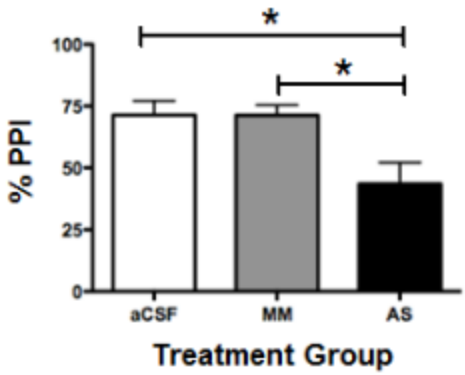

Figure 3. Synapsin II knockdown animals display impaired sensorimotor gating. AS animals with a synapsin II knockdown had lower percent prepulse inhibition (%PPI) calculated using the following equation: %PPI = [(average response magnitude to startle pulse only trials—average response magnitude to pre-pulse with startle pulse trials)/average response magnitude to startle pulse only trials] × 100. Significance was detected with Tukey's post hoc test and shown using * for $p<0.05$.

Cognitive symptom domains of SZ are present after synapsin II knockdown

5-choice serial reaction time test (5-CSRRT)

The 5-choice serial reaction time test (5-CSRRT) evaluates several elements that allow for simultaneous assessment attention, response inhibition, cognitive flexibility and processing speed (Amitai and Markou, 2010, 2008). Percent of correct responses is the most common measure of attention and is specifically calculated using the total number of correct responses divided by the sum of correct and incorrect responses. One-way ANOVA observed a significant effect of treatment was ($F(2,349)=45.34$, $p<0.0001$) with Tukey's post hoc testing reporting a significant effect between aCSF and AS (***$p<0.001$)as well as MM and AS groups (***$p<0.001$). Percent omissions were calculated such that the number of omitted responses was divided by the sum of correct and incorrect responses. This measure found a significant effect of treatment ($F2,349)=56.69$, $p<0.0001$), with similar significance observed after post-hoc testing between aCSF and AS (***$p<0.001$) and MM and AS (***$p<0.001$). The number of perseverative responses, defined as repeated responses committed following a cue light response prior to retrieving a

food pellet, is a common measure of cognitive inflexibility and compulsiveness. Perseverative responses were significantly affected by treatment ($F(2,348)=6.596$, $p=0.002$). Tukey's post hoc revealed significantly less perseverative responses made by aCSF compared to MM (**p<0.01) and AS (**p<0.01) groups. Indicating aCSF animals had better cognitive flexibility. Correct response latency is defined as the time (measured in seconds) between the presentation of a cue light and the time the response is registered. Treatment significantly affected correct response latency ($F(2,349)=7.981$, $p=0.0004$) with Tukey's post hoc testing demonstrating AS animals took significantly longer to make a correct response than both aCSF (***p<0.001) and MM (**p<0.01). Reward collection latency was another parameter investigated and was used as a measure of motivation. Reward collection latency was determined to be the time between the presentation of a reward pellet and collection of the reward pellet. Similar to correct response latency, SynII knockdown significantly affected reward collection latency ($F(2,329)=5.695$, $p=0.004$), with post hoc differences identified between AS and both aCSF (**p<0.01) and MM (*p<0.05) groups indicative of reduced motivation in AS rats.

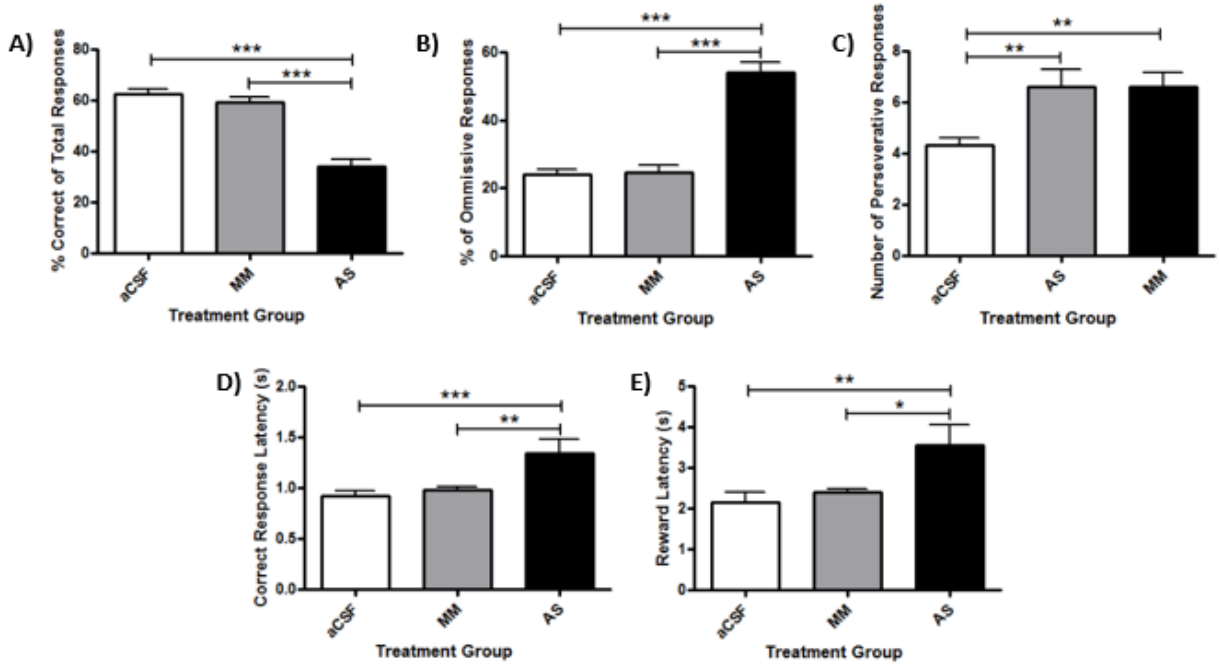

Figure 4. 5-CSRTT detected multiple cognitive deficits in synapsin II knockdown animals. Several parameters were evaluated using 5-CSRTT and together demonstrated animals with a synapsin II knockdown (AS) consistently made more errors, demonstrated attentional deficits and motivational impairments. Specifically, **4A)** shows the percent of correct responses made is reduced in synapsin II knockdown animals (AS), **4B)** The percent of omissions made was significantly higher in AS animals, **4C)** Synapsin II knockdown animals made more perseverative responses than aCSF animals, **4D)** Correct response latency indicated longer durations to make a correct response in synapsin II knockdown animals and **4E)** Time to collect reward known as reward latency was higher in synapsin II knockdown animals. Full descriptions of each parameter can be found in the methods section. In all graphs * represents $p<0.05$, ** represents $p<0.01$, and *** represents $p<0.001$.

8-Arm Radial Maze (8-ARM)

Cognitive symptoms of SZ encompass diverse domains of cognition. The 5-CSRTT demonstrated attention deficits and cognitive inflexibility, therefore another cognitive domain to be tested was memory using the 8-arm radial maze (8-ARM). The 8-ARM examines the cognitive domain of SZ as well however it explores the memory domain of cognition, specifically reference and working memory. A significant effect of treatment was found between shSynII and shCntrl treated animals using a 1-minute delay between forced and free runs, indicative of short-term memory impairment ($F(1, 3)=5.133$, $p=0.0314$).

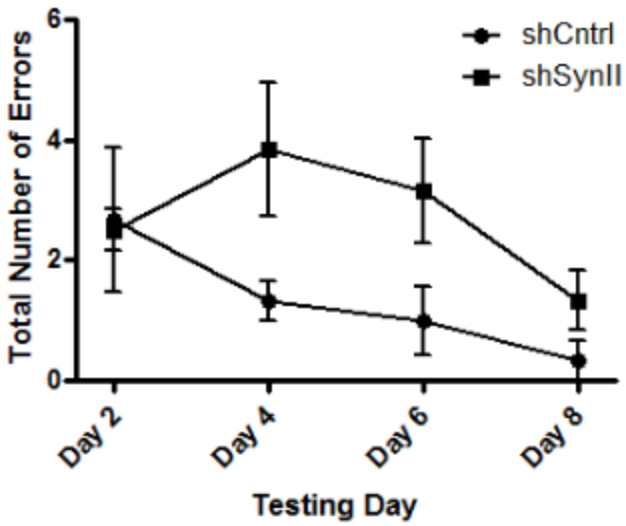

Figure 5. Synapsin II knockdown affected the number of errors made during 8-ARM testing. The total number of errors made during the 8-ARM was recorded over several sessions and a significant effect of treatment was found indicating synapsin II knockdown animals (shSynII) made more errors than shCntrl animals. Error bars depict SEM.

Negative symptoms of SZ can be recapitulated

The final symptom domain to be investigated was the negative symptom domain. To evaluate negative symptoms animals were tested using the social interaction paradigm which considers behaviours such as: sniffing another rat, climbing over or under another rat, aggression towards another rat and following another rat. Two-way ANOVA identified shSynII animals spent significantly less time interacting than shCntrl animals over the span of five minutes, echoing clinical social withdrawal ($F(3,3)=11.50$, $p=0.002$). Bonferroni post hoc testing found SynII knockdown animals spent significantly less time sniffing each other than the control animals (**$p<0.01$). Using this paradigm, this study then investigated predictive validity through the administration of olanzapine. Few therapeutics can efficiently remedy negative symptoms and therefore olanzapine, a current antipsychotic that shows some clinical relevance in treating negative symptoms was tested (Lecrubier et al., 2006). Following IP injection of olanzapine, no significant

differences were found between shCntrl and shSynII animals (F(1,3)=0.095,p=0.7595). The deficits previously identified in shSynII animals was ameliorated and animals had restored social behaviour.

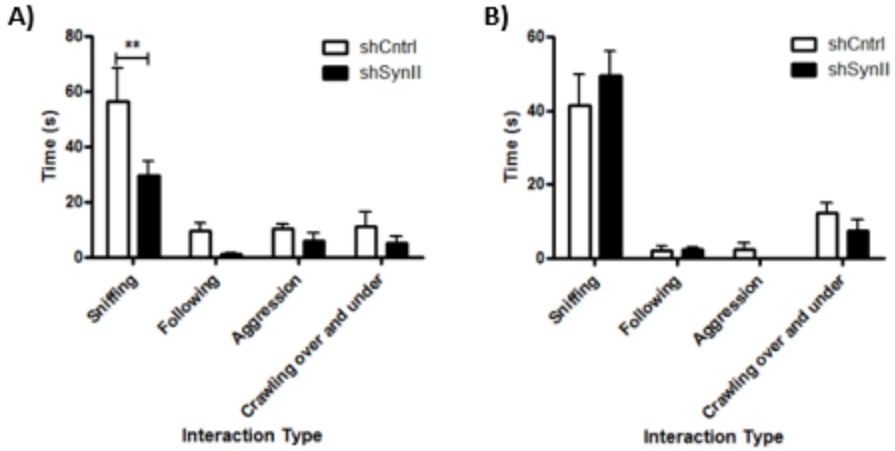

Figure 6. Social interaction is reduced in synapsin II knockdown animals but can be reversed using olanzapine. 6A) Social interaction was quantified for several interaction types. Synapsin II knockdown animals did not participate in sniffing behaviours as often as control animals, significance is shown using ** for p<0.01. **6B)** Administration of Olanzapine reversed the social deficits previously identified in the synapsin II knockdown animals. No significant difference was found between groups using two-way ANOVA.

Brain glucose utilization is increased after synapsin II knockdown

Metabolic brain activity was assessed spatially using [18]FDG mean uptake values using a combination of PET and CT fused imaging. In both SynII knockdown cohorts, global hyperactivation was observed. In the AS induced SynII knockdown cohort, treatment had a significant effect and increased overall brain metabolic activity compared to aCSF controls (F(1,17)=8.509, p=0.005). Bonferroni post hoc testing, however, did not reveal significant differences in any specific brain regions. Increased [18]FDG uptake measuring glucose utilization was replicated in the shSynII cohort compared to shCntrl rats. A significant effect of treatment was found by two-way ANOVA (F(1,17)=7.921,p=0.006) and a significant effect on

region (p=0.0001, F=21.04). Bonferroni post hoc testing was performed however no significant differences were found suggesting global hyperactivation.

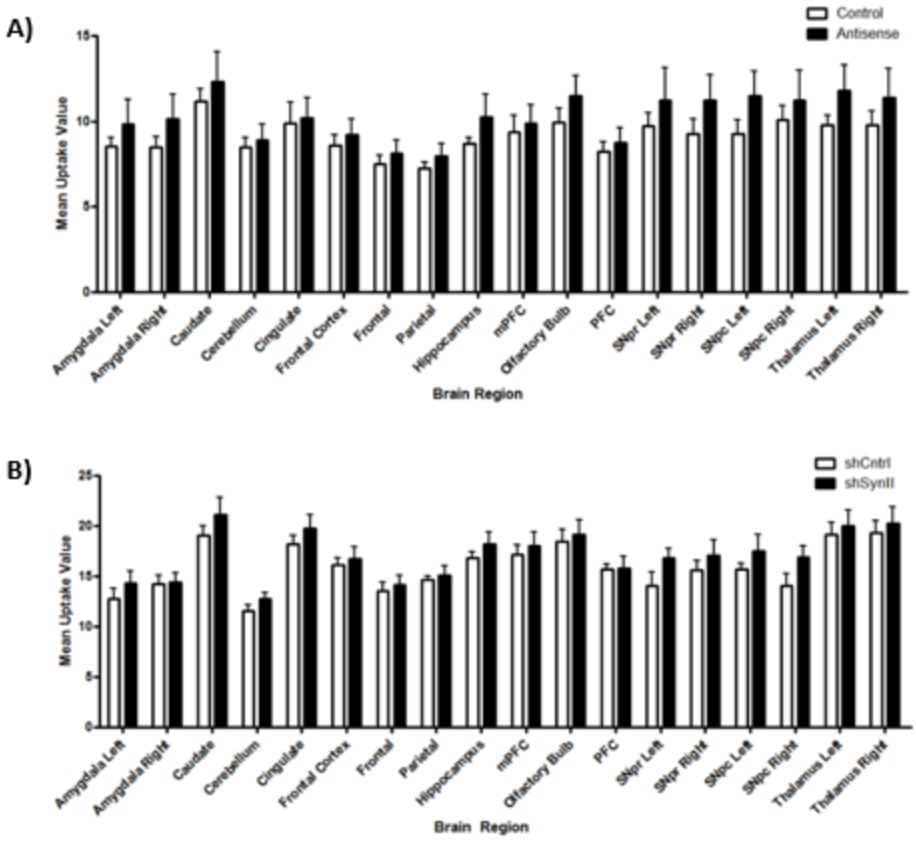

Figure 7. Functional PET and CT fused imaging show multiple brain regions consistently have higher glucose uptake in synapsin II knockdown animals. 7A) Mean uptake values of [[^{18}F]FDG analyzed in various brain regions identified a global hyperactivation phenomenon in synapsin II knockdown animals using oligonucleotides. **7B)** Mean uptake values for Synapsin II knockdown animals in the shRNA knockdown cohort are shown and again demonstrate global brain hyperactivation.

Discussion

The results of this study demonstrate that knockdown of SynII with both antisense oligonucleotides and shRNA, introduced to the mPFC of rats, can produce schizophrenic–like behaviours reflecting positive, negative and cognitive domains found in SZ. Administration of a current antipsychotic was also capable of ameliorating social deficits originally found in SynII knockdown animals and functional

imaging implicates SynII in neural activation with reduced SynII producing increased glucose uptake. Together, these results compile face validity, predictive validity and construct validity into one model and reinforce SynII reduction as a contributing factor to the presentation of SZ symptoms.

The significance of increased locomotor function found in SynII knockdown animals in this study is 2-fold. First, it phenotypically suggests a hyperdopaminergic state that can be translated loosely to hallucinations caused by increased dopamine in SZ patients. Second, it confirms that the SynII knockdown did not impair motor function in the animals and therefore changes found in other paradigms is not due to a lack of ambulation. While this test is limited in its ability to translate to clinical symptom manifestations, sensory-motor gating is able to be directly related. The impaired sensory-motor gating found in SynII animals is reminiscent of impaired PPI in clinical SZ (Brisch et al., 2014). Impaired PPI is repeatedly reported in glutamate antagonist models of SZ such as the phencyclidine (PCP), ketamine and MK-801 models (Moghaddam and Javitt, 2012; Wu et al., 2018). Of these models, SynII has been specifically investigated in the PCP model and found to be decreased after 14 day administration (Thomson et al., 2019). Glutamate signalling, known to be altered by SynII, therefore is likely involved in proper sensorimotor gating.

Due to the complexity of cognitive dysfunction in schizophrenia, it is necessary to access a broad range of cognitive domains to understand and create potential treatments for schizophrenia. The 5-CSRTT is an efficient method to access the various cognitive domains while allowing room for various parameter manipulations to mimic schizophrenia-like disruptions (Amitai and Markou, 2010). Amitai et al. (2010) proposed that increased reward latency and correct response latency can be indicators of lack of motivation, cognitive inflexibility or impaired motor function. This study can rule out motor deficits as SynII knockdown animals demonstrated increased locomotor activity. Percent accuracy was reduced in SynII knockdown animals which can be attributed to the schizophrenic like deficits in cognition and/or motivation. When the attentional load was increased using a shorter visual presentation of stimulus

duration, accuracy decreased across all groups. The greater deficits observed in the SynII knockdown group indicate impaired attention is the cause of disrupted performance. SynII knockdown rats also made significantly more preservative responses than aCSF-treated rats which may indicate cognitive inflexibility leading to compulsivity (Chamberlain et al., 2006). Looking at these parameters alone would suggest cognitive deficits rather than motivational deficits leading to 5-CSRTT impairment. However, synapsin II knockdown animals also committed significantly higher percent omissions which indicates a lack of motivation (Amitai and Markou, 2010). The same group also had significantly longer response times to both the stimulus and the reward, which again tends toward a motivational deficit (Amitai and Markou, 2008; Carli and Samanin, 1992; Semenova and Markou, 2007). Results of the 5-CSRTT should be interpreted in combination of each other. Therefore, to determine if lack of motivation played a role in latency response and omissions, the number of head entries and premature responses were considered. All groups performed a similar number of responses and therefore all groups can be deemed to be equally motivated and 5-CSRTT results can be attributed to cognitive deficits resulting from SynII knockdown.

Memory is another cognitive domain that clinically is affected in SZ patients, particularly visual and working memory (Keefe and Fenton, 2007; Nuechterlein et al., 2004; Spieker et al., 2012). Memory assessment using the 8-ARM revealed SynII knockdown animals displayed memory deficits. The behavioural results found here are directly translatable to a virtual 8-ARM paradigm used in humans and our results are comparable to a virtual 8-ARM performance where SZ subjects also demonstrate increased number or errors (Spieker et al., 2012). The well-established role of the hippocampus in memory led to the analysis of SynII levels within hippocampus tissue of schizophrenia patients and revealed a decreased in SynIIa and IIIa levels (Vawter et al., 2002). Our study provides evidence for a link between the pathology behind cognitive impairments, such as working memory, and reduced SynII levels found in SZ patients.

Social withdrawal is a common negative symptom of SZ that often goes untreated by current antipsychotics. The glutamate hypothesis proposes negative symptoms arise from decreased glutamate

in cortical regions of the brain. Glutamate antagonists support this as ketamine and PCP are able to manifest negative symptoms of SZ (Howes et al., 2015; Jones et al., 2011). SynII, specifically the SynIIa isoform, has been established as a primary player in maintaining the RP in glutamatergic neurons (Gitler et al., 2008). Knockdown of SynII has also been shown to decreased glutamate vesicular transporters VGLUT1 and VGLUT2 (Dyck et al., 2011). Therefore, loss of this regulatory protein in glutamatergic neurons likely produced irregularities in their neurotransmission and as a result manifested as social withdrawal behaviourally. Clinical studies have demonstrated that olanzapine holds some efficacy in ameliorating the negative symptoms of SZ, as indicated by the Positive and Negative Syndrome Scale (PANSS) total score, in addition to improvements in cognition when compared with haloperidol treatment (Lindenmayer et al., 2007). This study found olanzapine was able to ameliorate social withdrawal seen in SynII knockdown animals. How olanzapine was able to overcome SynII loss and ameliorate social withdrawal was not specifically investigated in this study. However, SynII has an established link to glutamatergic neurotransmission, and in pathological setting produces reduced activity. Ameliorating social withdrawal in SynII knockdown animals, likely due to reduced glutamatergic activity, may have been possible by olanzapine as this antipsychotic has been shown to increase glutamate release (Sacchi et al., 2017). Pre-clinically, studies have also shown that olanzapine administration attenuates NMDA receptor blocker ketamine-induced social withdrawal (Onaolapo et al., 2017)and decreased social interaction in the PCP model of SZ, thus acting on the glutamatergic system (Rademacher et al., 2002). Future research could elucidate the mechanism between SynII and olanzapine to establish how this occurs regarding SZ pathophysiology.

Hyperactive metabolic function was seen in SynII knockdown animals, revealing SynII as a key regulator of functional activity within the brain. The increased mean standard uptake values of [^{18}F]FDG found across various brain regions support the hyperactivity hypothesis of SZ. Hyperactivity has been suggested in unmedicated SZ populations with predominantly positive symptoms, as well as the MK-801

preclinical animal model of SZ. Therefore, the higher demand for glucose found may indicate increased effort during the working memory task or inefficient glucose utilization within the neurons. Neural inefficiency has been proposed in clinical studies of SZ patients during working memory through blood oxygenation level-dependent (BOLD) signals and functional magnetic resonance imaging (fMRI). Future studies should aim to investigate the effect of first- and second-generation antipsychotic drugs on brain metabolic function in the SynII knockdown animals to see if similarities are found in medicated and unmedicated patients

There are a few limitations to the current study that are important to highlight. First, the sample size was small and, despite this study replicating some previous behavioural work from our lab (Dyck et al., 2011, 2009b) it is still important to acknowledge. Another limitation of this study is the inability to distinguish if increased glucose demand is indicative of an increased effort, inefficient glucose utilization or a combination of the two. This should, therefore, be a focus of future studies.

In conclusion, this study supports the role of SynII in the etiology of SZ through the production of behavioural phenotypes and functional imaging like those displayed by patients with SZ. This study lends a particular focus on the negative and cognitive symptoms of SZ that are currently not sufficiently treated with current antipsychotics. Reduced SynII is able to manifest these SZ-like symptoms and alter brain glucose metabolism therefore, we propose that SynII is a potential therapeutic target for negative and cognitive symptoms of SZ.

Conflict of Interests and Financial Disclosures

Authors report so conflicts of interest. This work was supported by CIHR and NSERC.

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Chapter 5: Discussion and Conclusions

5.0 Discussion and Conclusions

Identifying molecular targets based on underlying disease pathology and pathogenesis is critical for translational research and drug efficacy in clinical trials. Without an understanding of specific mechanisms and pathways implicated in disease progression, target-based therapies cannot be developed, drug attrition rates at the clinical trial phase will be increased, and observed off-target/non-specific effects will be poorly understood (Gilbert, 2013). Drug-targeted drug discovery describes the identification of a molecule (often a protein) in the pathology of a disease and the changing of its functionality in order to restore "healthy" activity. Another approach to drug discovery is the phenotypic drug discovery approach, which proposes drug screening through phenotypic or observable changes in a system such as changes in behaviour. These two approaches should be combined in current treatment efforts and experimental methodologies. Without target-based knowledge, phenotypic changes produced using a compound give no insight into the underlying mechanisms of a disease and therapeutics cannot be effectively modulated or optimized. Without phenotypic-based knowledge, target validation can only provide theoretically based advantages. Drug discovery should involve an in-depth investigation into the functionality of potential targets within a given system in order to better understand how these targets may interact with one another in addition to the system as a whole; one target may not be sufficient (Swinney. David C and Anthony, 2011). Chapters presented in this thesis combine phenotypic and biochemical evidence to support specific pathways and targets proposing their role in disease pathophysiology. This provides a combination approach of phenotypic- and targeted-based drug discovery. A combination approach will lead to more effective treatment design by including molecular justification to overcome the limits of phenotypic based design and showing practically how targets are able to affect a system and change behaviour overcoming the limits of target-based design. The overall goal of this combination approach is to increase translatability in clinical settings.

Many unmet needs in various disease states are caused by unknown pathologies that drive observed symptoms, therefore, treatment needs align with the need for understanding molecular pathways to provide target-based efforts with phenotypic analysis. In PD, for example, there are no current neuroprotective therapeutics available, largely due to the lack of understanding around the etiology of its dopaminergic degeneration. Without a known cause, target-based strategies are difficult to develop, and phenotypic-based design strategies have not produced effective neuroprotective treatments. There are two hypotheses proposed to induce dopaminergic neuronal cell death: endoplasmic reticulum (ER) stress and oxidative stress. These two compensatory pathways are interconnected. Therefore, a more likely hypothesis is that the degeneration found in PD is due to a multi-hit phenomenon produced by a positive feedback-like system, where oxidative stress and ER stress are simultaneously leading to dopaminergic cell death.

This thesis used different techniques to investigate novel molecular targets. One focus used a knockdown approach to emphasize the role and function of a neurotrophic factor (MANF) by evaluating the biochemical and phenotypic changes that arise in its reduced presence. The study used a novel *in vivo* knockdown approach to reduce MANF that was specifically designed and implemented for this study. Another approach demonstrated the potential of CDK5/p25 hyperactivation in PD pathophysiology by identifying its existence in a well-established model of PD. Further, specific CDK5/p25 inhibition using a novel peptide, TP5, exemplified CDK5/p25 hyperactivity in the degenerative process as it provided protective qualities. Using the different methods, the two sides of the multi-hit hypothesis were able to be evaluated.

Investigation of both sides of the multi-hit hypothesis were completed using target-based and phenotypic-based approaches. This combined approach allowed for the validation of a specific molecular target in disease pathology that could phenotypically demonstrate disease modification. The two pathways of the multi-hit hypothesis, ER stress and oxidative stress, have been shown to reinforce one

another and are interconnected (refer to figure 4 in chapter 1). Therefore, initiation of one will lead to the other (Malhotra and Kaufman, 2007). Studies completed in this thesis modified one half of the equation at a time and assessed resulting apoptosis, thus demonstrating each can individually be implicated in PD neurodegeneration.

Manipulation of either oxidative stress or ER stress pathways was able to produce dopaminergic degeneration. In essence, this supports the multi-hit hypothesis and suggests that interventions should be aimed at reducing both of these stress processes to provide neuroprotection. MANF was investigated in relation to the ER stress half of the multi-hit hypothesis. Neuroprotective properties of the dopaminergic neurotrophic factor MANF have been proposed, and MANF has demonstrated efficacy as a neuroprotective therapy in the 6-OHDA and MPTP models of PD (Huang et al., 2016; Liu et al., 2018; Zhang et al., 2017). These studies have provided phenotypic-based evidence in support of neuroprotective properties; however, MANF specifically had not been investigated in the pathophysiology of PD. If MANF can provide neuroprotection, it stands to reason that it likely plays a vital role in maintaining physiological dopaminergic function. To the author's knowledge, the study included in chapter 2 is the first to investigate the specific target-based validity of MANF in neuroprotection. This was achieved by demonstrating that reduced MANF levels produce a chronic UPR state that favours CHOP-induced apoptotic processes and reduced tyrosine hydroxylase in the substantia nigra. Motor activity impairment and motor coordination deficits found were therefore attributed to the apoptosis in the substantia nigra. Apoptosis and motor deficits together resulting from reduced MANF highly implicate the neurotrophic factor in mechanisms that protect neurons from excessive ER stress. This adds target validation to current phenotypic-based research. Evidence for MANF in the other half of the multi-hit hypothesis has been demonstrated and suggests MANF can influence both neurodegenerative processes. Exogenous MANF was found to overcome oxidative stress induced by 6-OHDA, possibly through the AMPK/mTOR pathway (Zhang et al., 2017). MANF also inhibited oxidative stress in the MPTP model of PD, another oxidative

stress inducing model (Liu et al., 2018). Future studies should investigate if MANF reductions also lead to oxidative stress to complement this multi-hit hypothesis justification.

On the oxidative stress side of the proposed multi-hit hypothesis, the commonly used 6-OHDA model of PD was used to show target validity for CDK5/p25 hyperactivation. 6-OHDA is known to produce neurodegeneration through oxidative stress (Konnova and Swanberg, 2018; Smith and Cass, 2007). Increased CDK5/p25 was found in the substantia nigra of 6-OHDA treated animals, implicating its role in neuronal degeneration. CDK5/p25 hyperactivation in the 6-OHDA model provides target-based evidence for therapeutics to mitigate CDK5/p25 hyperactivation in neurodegeneration. This was then established through the specific inhibition of CDK5/p25 using TP5. TP5 administration prior to 6-OHDA was able to overcome the oxidative stress-related degeneration induced via 6-OHDA. TP5 administration specifically maintained tyrosine hydroxylase populations within the substantia nigra and protected animals from developing motor impairments. Phenotypic-based evidence can be extrapolated from the behavioural results and target based evidence from protected dopaminergic neuronal populations. TP5 is still in the preliminary testing phase and proof of concept experiments are still being completed.

Research into the further optimization of TP5 is being conducted, and preliminary findings are presented at the end of chapter 3. Briefly, a short fragment of TP5, termed Peptide A, showed similar CDK5/p25 inhibition and some preliminary behavioural benefits including reduced haloperidol induced catalepsy. Future studies using larger sample sizes and a more comprehensive assessment of dopaminergic specific neuroprotection will need to be conducted in order to effectively propose Peptide A as a potential therapeutic. The exploratory results of Peptide A thus far, add support for the therapeutic potential of CDK5/p25 inhibition as a neuroprotective target in PD. In relation to the multi-hit hypothesis being proposed, CDK5/p25 should be further evaluated in the ER stress pathway. Some evidence for CDK/p25 involvement in this pathway has been provided by Saito et al. showing ER stress causes calcium-dependent cleavage of p35 into p25 and induced CDK5/p25 hyperactivation (Saito et al., 2007). Other

evidence by Jiao et al. shows CDK5 is responsible for translocating XBP1 into the nucleus and therefore can regulate pathways in the UPR and ER stress(Jiao et al., 2017). In order to explicitly demonstrate a link between CDK5/p25 and ER stress however, further investigation is required.

Together, chapters 2 and 3 of this thesis indicate MANF as an ER stress-related molecular target for neuroprotective therapy development and CDK5/p25 inhibition as an oxidative stress-related target regarding neuroprotection. Together, manipulation of individual sides of the multi-hit hypothesis leads to neurodegeneration, and degeneration can be overcome via the aforementioned molecular targets. Neuroprotection in PD continuously remains out of reach; therefore, both processes should be considered in neurodegeneration of PD as each was separately capable of causing neurodegeneration. This approach will hopefully improved therapeutic translation into a clinical settings and help identify neuroprotective treatments.

The miscellaneous chapter 4 included in this thesis evidences the importance of target- and phenotypic-based drug design for another CNS disorder: schizophrenia (SZ). In SZ, negative and cognitive symptoms continuously go unmedicated because of the unknown etiology, and therefore a better understanding of how these symptoms arise can help drug discovery efforts to sufficiently overcome them. There is more opportunity to successfully mitigate negative and cognitive symptoms if treatment efforts incorporate both target-based and phenotypic evidence. Chapter 4 uses a target-based approach to support synapsin II as a molecular target for SZ. Face validity, another term for the presentation of phenotypic disease-like behaviours, was repeatedly found in synapsin II knockdown animals for negative and cognitive symptoms. More specifically, reduced synapsin II displayed all symptoms domains reminiscent of SZ. Predictive validity, the translatability of a model to a clinical setting, was established through olanzapine's ability to reverse previously identified social interaction deficits. Together, these demonstrate phenotypic evidence with a target-based approach to support synapsin II as a target for improved negative and cognitive symptom treatment. Functional brain imaging supported construct

validity, mechanistic/biochemical similarities to a disease. In the synapsin II knockdown animals, hyperactivation was depicted via increased glucose utilization as shown through PET imaging in the brains of synapsin II knockdown animals. Increased activation is translatable to clinically unmedicated SZ patients where PET imaging also found hyperactivation within the frontal cortex (Shinto et al., 2014). Synapsin II reduction used in this study is further translated to the molecular underpinnings of SZ through the identification of reduced levels of this phosphoprotein in the dorsolateral prefrontal cortex of SZ patients (Tan et al., 2014). Together, studies provide justification for synapsin II as a molecular target for alleviating the negative and cognitive symptoms of SZ. Future studies need to further investigate how pharmacological interventions can increase synapsin II levels in an attempt to mitigate negative and cognitive symptoms.

In conclusion, both ER stress and oxidative stress can lead to dopaminergic degeneration; therefore, potential neuroprotective therapies for PD should consider the involvement of both oxidative stress and ER stress pathways to improve efficacy in clinical settings. This thesis specifically identified reduced MANF in chronic UPR-induced apoptosis and resulting behavioural changes. CDK5/p25 hyperactivation was also identified in the 6-OHDA model of PD and inhibition of this complex was able to provide neuroprotective properties. Both are proposed neuroprotective targets based on their demonstrated involvement in preclinical modelling of PD pathophysiology. Preclinical modelling also supported the role of synapsin II in the pathophysiology of SZ and is therefore a molecular target identified for another CNS disorder. These work hopes to aid in the development of neuroprotective therapies for PD through the elucidation of the underlying pathophysiology of neurodegeneration and the development of therapies to overcome negative and cognitive symptom treatment hardships currently faced in SZ treatment.

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