INVESTIGATING THE ROLE OF MANF & CDNF IN THE PATHOPHYSIOLOGY OF PARKINSON'S DISEASE

INVESTIGATING THE ROLE OF CEREBRAL DOPAMINE NEUROTROPHIC FACTOR (CDNF) & MESENCEPHALIC ASTROCYTE-DERIVED NEUROTROPHIC FACTOR (MANF) IN THE PATHOPHYSIOLOGY OF PARKINSON'S DISEASE

By

Omar Shawaf, H.B.Sc

001140678

Principle Investigator: Dr. Ram K. Mishra

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TITLE: Investigating the Role of Cerebral Dopamine Neurotrophic Factor (CDNF) & Mesencephalic Astrocyte-Derived Neurotrophic Factor (MANF) in the Pathophysiology of Parkinson's Disease

AUTHOR: Omar Shawaf, H.B.Sc., McMaster University

SUPERVISOR: Dr. Ram K. Mishra

COMMITTEE: Dr. Bhagwati Gupta

Dr. Benicio Frey

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LAY ABSTRACT

CDNF and MANF are members of a recently discovered and evolutionarily conserved neurotrophic factor family implicated in supporting the survival and protection of midbrain dopaminergic neurons in the nigrostriatal pathway, which degenerate in Parkinson's Disease (PD). Increasing evidence demonstrated that MANF overexpression resulted in significant protection and repair of DA neurons in the substantia nigra (SN). Current literature suggests that CDNF and MANF are involved in regulating ER stress and are upregulated in cells and in rodents during the unfolded protein response (UPR). Thus, this study sought to investigate whether selective knockdown (K/D) of MANF and CDNF causes the underlying changes in the brain that lead to the behavioural manifestation of PD in preclinical models. 2 μL at 0.5 μL/minute of MANF, CDNF, MANF and CDNF combined, or a scrambled negative control (N=44) of rat lentiviral-mediated shRNA formulations were infused into the SN. Rats were tested on a battery of behavioural tests for the assessment of PD phenotypes, such as impairments in balance, gait and motor coordination. MANF K/D rats demonstrated PD phenotypes in the rearing duration, beam traversal, rotarod and cylinder test (P <0.05). These results were largely mirrored in the combined MANF and CDNF K/D group, however, CDNF K/D rats failed to demonstrate consistent motor deficits (P >0.05). Additionally, CDNF mRNA expression from the platelets of PD patients revealed no significant differences compared to healthy controls (P >0.05). In conclusion, the etiology of PD remains to be elucidated, and this is the first study to demonstrate that MANF K/D rats recapitulate key motor features of parkinsonism.

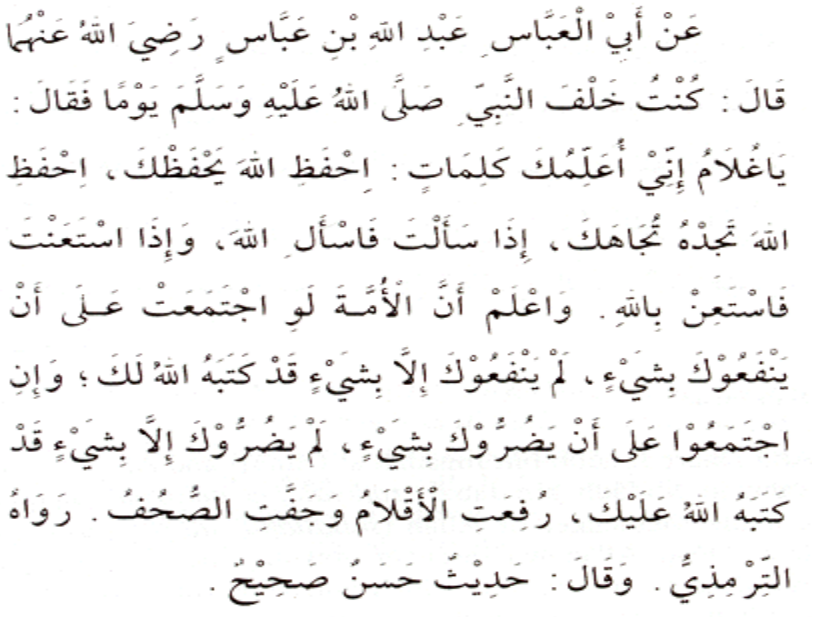
ABSTRACT

CDNF and MANF are members of a recently discovered and evolutionarily conserved neurotrophic factor family implicated in supporting the survival and protection of midbrain dopaminergic neurons in the nigrostriatal pathway, which degenerate in Parkinson's Disease (PD). Increasing evidence demonstrated that MANF overexpression resulted in significant protection and repair of TH+ cells and DA neurons in the substantia nigra (SN). In addition, continuous infusion of CDNF demonstrated greater protection of TH-positive neurons in the SNc and fibers in striatum than GDNF in the 6-OHDA neurotoxin model. Current literature suggests that CDNF and MANF are involved in regulating ER stress and are upregulated *in vitro* and *in vivo* during the unfolded protein response (UPR). Thus, this study sought to investigate whether selective knockdown (K/D) of MANF and CDNF causes pathophysiological conditions that lead to the behavioural manifestation of PD in preclinical models. Male Sprague-Dawley rats underwent stereotaxic surgery, whereby 2 µL at 0.5 µL/minute of MANF, CDNF, MANF and CDNF combined, or a scrambled negative control (N=44) of rat lentiviral-mediated shRNA formulations were infused into the SN in reference to bregma: Anterior/Posterior=-5.3 mm, Medial/Lateral=±2.3 mm, Dorsal/Ventral=-7.8 mm. Rats were tested on a battery of behavioural tests for the assessment of PD phenotypes, such as impairments in balance, gait and motor coordination. MANF K/D rats demonstrated PD phenotypes in the rearing duration, beam traversal, rotarod and cylinder test (P <0.05). These results were largely mirrored in the combined MANF and CDNF K/D group, however, CDNF K/D rats failed to demonstrate consistent motor deficits (P >0.05).

Additionally, CDNF mRNA expression from the platelets of PD patients revealed no significant differences compared to healthy controls (P >0.05). In conclusion, the etiology of PD remains to be elucidated, and this is the first study to demonstrate that MANF K/D rats recapitulate key motor features of parkinsonism.

Ibn Abbas reported: I was riding with the Messenger of God (Peace & Blessings Be Upon Him), and he (Peace & Blessings Be Upon Him) said:

"O young man, I will teach you some words. Be mindful of God and He will protect you. Be mindful of God and you will find Him before you. If you ask, ask from God. If you seek help, seek help from God. Know that if the nations gathered together to benefit you, they cannot benefit you unless God has written it for you, and if the nations gathered together to harm you, they cannot harm you unless God has written it for you. The pens have been lifted and the pages dried."



عَنْ أَبِيْ الْعَبَّاسِ عَبْدِ اللهِ بْنِ عَبَّاسٍ رَضِيَ اللهُ عَنْهُمَا
قَالَ : كُنْتُ خَلْفَ النَّبِيِّ صَلَّى اللهُ عَلَيْهِ وَسَلَّمَ يَوْمًا فَقَالَ :
يَاغُلَامُ إِنِّيْ أُعَلِّمُكَ كَلِمَاتٍ : اِحْفَظِ اللهَ يَحْفَظْكَ ، اِحْفَظِ
اللهَ تَجِدْهُ تُجَاهَكَ ، إِذَا سَأَلْتَ فَاسْأَلِ اللهَ ، وَإِذَا اسْتَعَنْتَ
فَاسْتَعِنْ بِاللهِ . وَاعْلَمْ أَنَّ الْأُمَّةَ لَوِ اجْتَمَعَتْ عَلَى أَنْ
يَنْفَعُوْكَ بِشَيْءٍ ، لَمْ يَنْفَعُوْكَ إِلَّا بِشَيْءٍ قَدْ كَتَبَهُ اللهُ لَكَ ؛ وَإِنِ
اجْتَمَعُوْا عَلَى أَنْ يَضُرُّوْكَ بِشَيْءٍ ، لَمْ يَضُرُّوْكَ إِلَّا بِشَيْءٍ قَدْ
كَتَبَهُ اللهُ عَلَيْكَ ، رُفِعَتِ الْأَقْلَامُ وَجَفَّتِ الصُّحُفُ . رَوَاهُ
التِّرْمِذِيُّ . وَقَالَ : حَدِيْثٌ حَسَنٌ صَحِيْحٌ .

Source: Sunan At-Tirmidhi 2516 (Sahih)

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A work of this size could only be accomplished through the blessings of God and the team that surrounded me throughout my journey. First and foremost, I would like to thank Dr. Ram Mishra for believing in my abilities and encouraging my progress throughout the research process. His wealth of knowledge has proven invaluable. The opportunity to work in his laboratory has not only enriched my graduate experience, but also afforded me the pleasure of working alongside dedicated undergraduate and graduate students. Secondly, I would like to thank Ashley Bernardo for her continuous support throughout this endeavor. I would also like to thank my committee members Dr. Benicio Frey and Dr. Bhagwati Gupta, as well as my committee chair Dr. Alexander K. Ball. Moreover, I would like to thank all the individuals who supported me and provided me with their expertise, time and company throughout this journey: Shreya Prashar, Hetshree Joshi, Sharon Thomson, Ritesh Daya, Jayant Bhandari, and Roohie Sharma.

I dedicate this thesis to my parents, Basil Shawaf and Raja Taifour, my sisters, Noor, Fatemah, Lamees, Sarah and Habiba Shawaf, and my brother, Omar Hashem.

I pray that the knowledge, skills, and relationships I gained in this process allow me to benefit humanity. American novelist Ernest Hemingway stated, “How little we know of what there is to know.” May the bounty of knowledge in this world and how little we have mastered always push us to remain curious and humble.

This work has been graciously funded by CIHR and NSERC.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

CDNF	Cerebral Dopamine Neurotrophic Factor
MANF	Mesencephalic Astrocyte-Derived Neurotrophic Factor
PD	Parkinson's Disease
K/D	Knockdown
SN	Substantia Nigra
STR	Striatum
RPM	Rotations Per Minute
NINDS	National Institute of Neurological Disorders and Stroke
DA	Dopamine
SNc	Substantia Nigra Pars Compacta
LBs	Lewy Bodies
MDS-UPDRS	Movement Disorder Society-Unified Parkinson Disease Rating Scale
Non-Motor Experiences of Daily Living	NM-EDL
Motor Experiences of Daily Living	M-EDL
Motor Examination	MEx
Motor Complications	MCompl
STN	Subthalamic Nucleus
GPi	Globus Pallidus Internus
GPe	Globus Pallidus Externus
SNr	Substantia Nigra Pars Reticulata
GABA	γ-Aminobutyric acid
NT	Neurotransmitter
PMC	Primary Motor Cortex
cAMP	Cyclic Adenosine Monophosphate
BBB	Blood–Brain Barrier
AAAD	Aromatic l-Amino Acid Decarboxylase
CNS	Central Nervous System
COMT	Catechol-O-Methyltransferase
MAO-B	Monoamine Oxidase Type B
6-OHDA	6-hydroxydopamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
VTA	Ventral Tegmental Area
NHP	Non-Human Primate
PPI	Prepulse Inhibition
PQ	Paraquat (N,N$^{'}$-dimethyl- 4-4-4$^{'}$-bypiridinium)
TH	Tyrosine Hydroxylase

UPS	Ubiquitin-Proteasome System
ALP	Autophagy-Lysosomal Pathway
Hip	Hsp70-Interacting Protein
ST13	Suppression of Tumorigenicity 13
Hsp70	Heat-Shock Protein 70
NTF	Neurotrophic Factor
NGF	Nerve Growth Factor
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
BDNF	Brain-Derived Neurotrophic Factor
RTK	Receptor Tyrosine Kinase
KO	Knockout
CNTF	Ciliary Neurotrophic Factor
CT-1	Cardiotrophin-1
LIF	Leukemia Inhibitory Factor
NPN	Neuropoietin
OSM	Oncostatin M
CLC	Cardiotrophin-Like Cytokine
IL-6	interleukin 6
GFL	GDNF Family Ligand
NRTN	Neurturin
ARTN	Artemin
PSPN	Persephin
TGF-b	Transforming Growth Factor-b
GPI	Glycolipid Glycosylphosphatidyl Inositol
GFRα	GDNF Family Receptor α
VEGF-A	Vascular Endothelial Growth Factor A
GDNF	Glial Cell line-Derived Neurotrophic Factor
ER	Endoplasmic Reticulum
UPR	Unfolded Protein Response
ARP	Arginine-Rich Protein
ERAD	ER-Associated Protein Degradation
UP	Unfolded Protein
IkB	Inhibitory Nuclear kappa B
PP1	Protein Phosphatase 1
PPP1R15A	Protein Phosphatase 1 Regulatory Subunit 15A
IRE1	Inositol-Requiring Enzyme 1
XBP1	X-box Binding Protein 1
CHOP	C/EBP Homologous Protein
AAV	Adeno-Associated Viruses (AAV)
RNAi	RNA interference
siRNA	short interfering RNA

shRNA	short hairpin RNAs
RISC	RNA-Induced Silencing Complex
qPCR	Real Time quantitative Polymerase Chain Reaction
CAF	Central Animal Facility
CCAC	Canadian Council on Animal Care
GFP	Green Fluorescent Protein
FSRR	Fixed Speed Rotarod
SEM	Standard Error of the Mean
SD	Standard Deviation
PERK	Pancreatic ER kinase-like ER kinase
ATF6	Activating Transcription Factor 6
eIF2	Eukaryotic Initiation Factor 2
TH-ir	TH immunoreactive
NMR	Nuclear Magnetic Resonance
Bax	Bcl-2-associated X

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Omar Shawaf, organized the experimental set-up, and performed all the animal handling, stereotaxic surgeries, behavioural testing habituation sessions, behavioural testing, behavioural testing analyses, and the qPCR.

I would like to specifically recognize Ashley Bernardo for her role in assisting in the stereotaxic surgeries, lentiviral-mediated shRNA infusions and behavioural tests. Additionally, I would like to acknowledge Raphaela Amarioarei and Isha Brown for their assistance in the footprint analysis, Shreya Prashar for optimizing the primer used for the qPCR, Hetshree Joshi and Michael Cruz for their assistance with the blood collection and running of the qPCR, and finally Joella Hoe, Sharon Thomson, Dima Malkawi, Brett McIntyre, Alicia Hanman, Helen Huynh and Safa Zargari for their support in running behavioural tests.

CHAPTER 1: INTRODUCTION

1.1 Parkinson's Disease

1.1.1 Prevalence, Incidence & Economic Burden

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting 1% of the global population over the age of 60 with its prevalence increasing with age (Connolly & Lang, 2014; Blesa & Przedborski, 2014). An estimated 55,000 Canadians aged 18 and older reported that they had been diagnosed with Parkinson's disease (Wong, Gilmour, & Ramage-Morin, 2014). A systematic review of 25 studies conducted by Hirsch et al. (2016) reported PD incidence rates of 17 per 100,000 person-years. Idiopathic as opposed to familial PD accounts for 95% of cases and is characterized by a slow disease progression that may result from a combination of environmental toxin exposure, aging, and genetic factors (Dehay & Bezard, 2011). Interestingly, between the ages of 60 to 79, males had significantly higher incidence rates of PD per 100,000 person-years than females (Hirsch, Jette, Frolkis, Steeves, & Pringsheim, 2016). Total healthcare costs associated with PD are expected to surpass $4 billion in Canada by the year 2031 (Finès, 2015). Approximately two-thirds of Canadians with PD reported annual out-of-pocket medical expenses that would not be reimbursed by insurance or government programs (Wong et al., 2014).

1.1.2 Symptomology & Pathophysiology

PD is a progressive motor disorder characterized by resting tremor, rigidity, akinesia or bradykinesia and postural instability (Brakedal, Tysnes, Skeie, Larsen, & Müller, 2014). In addition to motor impairments, non-motor symptoms such as cognitive

declines, autonomic perturbations, and psychiatric comorbidities contribute to the burden of illness of patients with PD (Hou & Lai, 2007).

A hallmark feature of PD is the degeneration of the dopamine (DA) neurons in the substantia nigra pars compacta (SNc) and the consequent deficit in striatal DA (Blesa & Przedborski, 2014). However, although heavily researched, the pathogenesis and etiology of PD remain elusive, and advancements in treatments are scarce (Blesa & Przedborski, 2014).

Increased oxidative stress and free radical production have also been suggested as contributing factors for the pathogenic mechanisms of PD. Currently, the most common hypothesis suggests that nigral neuronal death in PD is due to excessive oxidative stress resulting from auto-oxidation of catecholamines (Kim, Kim, Rhie, & Yoon, 2015). Additionally, a key feature of PD is the formation of Lewy bodies (LBs), which are primarily composed of α-synuclein aggregates (Bezard, Yue, Kirik, & Spillantini, 2013). LB further contribute to oxidative stress within the neuron.

1.1.3 Diagnosis & Classification

Although no definitive test for diagnosis exists, PD is diagnosed based on clinical criteria (Jankovic, 2008). These criteria are based on the presence of a combination of cardinal motor features, associated and exclusionary symptoms, and a patient's response to treatment, levodopa (Rao, Fisch, Srinivasan, & D'Amico, 2003). The National Institute of Neurological Disorders and Stroke (NINDS) diagnostic criteria for PD are summarized (Table 1)(Jankovic, 2008).

Table 1. National Institute of Neurological Disorders and Stroke (NINDS) diagnostic criteria for PD (Jankovic, 2008).

Group A Features (characteristic of PD)
- Resting tremor - Bradykinesia - Rigidity - Asymmetric onset
Group B features (suggestive of alternative diagnoses)
- Features unusual early in the clinical course - Prominent postural instability in the first 3 years after symptom onset - Freezing phenomenon in the first 3 years - Hallucinations unrelated to medications in the first 3 years - Dementia preceding motor symptoms or in the first year - Supranuclear gaze palsy (other than restriction of upward gaze) or slowing of vertical saccades - Severe, symptomatic dysautonomia unrelated to medications - Documentation of condition known to produce parkinsonism and plausibly connected to the patient's symptoms (such as suitably located focal brain lesions or neuroleptic use within the past 6 months)
Criteria for Definite PD
- All criteria for probable Parkinson's are met and - Histopathological confirmation of the diagnosis is obtained at autopsy
Criteria for Probable PD
- At least three of the four features in group A are present and - None of the features in group B is present (note: symptom duration ≥ 3 years is necessary to meet this requirement) and - Substantial and sustained response to levodopa or a dopamine agonist has been documented

The average age of individuals who reported symptoms was 64.4 years, with a diagnosis of PD 1.9 years later at 66.2. However, the period between symptom onset and diagnosis increased for younger individuals to an average of 7 years (Wong et al., 2014).

Progression of PD is followed using motor rating scales to standardize measurements. The Movement Disorder Society-Unified Parkinson Disease Rating Scale (MDS-UPDRS) is recognized as the most valid and reliable rating scales when following PD progression and evaluating medical interventions, and is the most widely used

(Brakedal et al., 2014). The MDS-UPDRS is composed of four sections: Part I: Non-Motor Experiences of Daily Living (NM-EDL), which includes items for clinician reporting and patient self-assessment; Part II: Motor Experiences of Daily Living (M-EDL); Part III: Motor Examination (MEx); and Part IV: Motor Complications (MCompl). Each item is scored on a scale from 0 (normal) to 4 (severe), whereby item scores are added to provide a total score for each part (Martinez-Martin et al., 2013).

1.1.4 Nigrostriatal Pathway

As mentioned previously, PD is characterized by marked loss of DA within the nigrostriatal system (Lewitt, 2012; More et al., 2013). The clinical features of PD only manifest after the death of 60-70% of DA cell bodies in the substantia nigra (SN) (Cheng, Ulane, & Burke, 2010). The nigrostriatal pathway is part of the basal ganglia motor loop, a DAergic pathway that originates in the SN sending axonal projections to the striatum. The basal ganglia are responsible for generating movement and the processing of sensory-motor information, whereby perturbations in this system result in the motor impairments associated with PD (Kandel, Schwartz, Jessell, Siegelbaum, & Hudspeth, 2014).

The basal ganglia are composed of four principle nuclei: the striatum (consisting of the caudate nucleus and the putamen), the globus pallidus, the subthalamic nucleus (STN) and the SN. The globus pallidus comprises the globus pallidus internus (GPi) and globus pallidus externus (GPe). The SN is similarly divided into two components: the pars compacta (SNc) and pars reticulata (SNr) (Figure 1)(Golan, Tashjan, Armstrong, & Armstrong, 2012).

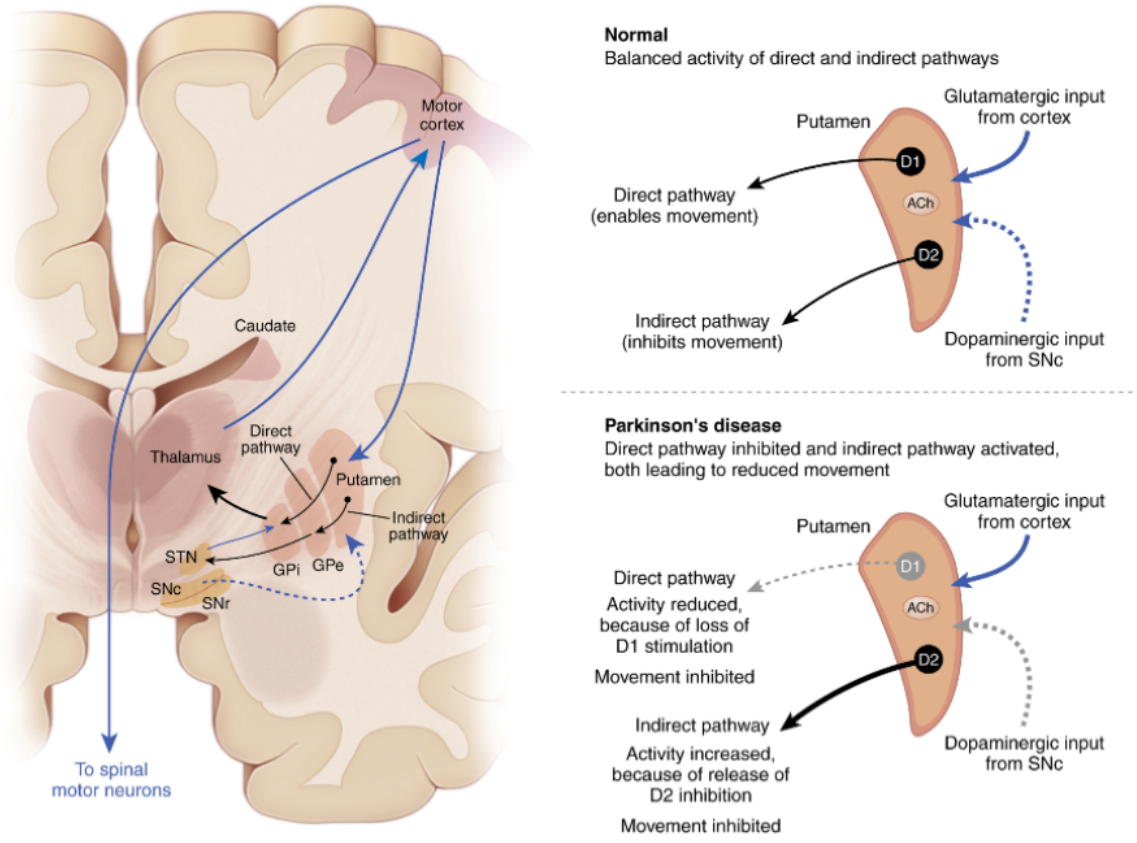

Figure 1. Perturbations to the nigrostriatal pathway in patients with PD (Golan et al., 2012).

In a healthy brain at rest, the GPi and SNr inhibit the thalamus through the release of γ-Aminobutyric acid (GABA), an inhibitory neurotransmitter (NT). The thalamus is unable to excite the primary motor cortex (PMC) due to the inhibition from the GPi and SNr and as a result no movement occurs (Visanji, 2014). The direct pathway of the basal ganglia leads to the disinhibition of the thalamus and stimulates muscle movement signalling through D1 receptors and an increase in cyclic adenosine monophosphate (cAMP) (Figure 1). During voluntary movement, the PMC sends an excitatory signal via the NT glutamate, to the putamen. Simultaneously, the putamen is also excited by DA NT

release from the SNc, which was activated by glutamatergic input from the PMC. The activation of the putamen raises its inhibitory GABAergic signal to the GPi and the SNr (Jung, Leem, & Kim, 2014). Inhibition of the GPi and SNr reduces their synaptic output, and as a result the thalamus is no longer inhibited, which allows it to send an excitatory glutamatergic signal to the PMC to stimulate a muscle movement (Kandel et al., 2014).

The indirect pathway works alongside the direct pathway to inhibit muscle movement that would otherwise conflict with the desired movement. At rest, the GPe inhibits the STN through the release of the NT GABA. This inhibition of the STN prevents its excitation of the GPi (Kandel et al., 2014).

The SNc modulates the direct and indirect pathways by regulating the amount of DA released. Striatal neurons of the direct pathway have DA D1 receptors to facilitate neurotransmission, whereas DA D2 receptors in the putamen inhibit the indirect pathway, the net result of both is cortical activation (Jung et al., 2014; Visanji, 2014).

In PD, the SNc is the site of selective degeneration of DAergic neurons. The loss of DA inputs from the SNc to the striatum leads to the depletion of DA in the striatum (Sullivan & Toulouse, 2011). The loss of striatal DA in the direct pathway causes decreased stimulation of D1 receptors in the putamen. The reduced excitation in the putamen reduces its inhibition to the GPi. In the indirect pathway, the reduced DA levels decrease inhibition of the putamen via the D2 receptors. Subsequently, the putamen is increased in its inhibitory output to the GPe, which results in a reduced inhibition to the STN. This leads to an increased excitatory output from the STN to the GPi (Kandel et al.,

2014). The cumulative effects of the increased activation of the GPi from the indirect pathway and the reduced inhibition of the GPi from the direct pathway causes a marked increase in the output of the GPi. Consequently, there is a substantial increase in the GPi's inhibitory output to the thalamus. This amplified inhibition decreases the corresponding excitatory output of the thalamus to the PMC, which then results in the decreased or uncontrolled motor activity seen in PD (Figure 1)(Golan et al., 2012; Kandel et al., 2014).

1.1.5 Levodopa: Gold Standard of Treatment

The motor impairments observed in PD arise from selective loss of pigmented midbrain neurons in the SNc that project to the putamen and caudate, releasing dopamine (Lewitt, 2012). This dopaminergic neurotransmission begins in the striatum and signals, in sequence, through the globus pallidus, the subthalamic nucleus, and the thalamus en route to the motor cortex (DeLong & Wichmann, 2009).

Key motor PD attributes manifest when more than half of the dopaminergic nerve terminals in the striatum have undergone degeneration (Figure 2) (Lewitt, 2012). Ground-breaking research by neuroscientists Arvid Carlsson and Oleh Hornykiewicz in the 1950s revealed that motor impairments in PD could be reversed temporarily by pharmacological interventions that restored striatal dopaminergic neurotransmission (Hornykiewicz, 2002). This reversal was accomplished through the oral administration of the agent, levodopa or L-DOPA, which is a precursor to the neurotransmitter dopamine and can directly stimulate postsynaptic striatal DA receptors (Lewitt, 2012).

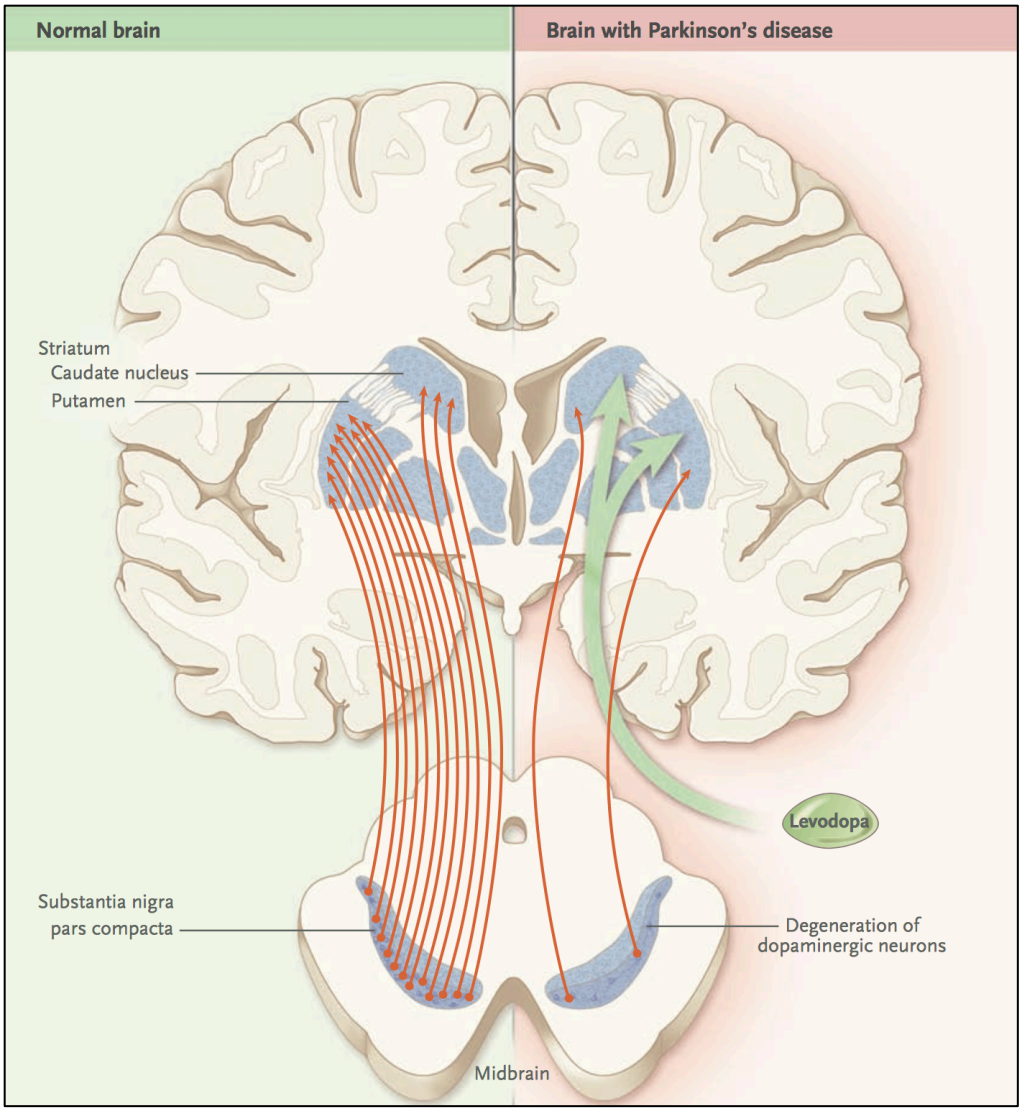

Figure 2. Loss of dopamine-synthesizing neurons in the midbrain seen in patients with PD.
A schematic comparison of coronal brain slices from a control subject (left) and a patient with PD (right) illustrates the major neurodegenerative loss of dopamine-synthesizing neurons in the SNc, projecting to striatum. Levodopa crosses the BBB and enhances striatal dopaminergic neurotransmission (Lewitt, 2012).

Levodopa (3,4-dihydroxy-l-phenylalanine) is a naturally occurring amino acid and an intermediate in the DA synthesis pathway (Figure 3). DA has a higher molecular weight than levodopa and as a result, cannot cross the blood–brain barrier (BBB),

rendering DA administration futile. Due to the metabolism and distribution of levodopa throughout the body, a small percentage of the drug reaches the brain after active transport across the BBB (John G. Nutt, 2008). Once in the brain, DA is rapidly formed from levodopa by aromatic l-amino acid decarboxylase (AAAD). Interestingly, drug co-administration can improve the efficacy of levodopa (Rascol, 2013). In order to limit the systemic side effects of levodopa conversion to DA outside the central nervous system (CNS), peripherally acting AAAD inhibitors, such as carbidopa or benserazide, are often combined with levodopa (Rascol, 2013; Lewitt, 2012). As orally administered levodopa has a clearance half-life of 1 to 3 hours, this can be extended by the inhibition of peripheral catechol-O-methyltransferase (COMT) with COMT inhibitors, such as entacapone or tolcapone (Nutt, 2000). Additionally, dopaminergic neurotransmission can be further improved with monoamine oxidase type B (MAO-B) inhibitors, selegiline or rasagiline, which impede DA metabolism from levodopa in the CNS (Lew, 2005; Pistacchi, Martinello, Gioulis, & Zambito Marsala, 2014).

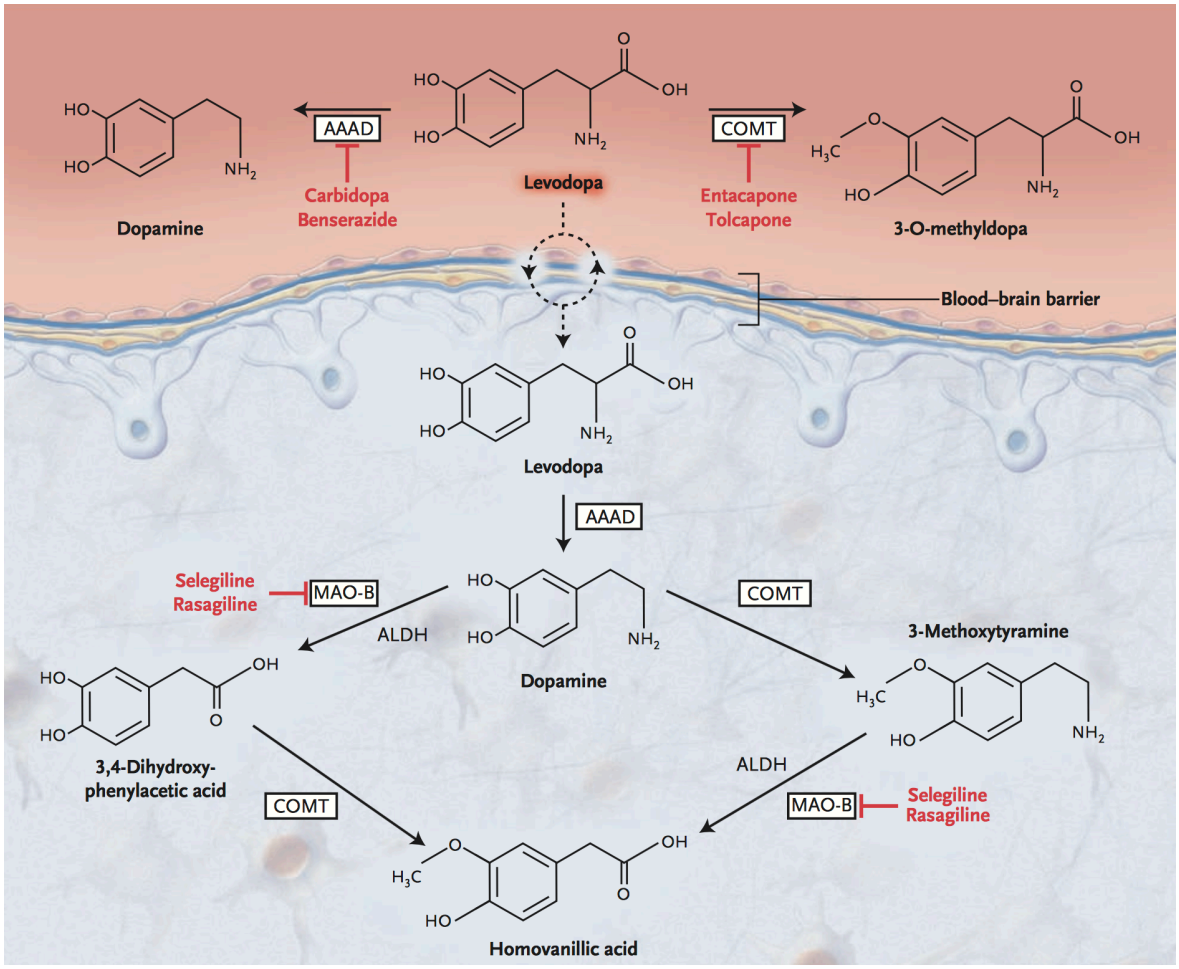

Figure 3. Pathways of levodopa and DA metabolism. A schematic illustrating the sites of action by Inhibitory drugs commonly used with levodopa. AAAD: aromatic l-amino acid decarboxylase; ALDH: aldehyde dehydrogenase; COMT: catechol-O-methyltransferase; MAO-B: monoamine oxidase type B (Lewitt, 2012).

Nevertheless, dyskinesia or abnormal involuntary movements and loss of motor control develop after long-term use or high-dose treatment with levodopa (Gray et al., 2014; Oertel & Quinn, 1997; Parkinson & Group, 2004). These motor complications are reported less often with the use of dopamine agonists and monoamine oxidase type B inhibitors (MAOBI) (Gray et al., 2014). Motor complications are seen less frequently with dopamine agonists and MAOBI than with levodopa, which advocates the use of an alternative long-term treatment regimen (Gray et al., 2014). However, non-motor

symptoms, which includes edema, nausea, hallucinations, and sleep disturbance occur more often with dopamine agonists than with levodopa (Ives, 2004; Stowe et al., 2008), and may potentially contribute to a greater burden of illness on the patient and caretaker than motor symptoms. The parameters evaluated in the UKPDRG study included safety and tolerability, which revealed higher mortality rates with the MAOBI, selegiline, than with levodopa alone (Gray et al., 2014), in contrast to prior studies (Ives, 2004). Interestingly, the DATATOP study reported that selegiline slowed functional decline in patients with PD (The Parkinson Study Group, 1989). Additionally, there are higher costs associated with dopamine agonists and MAOBIs than with levodopa (Gray et al., 2014). Taken together, the motor and non-motor side effects, safety and tolerability, and costs associated with the current therapeutic drugs for PD warrant the investigation of novel treatments that minimize motor side effects and demonstrate enhanced efficacy.

1.2 Animal Models of PD

1.2.1 Neurotoxin Models

The two most widely used neurotoxin agents used to model PD are 6-hydroxydopamine (6-OHDA) in rats and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice and monkeys (Blesa & Przedborski, 2014). Although the neurotoxic models recapitulate the hallmark nigrostriatal pathway degeneration, these models hold characteristics that differ drastically from clinical manifestations of PD. These differences include: the rapid degeneration of dopaminergic neurons in rodents compared to humans; lesions that are exclusively dopaminergic; preclinical models that lack the trademark PD proteinaceous inclusions known as Lewy bodies (LBs) (Halliday et al., 2009); and

inconsistencies in the behavioural perturbations observed (Table 2)(Blesa & Przedborski, 2014).

Table 2. Animal Models of PD. ↑↑↑ indicates severe loss, ↑↑ indicates moderate loss, and ↑ indicates mild loss of SNc neurons. All animal models represent manipulations in rat and mouse unless otherwise specified (Blesa & Przedborski, 2014).

	Animal model	Motor behavior	SNc neuron loss	Striatal DA loss	Lewy body/Syn pathology
Toxin-based	MPTP Mice	Reduced locomotion, bradykinesia	↑↑↑	↑↑↑	NO
	MPTP Monkeys	Reduced locomotion, altered behavior, tremor, and rigidity	↑↑↑	↑↑↑	NO
	6-OHDA rat	Reduced locomotion, altered behavior	↑↑↑	↑↑↑	NO
	Rotenone	Reduced locomotion	↑↑	↑↑↑	YES
	Paraquat/maneb	Reduced locomotion	↑↑	↑↑↑	YES
	MET/MDMA	Reduced locomotion	↑↑	↑↑↑	NO
Genetic mutations*	α-Synuclein	Altered behavior, reduced or increased motor activity	↑ Not consistent	↑	↑ (in old animals)
	LRKK2	Mild behavioral alteration	NO	NO	NO
	PINK1	No obvious alterations or reduced locomotion	NO	NO	NO
	PARKIN	No obvious locomotion or reduced locomotion	NO	↑	NO
	DJ-1	Decreased locomotor activity	NO	NO	NO
	ATP13A2	Late onset sensorimotor deficits	NO	NO	NO
Others	SHH	Reduced locomotion	↑↑	↑↑	NO
	Nurr1	Reduced locomotion	↑↑	↑↑	NO
	Engrailed 1	Reduced locomotion	↑↑	↑	NO
	Pitx3	Reduced locomotion	↑↑↑	↑↑↑	NO
	C-Rel-NFKB	Gait, bradykinesia, rigidity	↑↑	↑↑	YES
	MitoPark	Reduced locomotion, tremor, and rigidity	↑↑	↑↑	YES
	Atg7	Late onset locomotor deficits	↑↑	↑↑	YES
	VMAT2	Reduced locomotion and altered behavior	↑↑	↑↑	YES

1.2.1.1 MPTP

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is the common neurotoxin of choice for studies investigating the mechanisms of DA neurodegeneration in PD. MPTP is toxic in many species, such as primates and mice (Tieu, 2011), however rats have been shown to be resistant to MPTP-induced neurotoxicity (Johannessen, Chiueh, Burns, & Markey, 1985). MPTP is metabolized into MPP+ by MAO-B, whereby MPP+

interferes with complex I in the mitochondrial electron transport chain (Voutilainen, Arumäe, Airavaara, & Saarma, 2015). MPTP mainly leads to the reproducible and specific death of DA in the striatum and SNc and by extension, major disruptions in the nigrostriatal DA system (Blesa & Przedborski, 2014).

Furthermore, as seen in PD, MPTP causes greater localized loss of DA neurons in the SNc than in ventral tegmental area (VTA) and retrorubral field (Blesa et al., 2012; Blesa et al., 2011) and in non-human primates (NHPs) treated with low doses of MPTP, greater deterioration of DA nerve terminals in the putamen than in the caudate nucleus were also observed (Blesa et al., 2010; Snow et al., 2000). Nevertheless, clinical behavioural and motor similarities from preclinical rodent MPTP models of PD are not ubiquitously observed (Blesa & Przedborski, 2014).

1.2.1.2 6-OHDA

6-hydroxydopamine (6-OHDA), similar to MPTP, is a DA neurotoxin that is used to generate rapid lesions in the nigrostriatal DA neurons in rats (Deumens, Blokland, & Prickaerts, 2002)(Blesa & Przedborski, 2014). Since 6-OHDA cannot cross the BBB, it must be injected into the SNc, medial forebrain bundle or striatum (Blandini, Armentero, & Martignoni, 2008). 6-OHDA mirrors the exclusivity of cell loss attributed to DA neurons observed in PD, however, fails to produce the hallmark LB inclusions (Halliday et al., 2009). Behavioural assessments of motor impairments in the unilateral 6-OHDA model are commonly evaluated using drug-induced rotation tests (Dunnett & Lelos, 2010a). Drug-free behavioural paradigms can also be used to assess sensorimotor gating, such as in the prepulse inhibition (PPI) test (Glajch, Fleming, Surmeier, & Osten, 2012).

1.2.1.3 Paraquat

The widely used pesticide paraquat (N,N'-dimethyl- 4-4-4'-bypiridinium, PQ) generates reactive oxygen species that cause widespread oxidative stress (Berry, La Vecchia, & Nicotera, 2010). Epidemiological and preclinical studies have reported on PQ's ability to induce parkinsonism in humans and rodents, respectively (Berry et al., 2010). After PQ administration, mice demonstrated decreased motor activity and a dose-dependent degeneration of striatal tyrosine hydroxylase (TH) fibers, as well as SNc neurons (Blesa & Przedborski, 2014; A. I. Brooks, Chadwick, Gelbard, Cory-Slechta, & Federoff, 1999; Day, Patel, Calavetta, Chang, & Stamler, 1999; McCormack et al., 2002; Rappold et al., 2011). Paraquat models of PD are valuable because of their ability to stimulate the production of LB in DA neurons, a hallmark in patients with PD (Manning-Bog, McCormack, Purisai, Bolin, & Di Monte, 2003). Nevertheless, this model has shown conflicting reported values of midbrain DA loss upon experimental replication (Blesa & Przedborski, 2014).

1.2.2 Genetic Models

Genetic models of PD may help to elucidate the etiology of familial forms of PD, although reported pathological and behavioural phenotypes have raised concerns regarding the ability of genetic models to recapitulate key features of clinical PD (Blesa & Przedborski, 2014). A significant portion of genetic studies assessing the integrity of the nigrostriatal DA system reported an insignificant degeneration of DA neurons (Andres-Mateos et al., 2007; Hinkle et al., 2012; Sanchez et al., 2014; Blesa & Przedborski, 2014). However, fragmented and dysfunctional mitochondria (Matsui,

Uemura, Yamakado, Takeda, & Takahashi, 2014; Morais & De Strooper, 2007), altered mitophagy (Zhang, Duan, & Yang, 2014), ubiquitin–proteasome dysfunction (Dantuma & Bott, 2014), and changes in reactive oxygen species production and calcium handling (Gandhi et al., 2009; Joselin et al., 2012; Ottolini, Calì, Negro, & Brini, 2013) are amongst the perturbations reported. Transgenic rodent models that reproduce known mutations observed in familial PD patients are summarized (Table 2).

1.2.2.1 α-Synuclein

α-Synuclein is a presynaptic protein heavily associated with PD, whereby it promotes presynaptic SNARE-complex assembly. However, its molecular mechanism of action remains elusive (Diao et al., 2013). It was found that native α-synuclein stimulates the aggregation of synaptic-vesicle using a single-vesicle optical microscopy system (Diao et al., 2013). Therefore, it is conceivable that mutations in the *SNCA* gene may lead to perturbations in DA release.

α-Synuclein is also the main component of LB and was the first gene associated with an autosomal dominant type of familial PD, known as Park1 (Goedert, Spillantini, Del Tredici, & Braak, 2013). Gene missense mutations of α-synuclein (*SNCA*), encoding the substitutions A30P, A53T, and E46K, have been identified in familial forms of PD (Schapira, Olanow, Greenamyre, & Bezard, 2014; Vekrellis, Xilouri, Emmanouilidou, Rideout, & Stefanis, 2011). Interestingly, duplication or triplication of the SNCA gene resulted in overexpression levels of endogenous α-synuclein by 2–3 times, which was reported as being sufficient in replicating several features of PD, such as (1) changes in striatal neurotransmitter metabolism, (2) aggregation of α-synuclein in nigral DA

neurons, and (3) evidence of oxidative stress (Cannon et al., 2013). An increased mRNA copy number and elevated expression of wild-type α-synuclein (*SNCA*) have been shown to cause early-onset familial PD. Interestingly, the levels of *SNCA*-mRNA from the SN of sporadic PD patients revealed fourfold increases compared to control midbrains (Chiba-Falek, Lopez, & Nussbaum, 2006). Taken together, these results support the hypothesis that increased α-synuclein expression is associated with the degeneration of DA neurons in the SN and the development of sporadic PD (Chiba-Falek et al., 2006; Dimant, Ebrahimi-Fakhari, & McLean, 2012).

1.2.2.2 Hsp70-Interacting Protein

It is posited that molecular chaperones assist in cellular decisions involved in refolding misfolded protein, such as α-synuclein, or targeting them for degradation through either of the degradation pathways: the ubiquitin-proteasome system (UPS) or the autophagy-lysosomal pathway (ALP) (Dimant et al., 2012). The Hsp70-interacting protein (Hip), also known as cochaperone ST13 (suppression of tumorigenicity 13) stabilizes heat-shock protein 70 (Hsp70), a modifier of α-synuclein toxicity that also facilitates the refolding of misfolded *SNCA* proteins (Chu, Dodiya, Aebischer, Olanow, & Kordower, 2009; Kilpatrick et al., 2013; Scherzer et al., 2007). Hsp70 has been reported to protect against *SNCA* toxicity in *in vitro* (Klucken, Shin, Masliah, Hyman, & McLean, 2004), yeast (Flower, Chesnokova, Froelich, Dixon, & Witt, 2005), Drosophila melanogaster (Auluck, Chan, Trojanowski, Lee, & Bonini, 2002) and *in vivo* (Luk, Mills, Trojanowski, & Lee, 2008) models of PD. Hsp70 is active in both ATP- and ADP-bound forms; however, the ADP-bound form showed greater chaperone activity (Hartl & Hayer-

Hartl, 2002). Hip stabilizes the ADP state of HSP70 through interactions with its N-terminal domain, which has a high affinity for α-synuclein (Dimant et al., 2012; Höfeld, Minami, & Hartl, 1995; Nollen et al., 2001). In the SN of patients with PD, expression of Hsp70 was altered, revealing a three-fold change (Grünblatt, Mandel, Maor, & Youdim, 2001; Hauser et al., 2005). Not surprisingly, expression of Hsp70 ameliorated α-synuclein aggregation and rescued cells from α-synuclein–induced toxicity (Danzer et al., 2011), which further supports the protective function of molecular chaperones and Hip (Dimant et al., 2012).

A study that assayed the whole blood of 105 individuals found Hip mRNA copies to be lower in patients with PD in two independent populations compared to age-matched controls(Scherzer et al., 2007). This finding highlights the potential role of HSP70-interacting Hip in its ability to serve as an indicator for Hsp70 function and suggests perturbations in the expression of *Hip* may contribute to the etiology of PD (Scherzer et al., 2007).

The reported role of Hip in alleviating α-synuclein aggregation that contributes to the degeneration of dopaminergic neurons presents a novel avenue for elucidating the pathophysiology of PD and highlights the important role that molecular chaperones have in PD initiation and progression. A greater understanding of the cellular mechanisms underlying molecular chaperone activity may shed much needed-light on the etiology surrounding PD and may provide potential therapeutic targets to prevent the accumulation of the toxic α-synuclein aggregates common in many neurodegenerative diseases, including PD (Dimant et al., 2012).

1.3 Neurotrophic Factors

Neurotrophic factors (NTFs) are categorized into three main families and include peptides and proteins that are in charge of the regulation of neuronal differentiation, maturation, and survival, in addition to their ability to regulate neurite growth and branching that governs the ultimate number of neurons and the density of innervation during development (Voutilainen, Arumäe, Airavaara, & Saarma, 2015). In adulthood, NTFs regulate the metabolic maintenance of neurons, neuronal plasticity, and also protect and repair damaged neurons (Airavaara, Voutilainen, Wang, & Hoffer, 2012). It is because of these properties that research investigating the therapeutic potential of NTFs for neurodegenerative disorders, such as PD, has been growing.

The first to be discovered and most studied NTF family includes nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). These NTFs govern neuronal survival and plasticity through their activation of transmembrane receptor tyrosine kinases (RTKs) of the Trk family (Voutilainen et al., 2015). Additionally, these NTFs, along with their pro-forms bind to the p75 neurotrophic receptor to trigger neuronal apoptosis. Homodimeric BDNF activates TrkB tyrosine kinase receptors, which leads to the activation of PI3K/Akt, PLCc and MAP kinases (Figure 4) (Voutilainen et al., 2015). BDNF pretreatment was shown to support the survival of embryonic DAergic neurons in neurotoxin preclinical models of PD, but failed to protect and repair DA neurons when added to the SN after the neurotoxin (Voutilainen et al., 2015). Although BDNF has not been studied in clinical trials with PD patients, BDNF receptor knockout (KO) rodents revealed an unperturbed

nigrostriatal DA system (Voutilainen et al., 2015).

Neurokines represent the second family of NTFs, which are smaller secretory proteins that elicit their effects through the transmembrane gp130 receptor. This family includes ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF), neuropoietin (NPN), oncostatin M (OSM), cardiotrophin-like cytokine (CLC), interleukin 6 (IL-6), IL-11 and IL-27. Neurokines largely support the survival of motor neurons (Parkash et al., 2008). More specifically, CNTF promotes the survival of DA neurons and parasympathetic neurons, and LIF assist in the maintenance of sensory neurons (Voutilainen et al., 2015).

The most pertinent NTFs to DA neurons belong to the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs). GDNF and neurturin (NRTN), artemin (ARTN) and persephin (PSPN), are included in a distant family of the transforming growth factor-b (TGF-b) superfamily (Airaksinen & Saarma, 2002; Voutilainen et al., 2015). GFLs specifically bind to the membrane glycolipid glycosylphosphatidyl inositol (GPI)-anchored co-receptors of the GDNF Family Receptor α (GFRα) and the GFL–GFRα complex binds to and in turn activates RET receptor tyrosine kinase leading to the activation of PI3K/Akt, Src and MAP kinase pathways and effectively promoting the survival of DAergic neurons (Figure 4) (Voutilainen et al., 2015). KO of NRTN and its receptor GFRα2 showed impairments in the parasympathetic nervous system, but the nigrostriatal DA system remained intact. GDNF, GFRα1 and RET KO studies revealed that animals die at birth due to the absence of a kidney, but the DA system did not suffer any impairments (Airaksinen & Saarma, 2002). In experiments

that removed GDNF from adult mice using tamoxifen induction, significant loss of DA neurons in the midbrain resulting in adverse motor effects were reported, and thus, GDNF was concluded to be vital for DA neuron survival in adult animals (Pascual et al., 2008). Conversely, evidence from further studies showed that GDNF was in fact not necessary to maintain the nigrostriatal DA system in adulthood (Burke, 2006; Kopra et al., 2015). In an experiment that studied RET-deficient mice for 9 months, it was concluded that RET is also not essential for the maintenance of the midbrain DA system (Jain et al., 2006). GDNF and NRTN have been shown to protect and repair DA neurons in rodent and NHP models of PD (Hoffer et al., 1994; Rosenblad, Martinez-Serrano, & Björklund, 1998; Tomac et al., 1995), however they have only demonstrated modest clinical benefit in phase two clinical trials (Kordower & Bjorklund, 2013). Furthermore, GDNF failed to protect DAergic neurons in α-synuclein models of PD (Decressac et al., 2011; Lo Bianco, Déglon, Pralong, & Aebischer, 2004), which poses a challenge since α-synuclein aggregation is a hallmark in the pathophysiology of patients with PD (Voutilainen et al., 2015). Thus, a need exists to discover novel dopaminotrophic factors for PD treatment.

Vascular endothelial growth factor A (VEGF-A) and VEGF-B revealed neuroprotective and restorative in the 6-OHDA model of PD (Voutilainen et al., 2015). However, in the rodent 6-OHDA preclinical model of PD, VEGF-C showed modest neuroprotective effects (Piltonen et al., 2011).

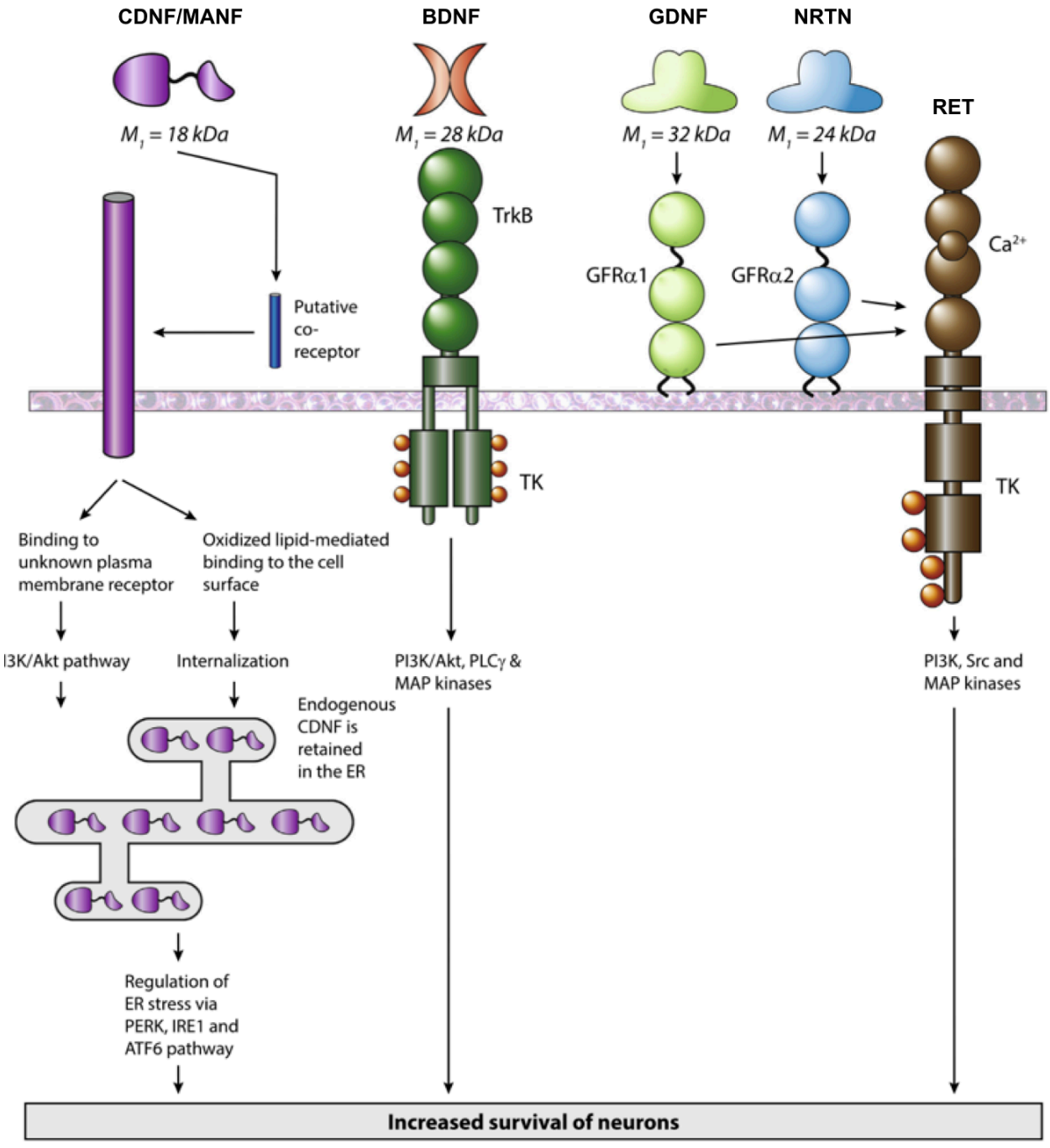

Figure 4. Hypothesized and established mechanisms of action for various classes of NTFs. Figure modified from Voutilainen et al., 2015.

1.3.1 MANF, CDNF & Parkinson's Disease

Mesencephalic astrocyte-derived neurotrophic factor (MANF) was the first discovered member of the cerebral dopamine neurotrophic factor (CDNF)–MANF family by John Commissiong from the culture medium of rat type-1 astrocyte ventral mesencephalic cell line as a novel NTF that supports the survival of embryonic DAergic neurons *in vitro* (Petrova et al., 2003).

MANF, also known as arginine-rich mutated in early stage tumors or ARMET, is an evolutionarily conserved protein present in vertebrate and invertebrate species, including fruit flies and Caenorhabditis elegans, which encode strikingly homologous proteins of comparable size and a conserved cysteine pattern (Petrova et al., 2003). Sequence analysis from a variety of organisms showed that vertebrates, such as mouse and zebrafish have orthologous genes for both CDNF and MANF. Human and zebrafish MANF proteins share 69 percent of their amino acid sequence, and CDNF proteins share 53 percent of their amino acid sequence. Human CDNF revealed 49 percent amino acid sequence similarity with fruit flies and 46 percent identity similarity with C. elegans MANF, whereas human MANF showed 53 percent sequence identity with fruit flies and 50 percent sequence similarity with C. elegans. Therefore, vertebrate and invertebrate CDNF and MANF form an evolutionarily conserved family of NTFs with 8 conserved cysteine residues (Voutilainen et al., 2015). CDNF (ARMET-like protein 1) is a paralog of MANF found in vertebrates (Lindholm et al., 2007). Amino acid sequence of CDNF/MANF family members reveals no homology with other proteins. MANF and CDNF have a molecular weight of 18 kDa (Hellman et al., 2011; Lindahl, Saarma, &

Lindholm, 2016). The primary sequence of MANF and CDNF contains an N-terminus signal peptide that directs them to the ER, which when cleaved becomes a mature protein that can be secreted (Lindahl et al., 2016; Lindholm et al., 2007; Petrova et al., 2003) (Figure 5). Sequence analysis of the active MANF protein revealed a homology to a predicted human arginine-rich protein (ARP) of 234 amino acids, with its putative arginine-rich region renamed MANF (Shridhar et al., 1996). Human MANF is 179 amino acids with a signal peptide of 21 amino acids, whereby its cleavage results in a mature protein of 158 amino acids (Lindahl et al., 2016; Petrova et al., 2003) (Figure 5). CDNF consists of 187 amino acids and contains a signal peptide of 26 amino acids, whereby its cleavage results in a mature protein of 161 amino acids. The amino acid sequence homology between mature human CDNF and MANF is 59% (Lindahl et al., 2016). CDNF and MANF proteins reportedly lack the pro sequence for enzymatic activation and contain eight cysteine residues in their primary structure, unlike NTFs, such as GDNF (Lindahl et al., 2016; Lindholm et al., 2007; Petrova et al., 2003) (Figure 5).

CDNF and MANF support the survival of midbrain DAergic neurons (Voutilainen et al., 2015), which are known to degenerate in PD (Lewitt, 2012). Nevertheless, CDNF and MANF are structurally and functionally different from the classical neurotrophic factors (NTFs) that are exclusively secreted proteins, such as GDNF. GDNF belongs to a family of growth factors that bind to transmembrane receptors and induce intracellular signalling cascades (Lindahl et al., 2016). Conversely, the mechanism of action of CDNF and MANF remains largely unknown. In cells, CDNF and MANF are localized in the endoplasmic reticulum (ER), and evidence implicates

MANF, and potentially CDNF, in the maintenance of ER homeostasis. MANF protein was found to be upregulated in several cell lines and additionally by cerebral ischemia of rat, as well as gene expression analysis of MANF mutants in Drosophila melanogaster led to the activation of the unfolded protein response (UPR), which is a signalling pathway activated to ameliorate ER stress (Apostolou, Shen, Liang, Luo, & Fang, 2008; Palgi, Greco, Lindström, Auvinen, & Heino, 2012). Mounting evidence suggest that CDNF and MANF, when administered as extracellular proteins or via viral vectors can prevent and repair midbrain DA neurons *in vivo* (Airavaara et al., 2012; Back et al., 2013; Cordero-Llana et al., 2015; Lindholm et al., 2007; Voutilainen et al., 2011).

It is theorized that during periods of ER-stress cells, monomeric CDNF and MANF bind to a putative co-receptor that stimulates the activation of unknown plasma membrane receptors, which lead to the triggering of intracellular signal transduction pathways, such as the PI3K/Akt pathway (Figure 4). Voutilainen et al. (2015) also hypothesized that conceivably in parallel, CDNF and MANF bind to oxidized lipids at the cell surface and are internalized via an undetermined mechanism leading to the regulation of ER stress and the UPR involving proteins PERK, IRE1, and ATF6 (Figure 4). Furthermore, intracellular CDNF or MANF can be retained in the ER where they have the ability to directly modulate ER stress via the UPR, unlike other classical NTFs (Voutilainen et al., 2015).

Studies have reported that the N-terminal domain of CDNF and MANF has the ability to interact with specific phospholipids, which is in agreement with the two stretches of conserved lysine and arginine residues on the surface of the molecules.

Moreover, crystal structure showed that the C-terminus of MANF is normally unfolded and, not unlike disulfide isomerases, the structure contains a CKGC motif with a disulfide bridge, which is in agreement with its indicated role in modulating ER stress. The functional importance of the CKGC sequence is further supported by the conclusion that mutating cysteine residues in this sequence inactivates the biological activity of MANF (Lindström et al., 2013; Voutilainen et al., 2015).

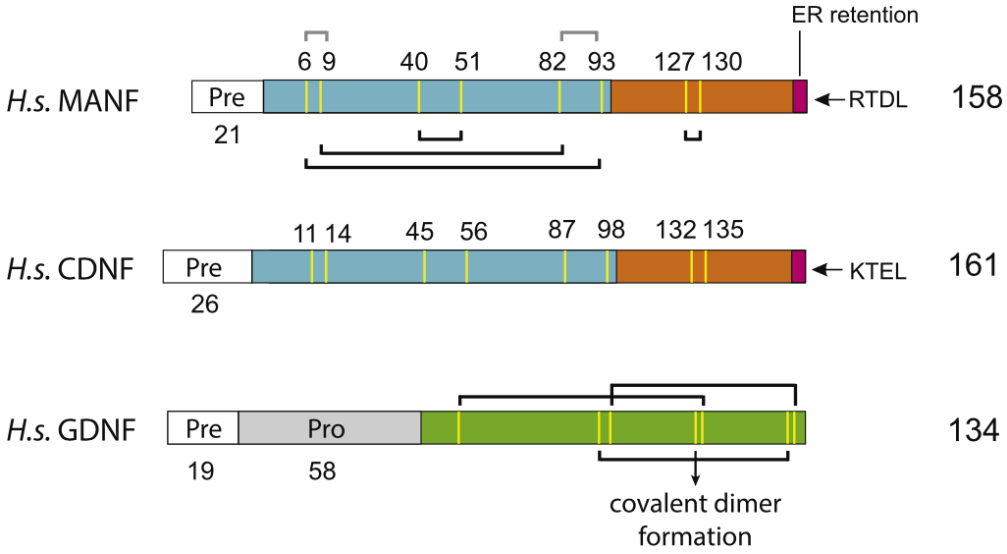

Figure 5. A schematic of the primary structure of human MANF and CDNF in comparison to that of GDNF. The saposin-like domain of MANF and CDNF is indicated in blue, and the SAP-like domain in orange. Cysteine residues are marked as yellow bars and numbered according to the sequence of mature protein. Disulfide bridges are indicated by black connecting lines. GDNF contains a pro-region which is enzymatically cleaved. Mature GDNF is indicated in green and disulfide bonds forming a cysteine knot motif indicated with black lines. Two GDNF molecules form a homodimer via a disulphide bridge and a cysteine residue involved is indicated arrows. H.s.; Homo sapiens (Lindahl et al., 2016).

CDNF expression has been reported in the SN, striatum, hippocampus, thalamus, olfactory bulb, hypothalamus, and cerebellum. Moreover, CDNF is also expressed in non-neuronal tissues, such as the heart, liver, lungs, skeletal muscles, spleen, testes, and

thymus (Cheng et al., 2013; Lindholm et al., 2007).

CDNF, not unlike MANF, is thought to assist in protein folding and ameliorate ER stress, thereby preventing neurodegeneration in PD and proving to be a potentially fundamental therapeutic in the maintenance of dopaminergic neurons in the midbrain (Lindholm & Saarma, 2010; Parkash et al., 2009). The accumulation of misfolded proteins is associated with perturbations in ER function, which is a characteristic of PD (Liu, 2014). These perturbations in the ER folding process leads to an accumulation of misfolded proteins and as a result ER stress, which under normal physiological conditions activates the unfolded protein response (UPR). The UPR is a cellular defense mechanism to alleviate the stress by suppression of protein translation, degrading misfolded proteins through ER-associated protein degradation (ERAD), and activating signalling pathways that lead to the production of molecular chaperones involved in the protein folding (Hetz, 2012; Lindahl et al., 2016; Szegezdi, Logue, Gorman, & Samali, 2006).

The hypothetical role of MANF during ER stress has been hypothesized and summarized (Figure 6)(Lindahl et al., 2016).

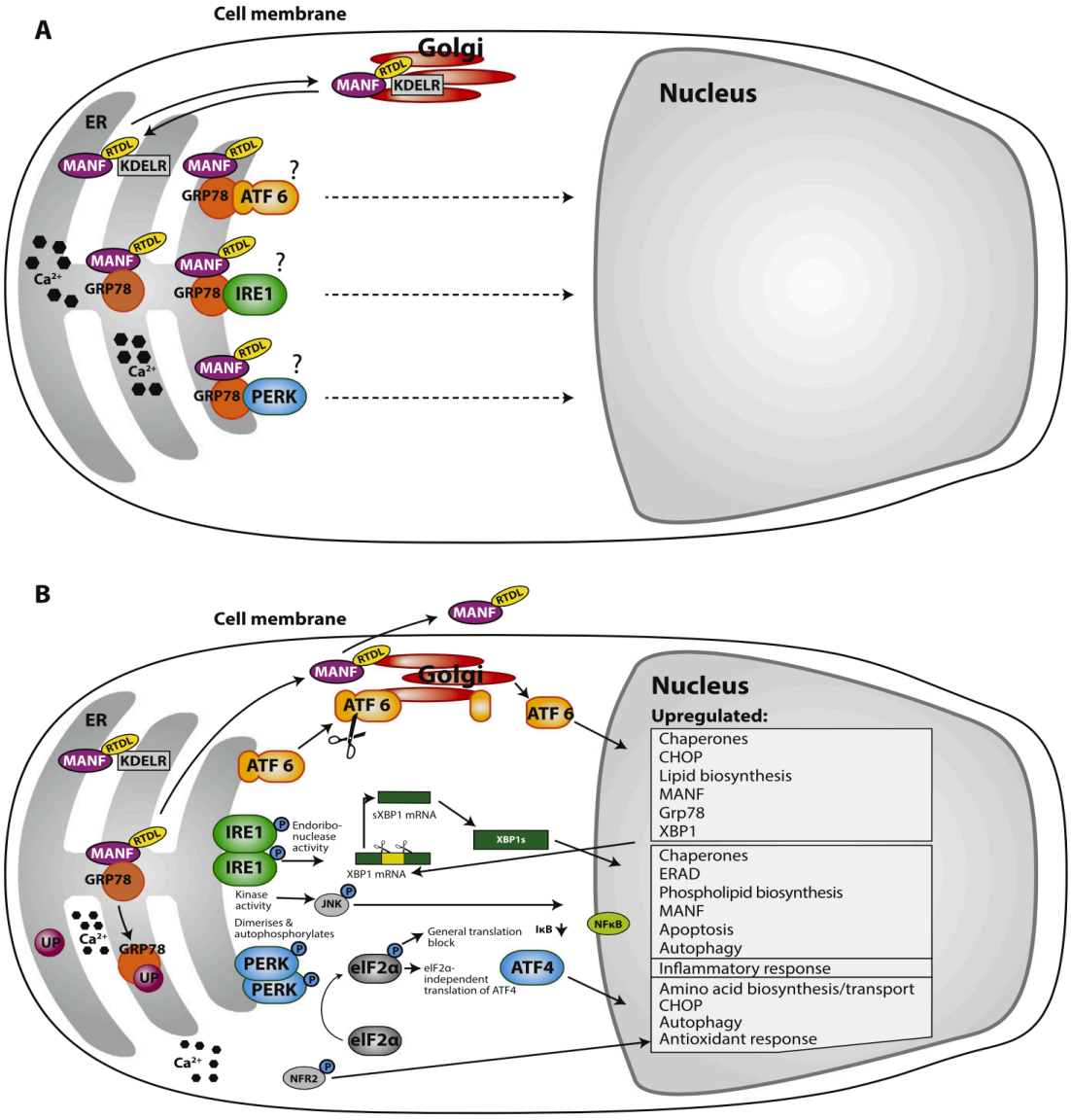

Figure 6. The UPR and MANF's potential role. (A) During periods of no ER stress, binding of GRP78 to the ER lumen of UPR sensors ATF6, IRE1 and PERK keeps them inactive. MANF is retained in the ER by KDELR via the C-terminal RTDL sequence, and by calcium dependent interaction with GRP78. KDELR may retrieve MANF from the Golgi back to the ER. The question marks indicate that MANF may bind GRP78, which is interacting with the UPR sensors that maintain their inactivity. **(B)** Activation of UPR during periods of ER stress. Binding of GRP78 to accumulating unfolded proteins (UP) leads to its dissociation from ATF6, IRE1, and PERK, resulting in their activation. ATF6 translocates to the Golgi, where it is cleaved by proteases. Active ATF6 functions as a transcription factor and induces genes involved in protein folding, and the expression of GRP78, MANF, proapoptotic transcription factor C/EBP homologous protein CHOP, and Xbp1 mRNA. It also directly controls genes encoding for components of ER-

associated protein degradation (ERAD). IRE1 is activated by dimerization and trans-autophosphorylation. Endoribonuclease activity of the cytoplasmic domain of active Inositol-requiring enzyme 1 (IRE1) removes an intron from XBP1, generating spliced X-box binding protein 1 (XBP1) transcription factor. XBP1s upregulates genes for chaperones, ERAD machinery, phospholipid synthesis and *Manf*. Dimerized and activated PERK inhibits general protein translation by phosphorylating translation initiation factor eIF2α subunit, while favouring the translation of ATF4 transcription factor. ATF4 induces pro-survival genes of redox-balance, amino acid metabolism, and protein folding. Marked translational inhibition may lead to decreased levels of inhibitory nuclear kappa B (IkB), leading to nuclear translocation of NF-κB and increased transcription of inflammatory response genes, and apoptotic cell death. ATF4 induces the expression of genes involved in restoring ER homeostasis, C/EBP homologous protein (CHOP), and protein phosphatase 1 regulatory subunit 15A (PPP1R15A) which in complex with protein phosphatase 1 (PP1) can relieve translation arrest by dephosphorylating eIF2α. Additionally, phosphorylation of transcription factor NRF2 by PERK results in upregulation of antioxidant response genes. In ER stress, Ca^{2+} is depleted from the ER, which may lead to dissociation of MANF from GRP78 and its secretion. However, MANF may remain in the ER through its interaction with KDELR. Alternatively, MANF interaction with KDELR is hypothesized to also regulate MANF secretion to the extracellular space during ER stress, which may influence a diverse set of signalling cascades initiated by activated UPR sensors (Lindahl et al., 2016).

1.4 Knockdown Models

1.4.1 Viral Vectors

Viral vectors, including lentiviruses and adeno-associated viruses (AAVs) have been employed in studying PD, such as in α-synuclein rodent models (Blesa & Przedborski, 2014). Rats are the ideal subjects for such experiments as their brain size allows for greater accuracy during viral vector delivery after stereotactic injections near neuronal cell bodies in the SNc (Lauwers et al., 2007). In contrast to α-synuclein transgenic mice, viral vector-mediated models of human WT or A53T mutant α-synuclein demonstrated marked and age-dependent DAergic neurodegeneration, motor impairments and α-synuclein cytoplasmic inclusions (Blesa & Przedborski, 2014; Decressac, Mattsson, Lundblad, Weikop, & Björklund, 2012; Klein, King, Hamby, & Meyer, 2002;

Lo Bianco, Ridet, Schneider, Deglon, & Aebischer, 2002). Although models employing viral vectors are prone to variability, they can serve as a great resource for elucidating the pathophysiology of PD and testing neuroprotective approaches.

1.4.2 Lentiviral Vector-Based Systems

Posttranscriptional gene silencing is better known as RNA interference (RNAi), which uses short hairpin RNAs (shRNAs) synthesized within the cell by DNA vector-mediated production. Similar to short interfering RNA oligonucleotides (siRNAs), shRNAs may be transfected with plasmid vectors encoding shRNAs transcribed by RNA polymerase III, however, they can also be transported into mammalian cells via viral vectors (Moore, Guthrie, Huang, & Taxman, 2010). While siRNA delivers the siRNA duplex directly to the cytosol, shRNAs are capable of DNA integration and consist of two complementary 19 to 22 base pairs RNA sequences linked by a short loop of 4 to 11 nucleotides. Following transcription by DNA polymerase III in the nucleus, Exportin 5 exports the shRNA sequence to the cytosol where it is recognized by an endogenous enzyme, Dicer, which cleaves the shRNA short loop creating siRNA duplexes. Like the exogenously delivered synthetic siRNA oligonucleotides, this endogenously derived siRNA binds to the target mRNA and is included into the RNA-induced silencing complex (RISC)/AGO2 complex for target-specific mRNA degradation (Figure 7)(Moore et al., 2010). The passenger or sense strand is degraded. Subsequently, the antisense or guide strand directs the RISC complex to complementary mRNA. In the event of complete complementarity, RISC cleaves the mRNA, however upon imperfect

complementarity, RISC blocks translation of the mRNA. In both events, the shRNA leads to targeted gene silencing.

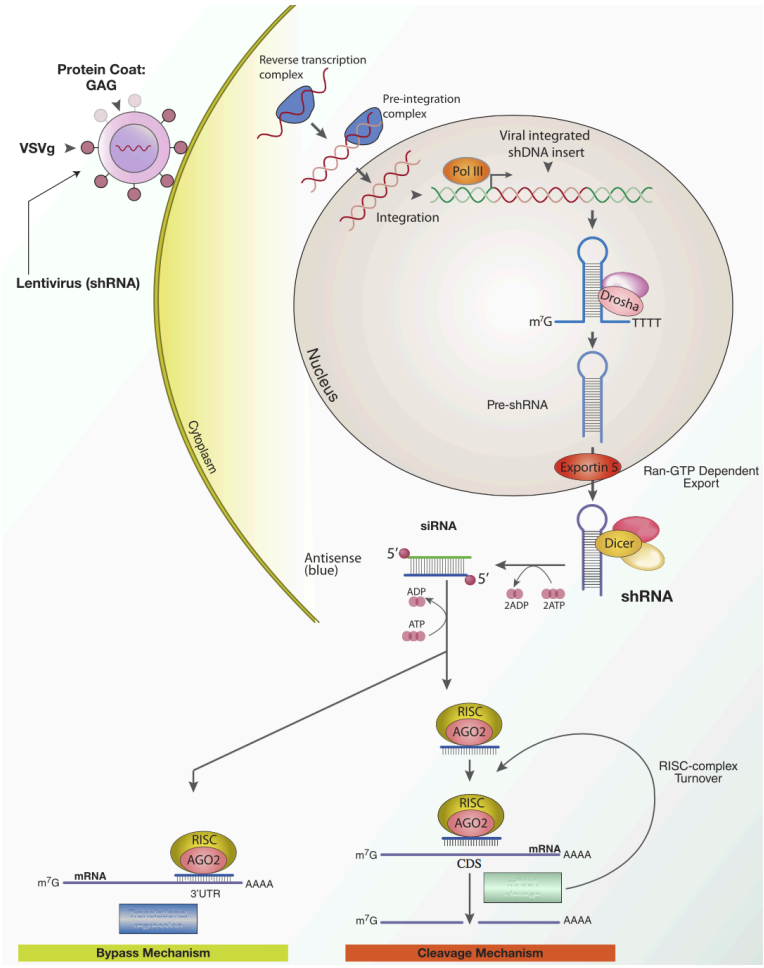

Figure 7. Lentiviral delivery of shRNAs and the mechanism of RNA interference in mammalian cells. shRNA is processed endogenously by Exportin 5, Dicer, and the RISC/AGO2 complex (Dan Cojocari, Department of Medical Biophysics, University of Toronto 2010).

In contrast to siRNA, shRNA, on the other hand, may be used to generate stable knockdown cell lines, thereby eliminating the need for multiple rounds of transfection and greatly increasing reproducibility of results (Moore et al., 2010).

There are multiple methods of introducing shRNA into cells, however, lentiviral-mediated transduction is a common and convenient method of introducing shRNA into dividing or non-dividing cells, and is reportedly less toxic to cells than adenoviral-mediated transduction. Lentiviruses integrate a large volume of viral RNA into DNA of the host cell and can efficiently infect non-dividing cells as well, which establishes lentiviral vectors as one of the most efficient methods of gene delivery and silencing (Cockrell & Kafri, 2007). Permanent suppression of gene expression in adult organisms by shRNA is a powerful method to study gene function, disease pathophysiology and develop gene-specific therapeutic interventions.

CHAPTER 2: OBJECTIVES, AIMS & HYPOTHESES

CDNF and MANF have been implicated in supporting the survival and protection of midbrain DAergic neurons in the nigrostriatal pathway, which are known to degenerate in PD (Lewitt, 2012). Nevertheless, CDNF and MANF are structurally and functionally different from the classical NTFs, such as GDNF, which has shown to be a potent NTF that enhances survival of midbrain dopaminergic neurons (Lin, Doherty, Lile, Bektesh, & Collins, 1993). Interestingly, intrastriatal lentiviral vector-mediated expression of CDNF in the 6-OHDA model of PD decreased amphetamine-induced rotations and increased tyrosine hydroxylase (TH) striatal fiber density, however with no effects on TH^+ cell numbers in the SN (Cordero-Llana et al., 2015). In addition, it was demonstrated that CDNF delivery into the striatum before and 4 weeks after 6-OHDA lesion resulted in a dose dependence preventative loss and increase in TH^+ neurons in the SN, respectively (Lindholm et al., 2007). Conversely, MANF overexpression in the SN revealed no effects on amphetamine-induced rotations or TH striatal fiber density, however interestingly resulted in significant maintenance of TH^+ cells (Cordero-Llana et al., 2015). Moreover, combined CDNF and MANF overexpression resulted in significant decreases in amphetamine-induced rotations, large increase in striatal TH-fiber density and substantial protection of TH^+ cells in the SN (Cordero-Llana et al., 2015). Thus, as demonstrated by previous studies, CDNF, MANF and combinational delivery of these NTFs into the SN resulted in synergistic neuroprotective effects, which warrants additional studies in order to further characterize their effects, especially in knockdown (K/D) models.

Aim 1: MANF Knockdown Model

A. K/D gene expression of MANF using shRNA lentiviral vectors infused unilaterally into the SN of rats.
B. Evaluate whether MANF K/D rats recapitulate key behavioural and motor features of PD as measured by: the beam traversal test, footprint analysis, the fixed speed rotarod test, the cylinder test and rearing and total distance parameters.
C. Quantify the mRNA expression of MANF using Real Time quantitative Polymerase Chain Reaction (qPCR), the levels of DA in the striatum (STR), the TH striatal fiber density, and the number of TH+ cells in SN at various time points post lentiviral shRNA infusion.

Aim 1 Hypotheses: It is hypothesized that MANF K/D will result in:

A. Increased latency and traversal times, as well as increased number of contralateral errors in the beam traversal test compared to control rats.
B. Changes in gait as measured by: greater footstep overlap and shorter hindlimb stride lengths compared to control rats.
C. Decreased time on the rotarod compared to control rats.
D. Increased ipsilateral forelimb preference compared to control rats.
E. Decreased rearing duration compared to control rats.
F. Decreased total distance travelled compared to control rats.
G. Decreased mRNA expression of MANF, DA and TH in the SN and striatum compared to control rats.

Aim 2: CDNF Knockdown Model

A. K/D gene expression of CDNF using shRNA lentiviral vectors infused unilaterally into the SN of rats.
B. Evaluate whether CDNF knockdown rats recapitulate key behavioural and motor features of PD as measured by: the beam traversal test, footprint analysis, the fixed speed rotarod test, the cylinder test and rearing and total distance parameters.
C. Quantify the mRNA expression of CDNF using Real Time quantitative Polymerase Chain Reaction (qPCR), the levels of DA in the striatum (STR), the TH striatal fiber density, and the number of TH+ cells in SN at various time points post lentiviral shRNA infusion.

Aim 2 Hypotheses: It is hypothesized that CDNF K/D will result in: See Aim 1 Hypotheses.

Aim 3: MANF and CDNF Knockdown Model

A. K/D gene expression of MANF and CDNF using shRNA lentiviral vectors infused unilaterally into the SN of rats.
B. Evaluate whether MANF and CDNF knockdown rats recapitulate key behavioural and motor features of PD as measured by: the beam traversal test, footprint analysis, the fixed speed rotarod test, the cylinder test and rearing and total distance parameters.
D. Quantify the mRNA expression of MANF, CDNF, and levels of DA, and TH using qPCR at various time points post lentiviral shRNA infusion.

Aim 3 Hypotheses: It is hypothesized that MANF and CDNF K/D will result in: See Aim 1 Hypotheses.

Aim 4: Evaluate the Extent of Gene Silencing of CDNF and MANF

A. Quantify the mRNA expression using qPCR at 1 month after the completion of treatment studies in the SN and striatum compared to control rats.

Aim 4 Hypotheses: It is hypothesized that there will be a significant decrease (~70%) in mRNA expression of MANF and CDNF in the SN and striatum compared to controls.

Aims 5: Quantify MANF and CDNF mRNA Expression using qPCR from the Platelets of PD Patients.

Aim 5 Hypotheses: It is hypothesized that there will be a significant decrease in mRNA expression of MANF and CDNF from the platelets of PD patients compared to healthy controls.

CHAPTER 3: METHODOLOGY

3.1 Animals

Male Sprague-Dawley rats were obtained at a weight of 250 to 300 grams from Charles River Laboratories (St. Constant, QC, Canada), and individually housed at the McMaster University Central Animal Facility (CAF) in accordance with the Canadian Council on Animal Care (CCAC). Male rats were chosen to avoid the effects of the female estrus cycle, and to relate findings from the present study to previous research from our lab, which has focused on male rats. Animals were housed individually in standard specific pathogen free cages on a reverse 12:12 light/ dark cycle in a room maintained at 22 degrees Celsius with 50% humidity, and food-restricted to 90% of their free-feeding body weight, but with access to water *ad libitum*. General health and weight of the rats were monitored daily.

3.2 Stereotaxic Surgery

Animals were anaesthetized (Isoflurane, Pharmaceutical Partners of Canada Inc., Richmond Hill, ON) and mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, California, USA). An analgesic (buprenorphine), a local anesthetic (bupivacaine), an antibiotic (enrofloxacin), an anti-inflammatory drug (ketoprofen) and saline were administered subcutaneously prior to incision. An incision, approximately 2 cm in length, was made midline on the dorsal surface of the skull and a guide cannula was used to accurately mark the coordinates of the SN in reference to bregma Anterior/Posterior=-5.3 mm, Medial/Lateral=±2.3 mm, Dorsal/Ventral=-7.8 mm (Klein et al., 2002; Paxinos & Watson, 1986).

3.3 Lentiviral Vector Mediated shRNA Knockdown

2 μL at 0.5 μL/minute of MANF (ARMET), CDNF (ARMETL1), a scrambled control or a combination of both MANF and CDNF (1 μL each) rat shRNA lentiviral particles (Table 3, 29mer target-specific shRNA; OriGene Technologies, Incorporated; Rockville, MD) were infused unilaterally into the SN using the UMP3 UltraMicroPump (World Precision Instruments, Inc.). The scrambled shRNA has the same nucleotide composition, but not the same sequence, as the test shRNA and thus, does not target any known gene in the target organism and effectively serves as a negative control. The scrambled control stimulates RNAi machinery, which allows for the evaluation of the baseline effect after the introduction of duplex RNA on gene expression.

Subsequently, the incision was closed using stainless steel wound clips (Durect Corporation, Cupertino, CA). Animals were monitored for seven consecutive days and staples were removed one week post-surgery. One week was allotted for recovery before the start of behavioural testing.

The four gene-specific shRNA lentiviral particles are packaged from a pGFP-C-shLenti vector (Figure 8), which is a third generation Lentivector that requires the viral components to be carried in vectors to produce viral particles. There are three major functional elements within the 5'LTR and 3'LTR that can be transduced to many cell lines: 1) an shRNA expression cassette driven by an U6 promoter; 2) a puromycin resistant gene driven by an SV40 promoter; 3) and a tGFP gene driven by a CMV promoter. The bacterial selection marker for the vector is Chloramphenicol. The pGFP

plasmid generates tagged proteins in mammalian cells through the green fluorescent protein (GFP), which allows for the visualization of the spatial and temporal localization of the protein of interest by fluorescence microscopy.

The shRNA expression cassette consists of a 29 base pair target gene specific sequence, a 7 base pair loop, and a 29 base pair reverse complementary sequence, which are all under human U6 promoter (Figure 9). A termination sequence (TTTTTT) is situated downstream of the second 29 base pair reverse complementary sequence in order to terminate the transcription by RNA Polymerase III. The gene-specific shRNA cassette is sequence-verified to ensure its match to the MANF and CDNF target gene.

Figure 8. Nucleotide Sequence for OriGene's pGFP-C-shLenti shRNA-29 Expression Vector (OriGene Technologies, Incorporated; Rockville, MD).

GTCGACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGT
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AATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGAC

Features for pGFP-C-shLenti vector:

Start	End	Description
835	1015	5LTR
2618	2624	EcoR1
2693	2949	U6 promoter
3039	3230	SV40 promoter
3294	3893	Puromycin-N-acetyl transferase
4269	4275	Xba1
4280	4985	CMV promoter
5034	5738	tGFP
7070	7250	sinLTR
7217	7836	pBR322 origin replication
7896	8555	CAM^r for Chloramphenicol resistance

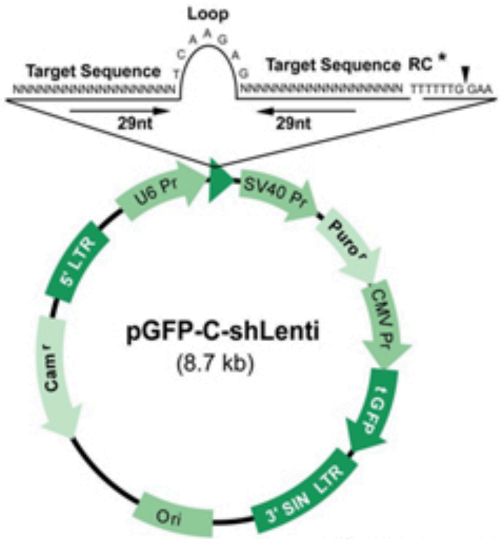


Figure 9. A schematic of the major functional components of the pGFP-C-shLenti vector. RC *: Reverse Complement (OriGene Technologies).

Table 3. CDNF and MANF shRNA nucleotide sequences used (OriGene Technologies, Incorporated; Rockville, MD).

Armetl1 (CDNF), Rat	
Catalog Number	**Sequence**
TL703512VA	AAC TCC TTG CTA ACC AGA GGA ATA GAC TT
TL703512VB	TGC GCG GAG AAG CAT GAC TAC GTG AAC CT
TL703512VC	TTG GAT CTC CAA CCA TGT GCT GGC ACA GG
TL703512VD	GCT GAC TGT GAA GTA TGC AAA GAG TTC TT
Armet (MANF), Rat	
Catalog Number	**Sequence**
TL706724VA	AAT CGG TTG TGC TAC TAC ATT GGA GCC AC
TL706724VB	CCA CCA AGA TCA TCA ATG AGG TAT CGA AG
TL706724VC	TTC ACC AGC CAC TAT TGA AGA AGA ACT TA
TL706724VD	AGG ACC TCA AAG ACA GGG ATG TCA CAT TT

3.4 Behavioural Testing

The contralateral limb refers to the side of the body that is opposite to the site of the shRNA infusion. The ipsilateral limb refers to the side of the body that is on the same side as the site of the shRNA infusion.

3.4.1 Locomotor Activity: Total Distance and Rearing Duration

Locomotor testing, which measures horizontal and vertical activity as well as the pattern of activity, was performed during the dark period of the dark/light cycle as rats show maximum activity during these hours. Bradykinesia and akinesia are characteristic symptoms in PD and are assessed in rodents through locomotor activity, whereby

hindlimb motor impairments lead to reduced rearing and a decrease in total distance travelled (Dunnett & Lelos, 2010a). AccuScan computerized cages (AccuScan Instruments, Columbus, OH, USA) were used, and multidirectional movements were recorded for a two hour period. Rats were habituated to the test chambers for two weeks prior to testing. The first 20 minutes was a recorded habituation period. Each animal cage consisted of a transparent acrylic chamber (16 inches in length, 8 inches in height and 5 inches in width) surrounded by horizontal and vertical infrared sensor beams. The interruptions to the horizontal infrared sensors caused by the rat's movement were taken as a measure of total distance travelled, whereas the interruptions of vertical sensors measured rearing activity. When the animal activates the vertical sensor by rearing, this variable starts incrementing time (s) and continues to increment until the animal goes below the level of the vertical sensor.

3.4.2 Narrow Beam Traversal

Animals were trained to traverse a beam that allows for the measurement of skilled walking and hindlimb errors, which reflect impaired coordination. This test reliably measures basal ganglia disturbances in genetic models with minimal nigrostriatal damage (Brooks & Dunnett, 2009). It has been shown to detect changes in DA function in older animals and may consistently detect nigrostriatal DA loss because it measures skilled walking (Meredith & Kang, 2006). The test measures latency time, ipsilateral and contralateral hind limb stepping errors, and time to traverse. The beam test requires three trials and ends in a home cage. The test features two raised platforms 60 cm above the ground and 1 meter apart. The starting platform is a flat surface and the ending platform

features a 20 cm high, 20 cm in length x 20 cm wide enclosure. Between the two platforms is a 1.5 cm wide beam that the rat must traverse in order to enter the sheltered home cage opposite the starting platform. Rats were trained using a 9 cm wide beam and gradually trained to cross the 1.5 cm beam over the course of 3 weeks.

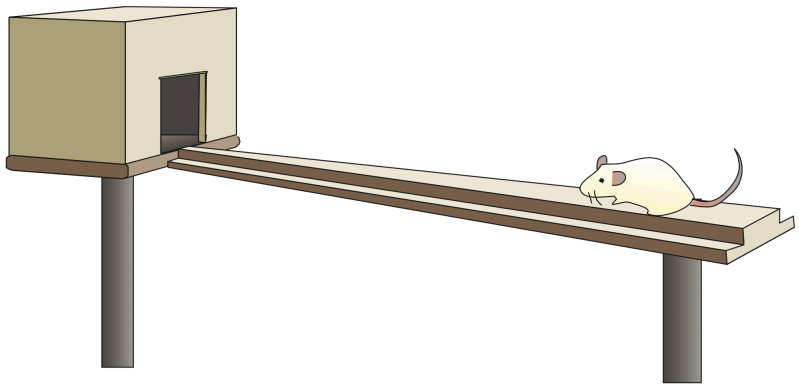


Figure 10. A schematic of the narrow beam traversal task (Brooks & Dunnett, 2009).

3.4.3 Footprint Analysis

Footprint analysis is a test of motor function specifically looking at gait. Reduced stride length in the 6-OHDA rats is similar to the shuffling gait observed in patients with PD. Additionally, the compensatory shift of weight support to the unaffected side, as measured by contralateral overlap, is seen in PD patients (Klein, Wessolleck, Papazoglou, Metz, & Nikkhah, 2009). To obtain footprints, the hind- and forefeet of the rats were coated with blue and orange nontoxic paints, respectively. The rat was then prompted to walk along a 172 cm runway, with 25.5 cm high walls and 12.5 cm wide over a blank sheet of white paper. All rats had 8 training runs, followed by biweekly test runs. The footprint patterns were analyzed for four step parameters (all measured in centimeters).

(1) Contralateral hindlimb overlap measured as the horizontal overlapping distance between the fore- and hindlimb of the side of the body that is opposite to the infusion; (2) Ipsilateral hindlimb overlap measured as the horizontal overlapping distance between the fore- and hindlimb of the side of the body that is the same side as the infusion; (3) Contralateral hindlimb stride length as the distance between the two consecutive steps of the same paw; (4) and ipsilateral hindlimb stride length as the distance between the two consecutive steps of the same paw. Three values from each run were taken for each parameter. The mean value of each set of three values was used in subsequent analysis (Carter et al., 1999). The initial 15 cm were excluded from each footprint sheet.

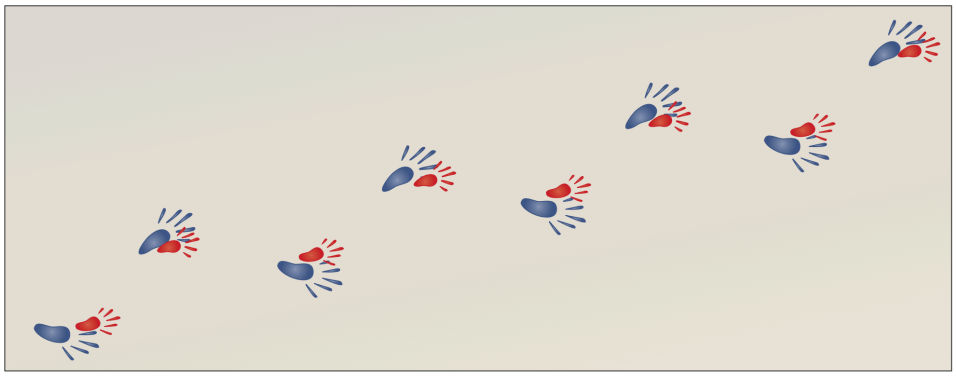


Figure 11. A schematic of the footprint task (Brooks & Dunnett, 2009).

3.4.4 The Fixed Speed Rotarod Test

The fixed speed rotarod (FSRR) test apparatus (Digital Rota Rod, Bluefic Rotarod, India) is a motor-driven revolving rod used to measure fore- and hindlimb motor coordination and balance. The rotarod instrument was designed as a way 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 & Dunnett, 2009). The FSRR test has been reported to be more sensitive in detecting small lesions (Monville, Torres, & Dunnett, 2006).

A rat is placed on a rotating rod approximately 2.75 inches in diameter in the opposite direction of rotation and has to maintain its balance, whereby switch on the floor below records the latency until the rat falls. Rats are tested at 10, 20 and 35 rotations per minute (RPM) for three trials at each fixed speed. During the four day training period, each rat was placed on the rotarod at a constant speed (16 RMP) for a maximum of 60 seconds for three consecutive trials. The mean latency to fall off the rotarod (for the two trials at each speed level) was recorded and used in subsequent analysis (Carter et al., 1999).

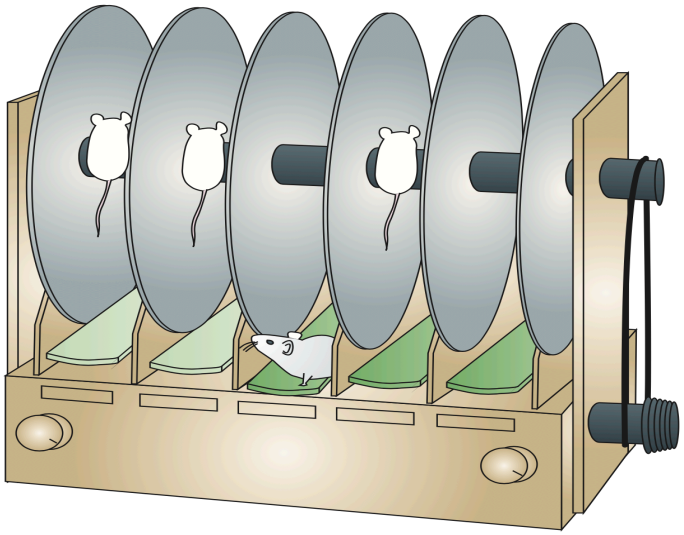


Figure 12. A schematic of a rotarod (Brooks & Dunnett, 2009).

3.4.5 The Cylinder Test

The cylinder test was initially created in order 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 voluntary forelimb use (Barker, 2001; Cenci & Lundblad, 2005).

The cylinder test uses the exploratory behaviour of rodents placed in an unfamiliar environment, whereby the rodents rear by standing on their hindlimbs and the press their forelimbs against the walls of the cylinder to support their bodies as they survey their surroundings (Schallert, Fleming, Leasure, Tillerson, & Bland, 2000). The asymmetry in a rodent's forelimb use provides a measure for forelimb akinesia after the rodent is placed in a transparent cylinder 31 cm in height and 30 cm in diameter (Kriks et al., 2011). The independent use of the left or right forelimb to contact the cylinder during a rear was scored for a total of 20 independent forelimb contacts. The initial limb to make contact with the cylinder wall was recorded and the rodent had to remove both forepaws from the cylinder wall before another independent forepaw wall contact was scored. Only wall contacts with fully extended digits are counted. Data are expressed as the number of ipsilateral forelimb wall contacts per 20 wall contacts (Kriks et al., 2011).

Rodents with a unilateral 6-OHDA lesion showed a decrease in the utilization of the contralateral forepaw when rearing to explore the cylindrical environment (Iancu, Mohapel, Brundin, & Paul, 2005). This test requires no training (Brooks & Dunnett, 2009). Due to the asymmetry between the affected and unaffected forelimbs, each animal can serve as its own control for individual differences in motivation and exploration,

however, a control group was used for additional comparison of ipsilateral paw preference.

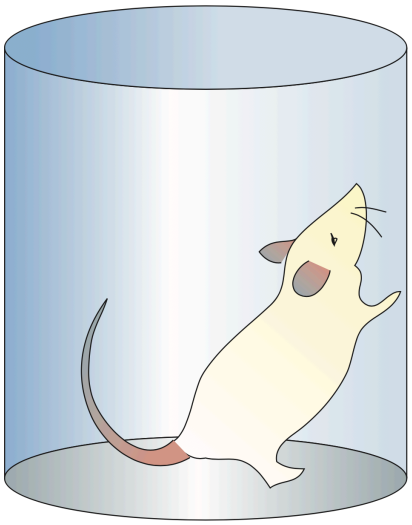


Figure 13. A schematic of the cylinder test (Brooks & Dunnett, 2009).

3.5 Quantification of CDNF in the Platelets of PD Patients

3.5.1 Participant Enrollment

Before participant recruitment, ethical approval to collect blood samples from subjects for the purpose of CDNF quantification in platelets was obtained from the Hamilton Integrated Research Ethic Board in Hamilton, Ontario, and the Research Ethics Board at McMaster University. More specifically, patients with PD were recruited through collaboration with the movement disorder clinics Rathborne Clinic and Meyer's Clinic in Hamilton, Ontario. Additionally, a physical examination from a general practitioner and a follow-up confirmation by a neurologist confirmed the diagnosis of PD. The study's inclusion criteria included a primary diagnosis of PD with no additional movement disorders, such as essential tremor or amyotrophic lateral sclerosis. Exclusion

criteria included patients with a history of renal failure and/or on dialysis, currently on dopamine blockers and/or comorbid for neuropsychiatric illness. Healthy subjects were recruited and matched for age and sex with the PD group were recruited at from the Medical Ambulatory Care outpatient clinic at Juravinski Hospital and from McMaster University in Hamilton, Ontario. The healthy controls had no diagnosis of PD and any of the exclusion criteria, and were included if they had diabetes, high or low blood pressure and headaches. All subjects provided written informed consent and were not given compensation for their involvement in this study.

3.5.2 Blood Collection

Venous blood samples (approximately 20 mL) were collected via phlebotomy into two 10 mL BD vacutainer tubes with 1.42 mL of acetate citrate dextrose.

3.5.3 Platelet Preparation

The collected blood was centrifuged at 980 × g for 2 minutes in an Eppendorf 5810R centrifuge (Hamburg, Germany) at 22°C. After centrifugation, red blood cells collect at the bottom of the vial and the pale yellow blood serum forms the upper layer. Subsequently, the upper two-thirds of the blood serum is removed using a siliconized Pasteur pipette and placed into a 15 mL conical centrifuge tube. The sample was then centrifuged further at 1200 × g for 7 minutes after which the supernatant was removed and the pellet was resuspended in 7 mL of cold PEB solution. The supernatant was removed once again and the pellet was resuspended in 1 mL of cold PEB and transferred to a 1.5 mL Eppendorf tube and centrifuged at 1200 × g for 7 minutes in an Eppendorf 5415R microcentrifuge (Hamburg, Germany). The supernatant was carefully removed

and the pellet was stored at -80°C for future use.

3.5.3 RNA Isolation from Platelets & cDNA Synthesis

RNA was extracted using TRIzol reagent (Thermo Fisher Scientific Incorporated, Waltham, Massachusetts, USA). Pellets were suspended in 1 mL of TRIzol solution and incubated at room temperature for 5 minutes. 0.2 mL of chloroform was added to each mL of Tri-pure isolation reagent, and then agitated. Samples were incubated for 20 minutes at room temperature and then centrifuged at 12 000 × g for 15 minutes. The colourless RNA phase was then removed and transferred to a 1.7 mL Eppendorf, after which 0.5 mL of iso-propanol was added. Samples were again incubated at room temperature for 10 minutes and then centrifuged for 10 minutes at 12 000 × g. The supernatant was promptly discarded and 1 mL of 75% ethanol was added to each sample, vortexed and subsequently centrifuged at maximum speed for 15 minutes. The supernatant was then discarded and the RNA was resuspended in 20 µL of DEPC-Treated RNase-free water and heated to 55°C for 10 minutes. Afterwards, a DNase I kit (Invitrogen Life Technologies, Burlington, Ontario, Canada) was used to remove any contaminating DNA. RNA purity and quantity were determined using a Beckman Spectrophotometer (DU-640) measuring absorption at 260 nm and 280 nm. The isolated RNA was used to synthesize cDNA whereby all RNA samples were diluted to 25 ng/µL and 16 µL of RNA along with 4 µL of the qScript cDNA SuperMix were used (Quanta Biosciences, Gaithersburg, MD).

3.5.4 mRNA Quantification Using qPCR

Real-Time quantitative PCR (qPCR) was used to determine mRNA copy numbers of CDNF in platelets. qPCR was performed, for each sample, in triplicates using the MX3000P qPCR and OneStep qPCR kit (QIAGEN Incorporated, Toronto, Ontario, Canada). CDNF primers were designed using OligoPerfectTM Designer software (Invitrogen Life Technologies, Burlington, Ontario, Canada) and were synthesized at MOBIX (McMaster University, Hamilton, Ontario, Canada). The PCR primers used are described (5 μM) (Table 4). Each reaction comprised of 12.5 μL QuantiFast SYBR Green (Qiagen), 5 μL each of the 5μM CDNF primers and nuclease-free water to a final volume of 25 μL and contained 40 ng of total cDNA. A standard curve was generated using varying concentrations of cDNA ranging from 1 attogram to 1 picogram. The thermal profile for the PCR protocol included: heat activation of 95°C for 5 minutes and cycle conditions were 95 degrees for 10 seconds and 60 degrees for 30 seconds as the annealing and extension steps were combined with the QuantiFast SYBR Green PCR Kit (Qiagen). Real Time qPCR conditions were optimized to ensure amplifications occurred in the exponential phase and efficiencies remained constant throughout and that no primer-dimers were detected over the course of the run.

MX3000P software performs analysis of data obtained to quantify the mRNA copy number of the target sequence. Sample values run in triplicates were averaged prior to analysis. All qPCRs were analyzed through an absolute quantification method based on the standard curve. Cycle threshold values and the standard curve generated by the MX3000P software produce a copy number that is indicative of the number of copies of

cDNA transcript originally present in the reaction. All samples were multiplied by a standardizing factor which set the average of the controls to be approximately 100 for analysis purposes. An unpaired Student's *t*-test was used to determine the difference in CDNF mRNA expression between PD patients and healthy control subjects.

Table 4. Primers used and to be used for Real Time qPCR quantification of GAPDH and CDNF mRNA expression in platelets of PD patients and from the striatum and SN of rats, respectively.

Gene	Primer	Sequence
Human *GAPDH*	Forward	5'-GAG TCA ACG GAT TTG GTC GT-3'
	Reverse	5'-TTG ATT TTG GAG GGA TCT CG-3'
Human *CDNF*	Forward	5'-GGG CCG ACT GTG AAG TAT GT-3'
	Reverse	5'-GGC ATA TGC ACA CTC ATT GG-3'
Rat *Cdnf*	Forward	5'-AAA GAA AAC CGC CTG TGC TA-3'
	Reverse	5'-TCA TTT TCC ACA GGT CCA CA-3'

3.6 Sacrifice

Following behavioural testing, rats will be anaesthetized with isofluorane (Pharmaceutical Partners of Canada Inc., Richmond Hill, ON) and decapitated in accordance with McMaster University's Central Animal Facility (CAF). Both the left and right hemisphere's striatum and SN will be collected and stored at –80 degrees Celsius for future analyses. A separate cohort of 12 rats infused bilaterally with MANF, CDNF or both MANF and CDNF lentiviral-mediated shRNA were sacrificed 1 month post surgery and the striatum and SN regions of both sides of the brain were collected in order to evaluate the mRNA expression of these genes.

3.7 Statistics

All statistical analyses were carried out using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Before analyses, outlier detection was performed using the GraphPad Outlier Tool. Results were analyzed using an unpaired Student's *t*-test as well as a Two Way ANOVA followed by Tukey's multiple comparison, whereby significance was defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

Results are presented as plus or minus the standard error of the mean (SEM) as it takes into account the standard deviation (SD) as well as the sample size in quantifying the precision of the mean, which is indicative of how far the result means are likely to be from the true population mean. The SD simply quantifies variability in terms of how much values differ from one another.

CHAPTER 4: RESULTS

The following is a table summarizing the effects of MANF and CDNF on tested behavioural parameters.

Table 5. Behavioural Testing Results Summary. ✔ and ✗ denote significant (in at least two time points) and no significant differences compared to control rats, respectively.

Behavioural Test	MANF	CDNF	MANF & CDNF
Total Distance	✗	✗	✗
Rearing Duration	✔	✔	✔
Beam Traversal			
Latency Time	✔	✗	✔
Traversal Time	✔	✔	✔
Contralateral Errors	✔	✗	✗
Total Errors	✔	✔ (inconsistent)	✗
Footprint			
Contralateral Overlap	✗	✗	✗
Ipsilateral Overlap	✔ (inconsistent)	✗	✔ (inconsistent)
Contralateral Hindlimb Stride Length	✗	✗	✗
Ipsilateral Hindlimb Stride Length	✗	✗	✗
Fixed Speed Rotarod			
4 Months	✔	✗	✔ (35 RPM)
5 Months	✗	✔	✔ (35 RPM)
Cylinder	✔	✗	✗

4.1 Locomotor Activity: Total Distance Travelled

4.1.1 MANF K/D

After rats were unilaterally infused with lentiviral-mediated MANF shRNA into the SN (N=8), the total distance travelled did not differ compared to control rats (N=8) (*P*

> 0.05), however time did have an effect on total distance travelled within each treatment group ($P < 0.05$).

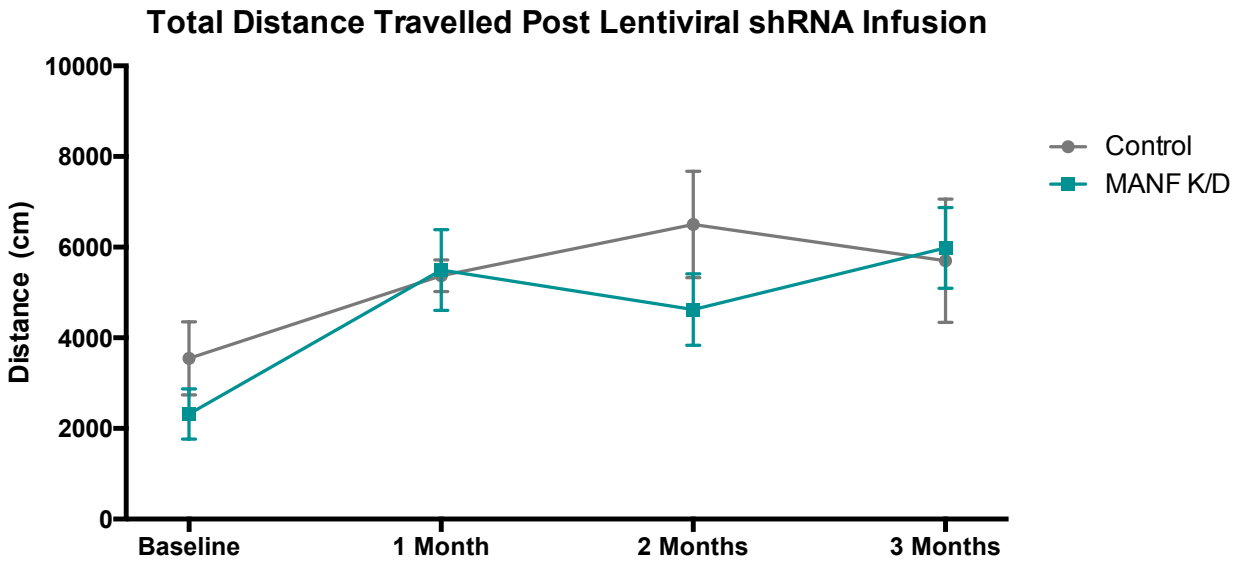


Figure 14. The effect of lentiviral-mediated MANF shRNA on the total distance travelled (cm) in rats. Rats (N=16) were tested for locomotor activity for 2 hours in 10 minute intervals. A Two Way ANOVA followed by Tukey's multiple comparison test revealed that treatment did not have a statistically significant effect: F (1, 47) = 1.069; $P = 0.3064$. Time had a statistically significant effect: F (3, 47) = 4.378; $P = 0.0085$. MANF K/D at 3 months showed a significant increase compared to baseline MANF K/D levels ($P < 0.05$). Data are represented as the mean ± SEM values.

4.1.2 CDNF K/D

After rats were unilaterally infused with lentiviral-mediated CDNF shRNA into the SN (N=8), the total distance travelled did not differ compared to control rats (N=8) ($P > 0.05$), however time did have an effect on total distance travelled within each treatment group ($P < 0.05$).

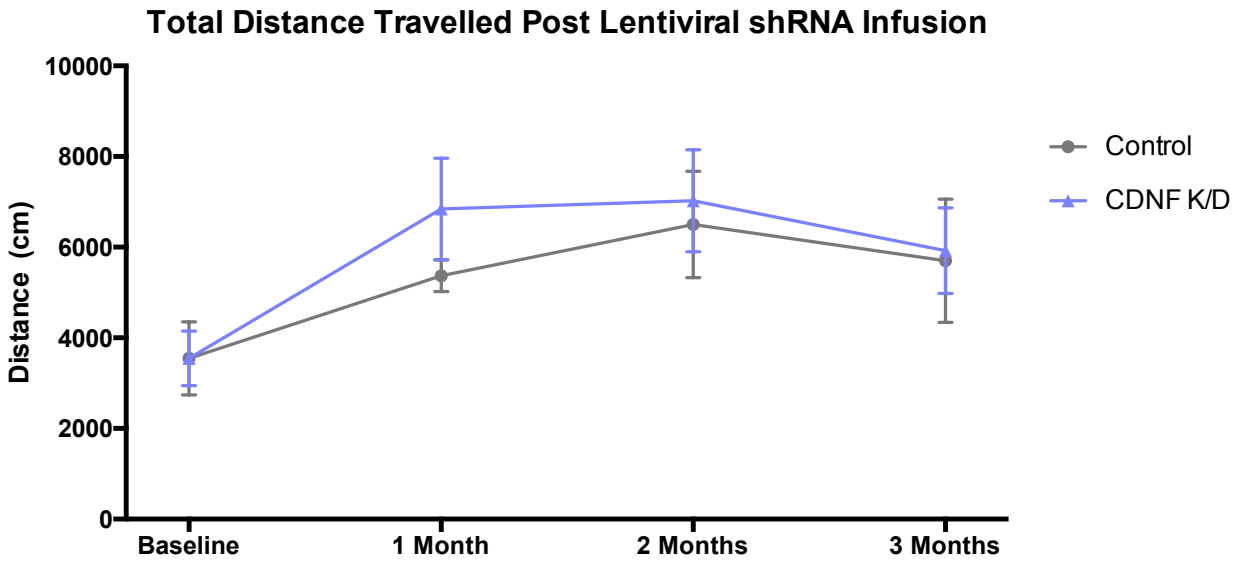


Figure 15. The effect of lentiviral-mediated CDNF shRNA on the total distance travelled (cm) in rats. Rats (N=16) were tested for locomotor activity for 2 hours in 10 minute intervals. A Two Way ANOVA followed by Tukey's multiple comparison test revealed that treatment did not have a statistically significant effect: F (1, 47) = 0.6105; *P* = 0.4385. Time had a statistically significant effect: F (3, 47) = 3.927; *P* = 0.0140. Data are represented as the mean ± SEM values.

4.1.3 MANF & CDNF K/D

After rats were unilaterally infused with lentiviral-mediated MANF and CDNF shRNA into the SN (N=8), the total distance travelled did not differ compared to control rats (N=8) ($P > 0.05$), however time did have an effect on total distance travelled within each treatment group ($P < 0.05$).

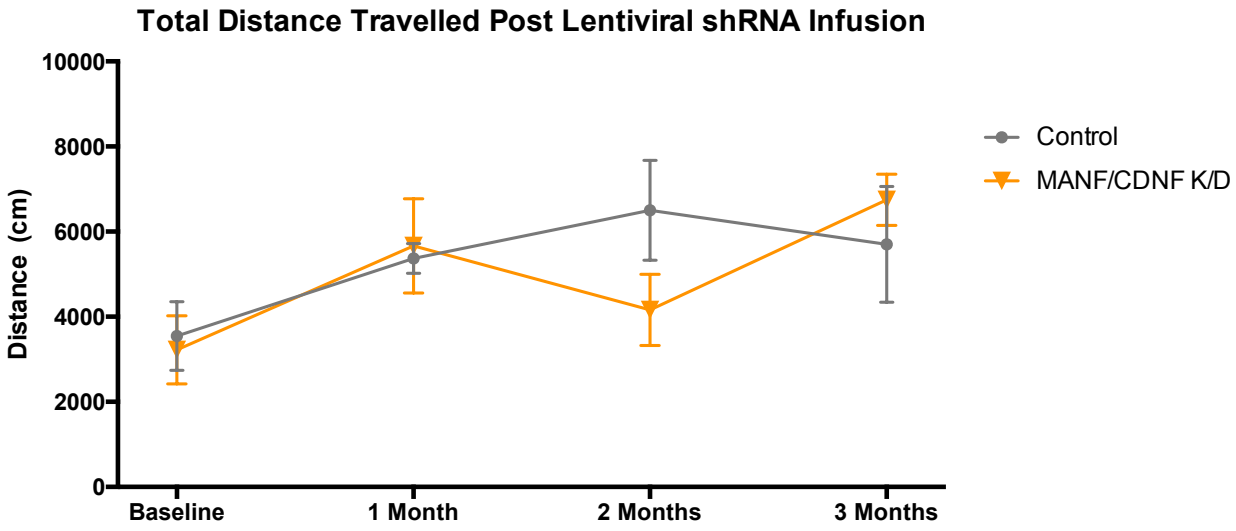


Figure 16. The effect of lentiviral-mediated MANF and CDNF shRNA on the total distance travelled (cm) in rats. Rats (N=16) were tested for locomotor activity for 2 hours in 10 minute intervals. A Two Way ANOVA followed by Tukey's multiple comparison test revealed that treatment did not have a statistically significant effect: F (1, 46) = 0.2357; $P = 0.6296$. Time had a statistically significant effect: F (3, 46) = 3.239; $P = 0.0305$. Data are represented as the mean ± SEM values.

4.2 Rearing Duration

4.2.1 MANF K/D

2.5, 3 and 5 months after rats were unilaterally infused with lentiviral-mediated MANF shRNA into the SN (N=8), the rearing duration increased compared to control rats (N=8) ($P < 0.05$).

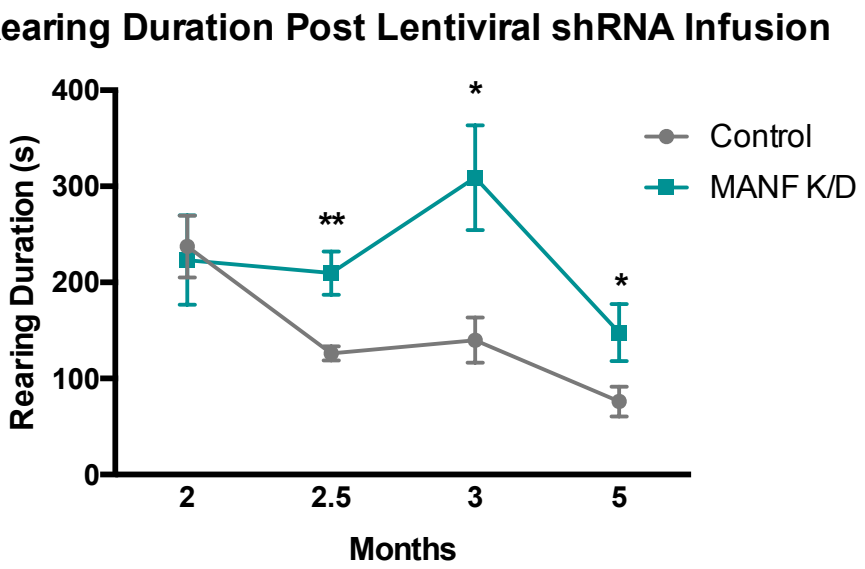


Figure 17. The effect of lentiviral-mediated MANF shRNA on the rearing duration (s) in rats. Rats (N=16) were tested for locomotor activity for 2 hours in 10 minute intervals. An unpaired t-test revealed that MANF K/D rats showed significant increases in rearing duration at 2.5, 3 and 5 months after lentiviral-mediated shRNA infusion ($P < 0.05$). Data are represented as the mean ± SEM values.

4.2.2 CDNF K/D

2.5, and 3 months after rats were unilaterally infused with lentiviral-mediated CDNF shRNA into the SN (N=8), the rearing duration increased compared to control rats (N=8) ($P < 0.05$).

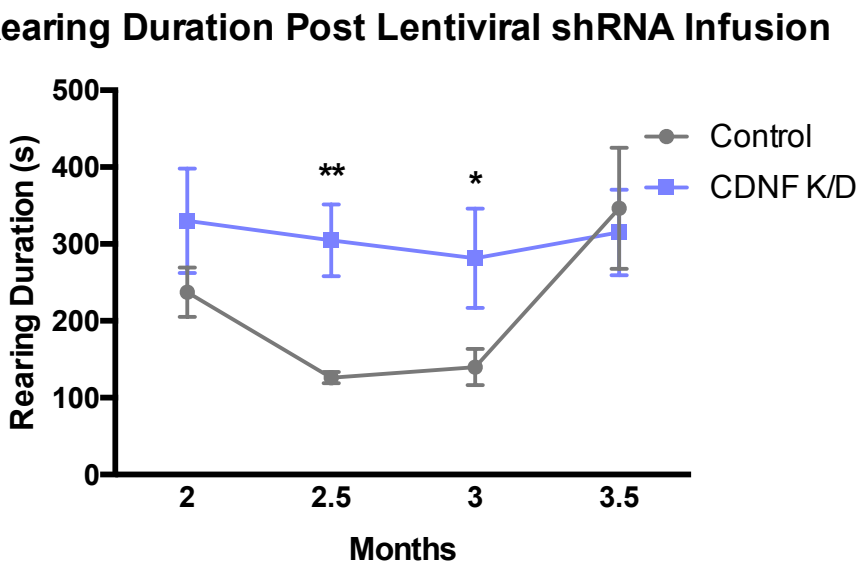


Figure 18. The effect of lentiviral-mediated CDNF shRNA on the rearing duration (s) in rats. Rats (N=16) were tested for locomotor activity for 2 hours in 10 minute intervals. An unpaired t-test revealed that CDNF K/D rats showed significant increases in rearing duration at 2.5, and 3 months after lentiviral-mediated shRNA infusion ($P < 0.05$). Data are represented as the mean ± SEM values.

4.2.3 MANF & CDNF K/D

2.5, and 3 months after rats were unilaterally infused with lentiviral-mediated MANF and CDNF shRNA into the SN (N=8), the rearing duration increased compared to control rats (N=8) ($P < 0.05$).

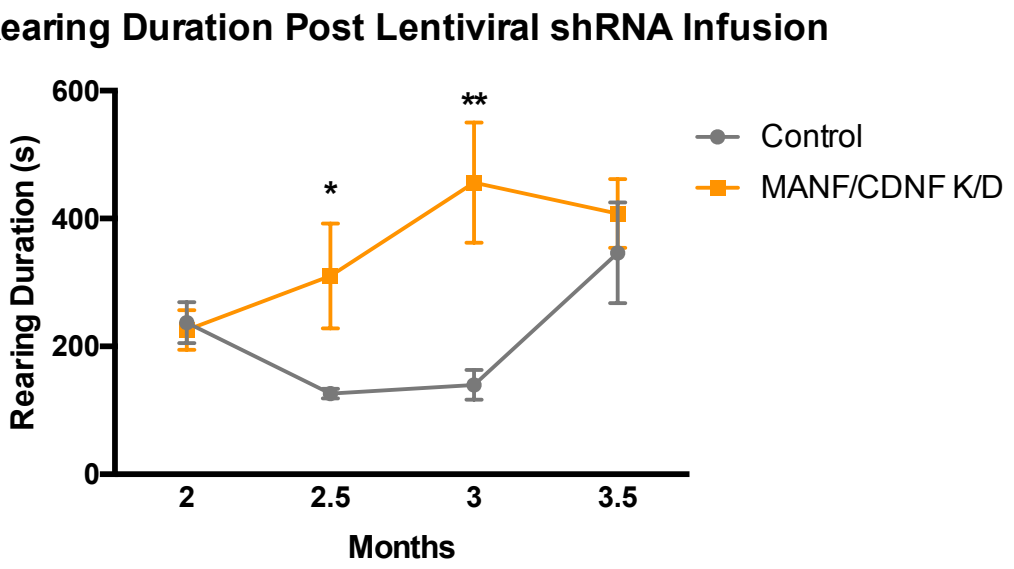


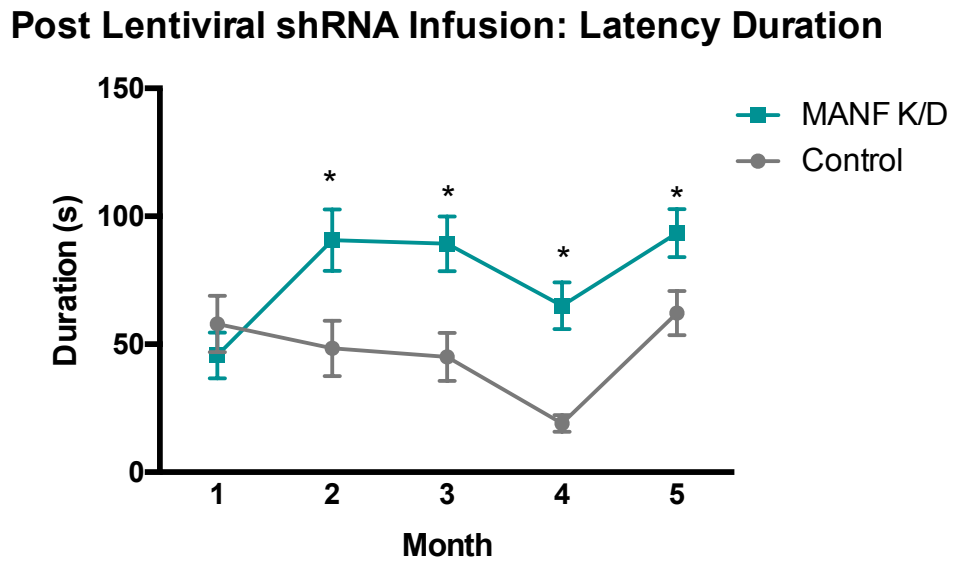
Figure 19. The effect of lentiviral-mediated MANF and CDNF shRNA on the rearing duration (s) in rats. Rats (N=16) were tested for locomotor activity for 2 hours in 10 minute intervals. An unpaired t-test revealed that MANF and CDNF K/D rats showed significant increases in rearing duration at 2.5, and 3 months after lentiviral-mediated shRNA infusion ($P < 0.05$). Data are represented as the mean ± SEM values.

4.3 Narrow Beam Traversal Test

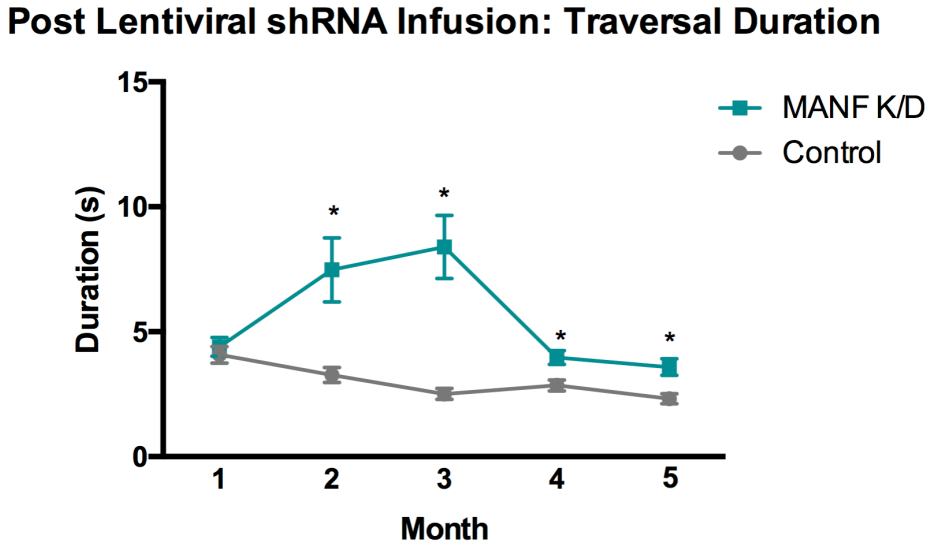
4.3.1 MANF Knockdown Results

2, 3, 4 and 5 months after rats were unilaterally infused with lentiviral-mediated MANF shRNA into the SN, the beam traversal test revealed a significant increase in the latency and traversal durations, as well as contralateral and total slips compared to control rats ($P < 0.05$).

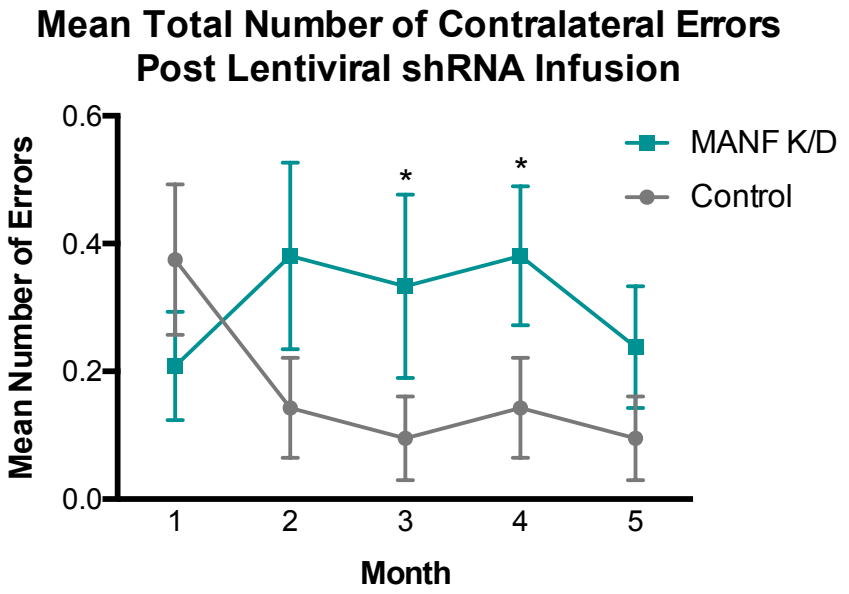
A.


Post Lentiviral shRNA Infusion: Latency Duration
MANF K/D
Control
Duration (s)
150
100
50
0
1
2
3
4
5
Month
*
*
*
*

B.


Post Lentiviral shRNA Infusion: Traversal Duration
MANF K/D
Control
Duration (s)
15
10
5
0
1
2
3
4
5
Month
*
*
*
*

C.



D.

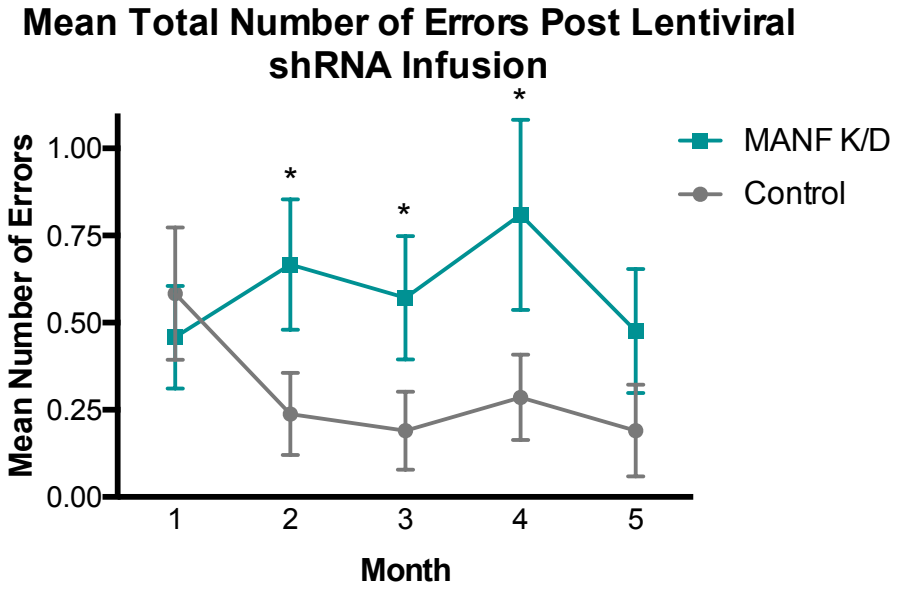
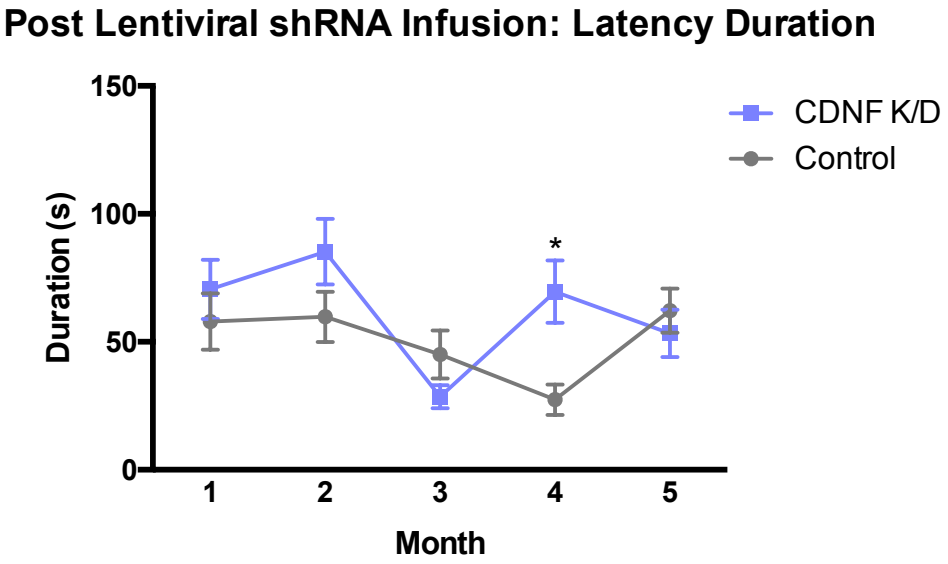


Figure 20. The effect of lentiviral-mediated MANF shRNA on the latency (A) and traversal (B) duration (seconds), contralateral slips (C) and total slips (D) in rats 1 to 5 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter. An unpaired Student *t*-test revealed that treatment did have a statistically significant effect in the latency and traversal duration, as well as contralateral and total slip parameters compared to control rats, $P < 0.05$. Data are represented as the mean ± SEM values.

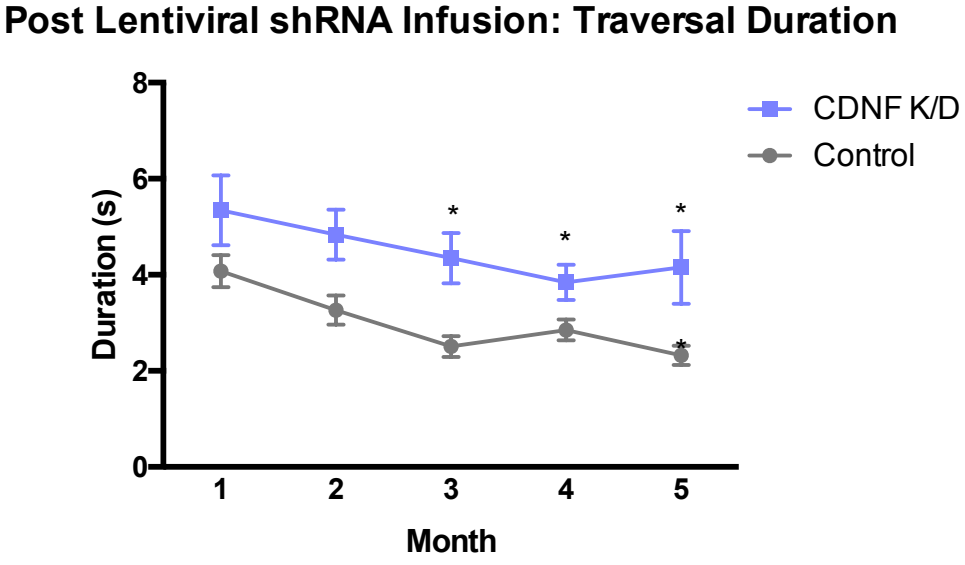
4.3.2 CDNF Knockdown Results

2, 3, 4 and 5 months after rats were unilaterally infused with lentiviral-mediated CDNF shRNA into the SN, the beam traversal test revealed a significant increase in the traversal duration, as well as total slips compared to control rats ($P < 0.05$).

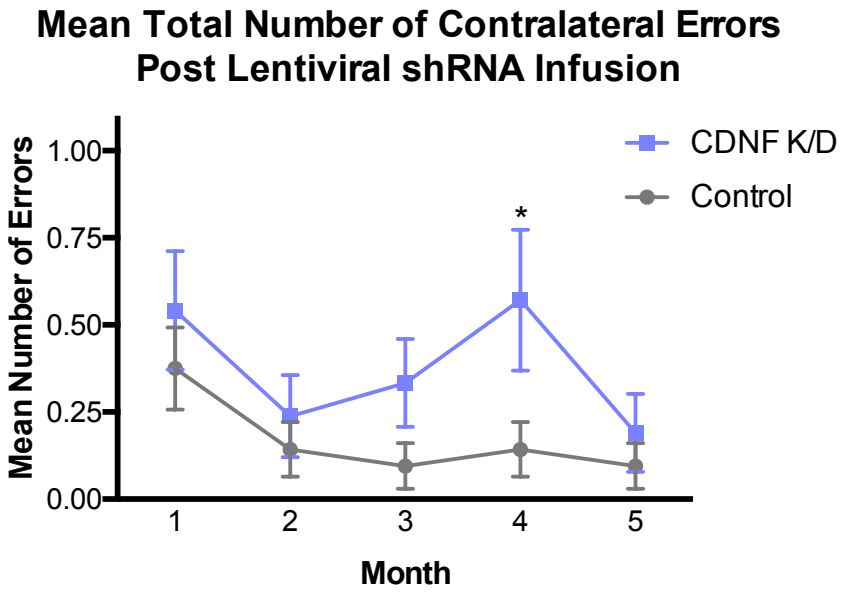
A.



B.



C.



D.

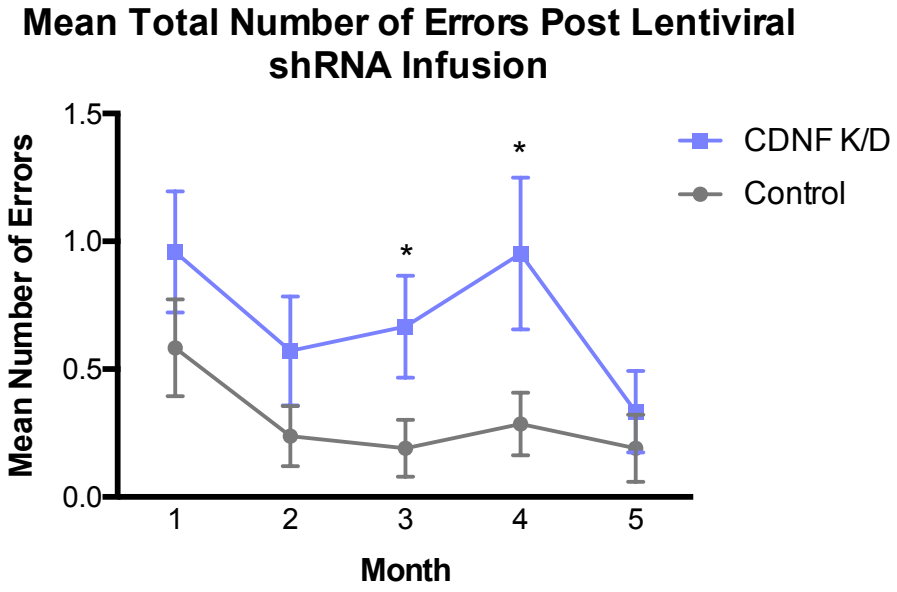
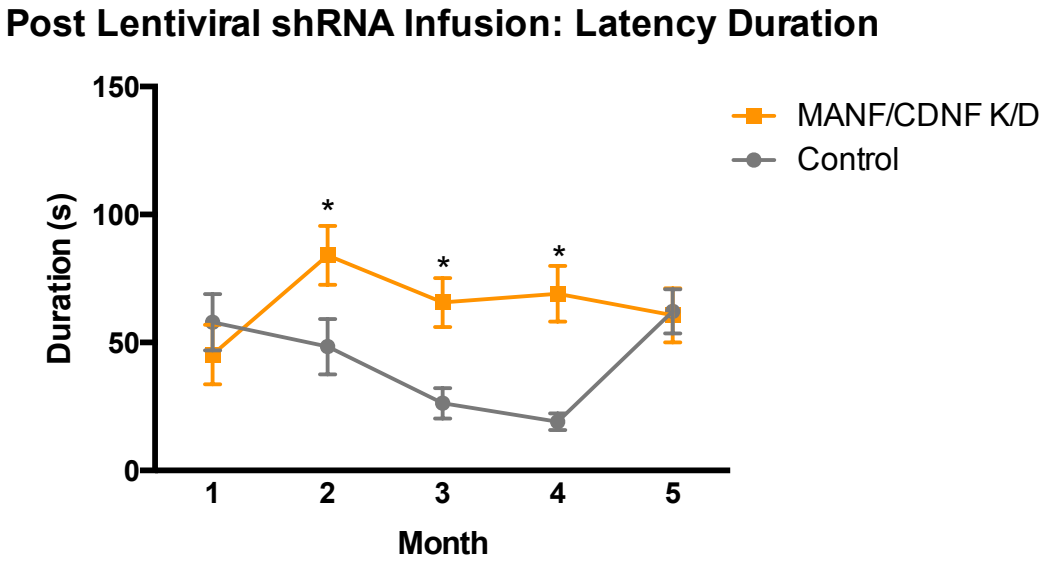


Figure 21. The effect of lentiviral-mediated CDNF shRNA on the latency (A) and traversal (B) duration (seconds), contralateral slips (C) and total slips (D) in rats 1 to 5 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter. An unpaired t-test revealed that treatment did have a statistically significant effect in the traversal duration and total errors parameters compared to control rats, $P < 0.05$. Data are represented as the mean ± SEM values.

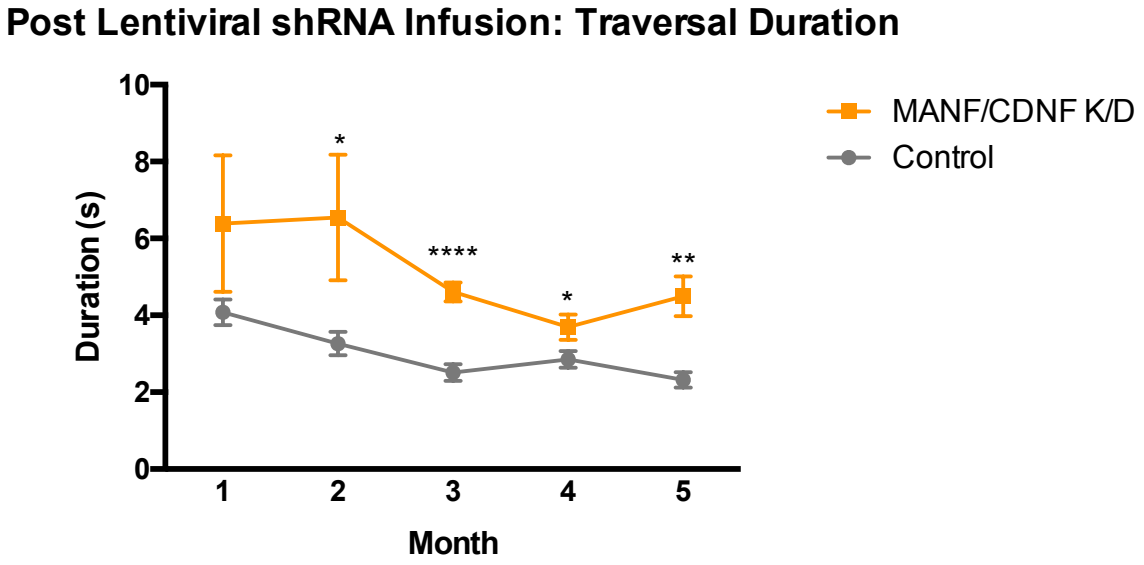
4.3.3 CDNF & MANF Knockdown Results

2, 3, 4 and 5 months after rats were unilaterally infused with lentiviral-mediated MANF and CDNF shRNA into the SN, the beam traversal test revealed a significant increase in the latency and traversal durations compared to control rats ($P < 0.05$).

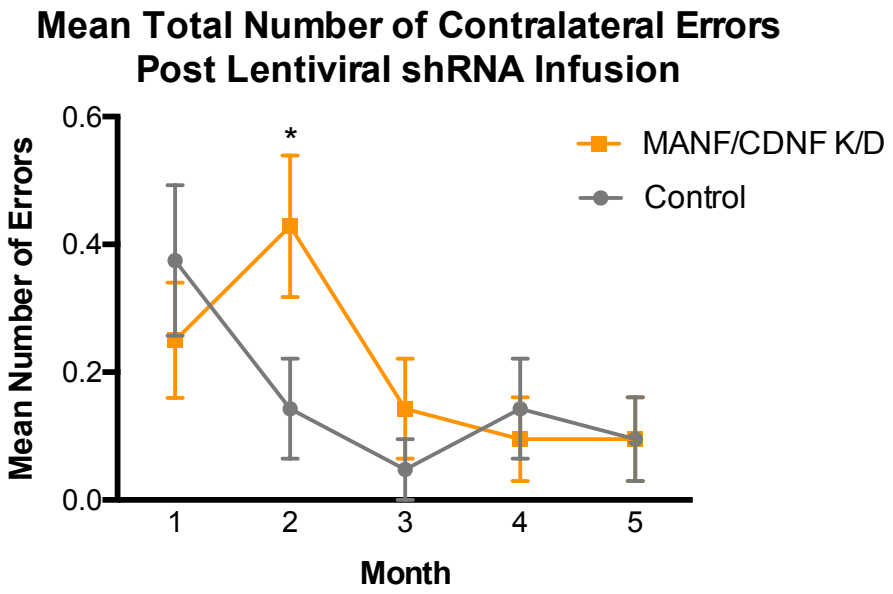
A.



B.



C.



D.

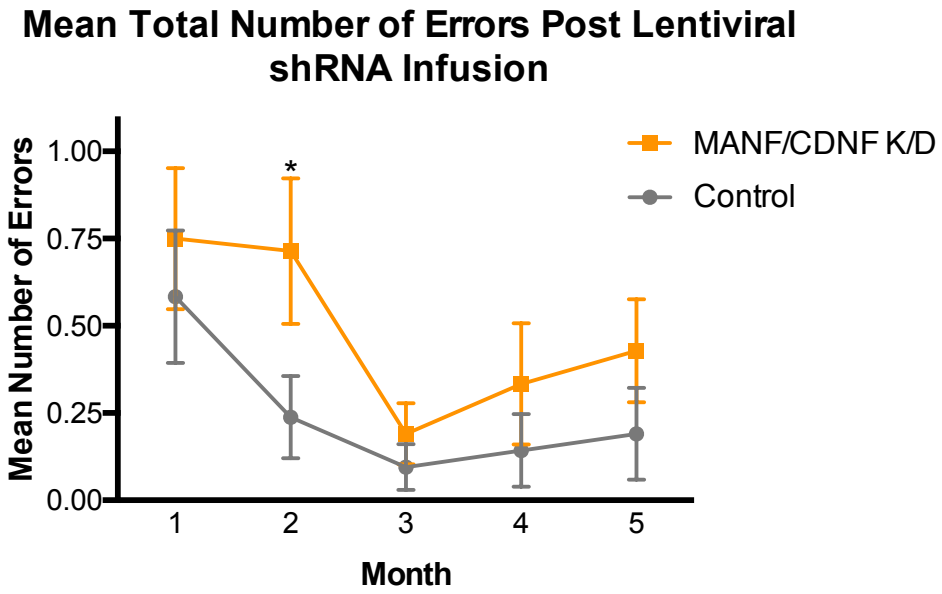


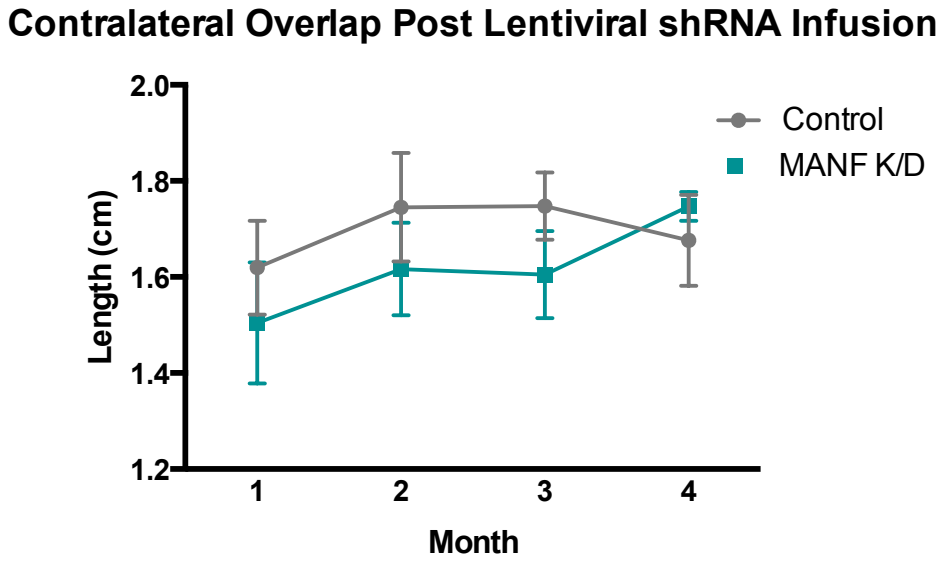
Figure 22. The effect of lentiviral-mediated MANF and CDNF shRNA on the latency (A) and traversal (B) duration (seconds), contralateral slips (C) and total slips (D) in rats 1 to 5 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter. An unpaired t-test revealed that treatment did have a statistically significant effect in the latency and traversal durations parameters compared to control rats, $P < 0.05$. Data are represented as the mean ± SEM values.

4.4 Footprint Analysis

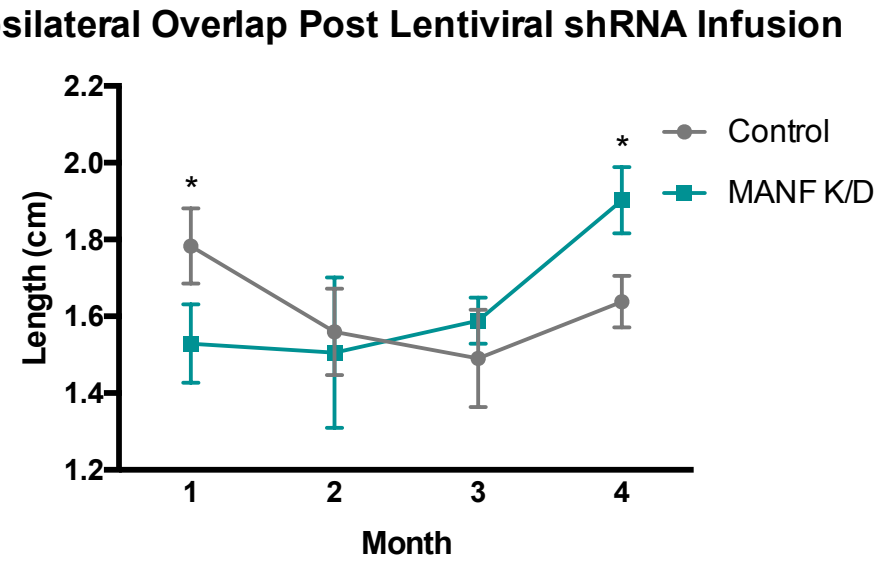
4.4.1 MANF K/D

1, 2, 3, and 4 months after rats were unilaterally infused with lentiviral-mediated MANF shRNA into the SN, footprint analysis revealed significant increases in ipsilateral overlap compared to control rats ($P < 0.05$). The contralateral overlap, and contralateral and ipsilateral hindlimb stride lengths did not reveal any significant changes compared to control rats ($P>0.05$).

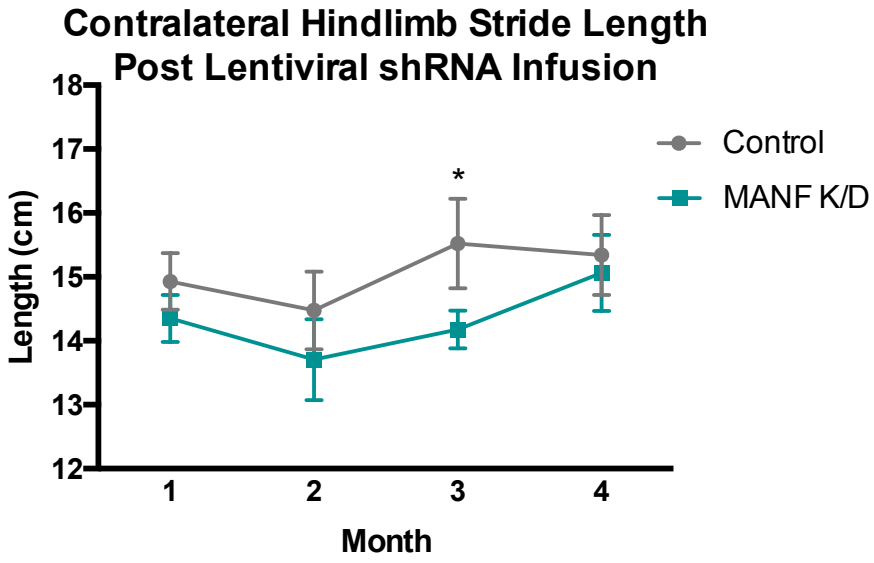
A.



B.


Ipsilateral Overlap Post Lentiviral shRNA Infusion
Length (cm)
2.2
2.0
1.8
1.6
1.4
1.2
1
2
3
4
Month
*
*
Control
MANF K/D

C.


Contralateral Hindlimb Stride Length
Post Lentiviral shRNA Infusion
Length (cm)
18
17
16
15
14
13
12
1
2
3
4
Month
*
Control
MANF K/D

D.

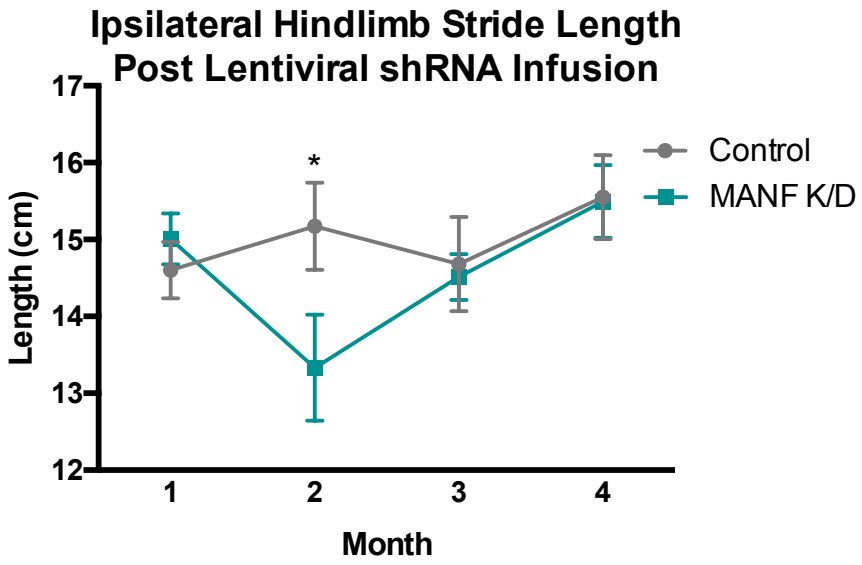
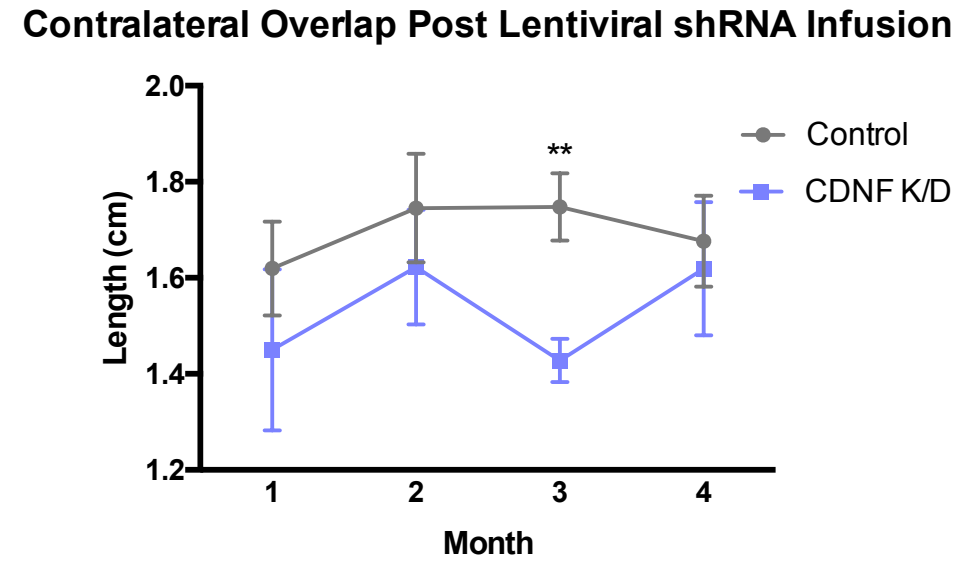


Figure 23. The effect of lentiviral-mediated MANF shRNA on the contralateral overlap (A), ipsilateral overlap (B), contralateral hindlimb stride length (C) and ipsilateral hindlimb stride length (D) in rats 1 to 4 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter. An unpaired t-test revealed that treatment did have a statistically significant effect in the ipsilateral overlap parameter compared to control rats, $P < 0.05$. Data are represented as the mean ± SEM values.

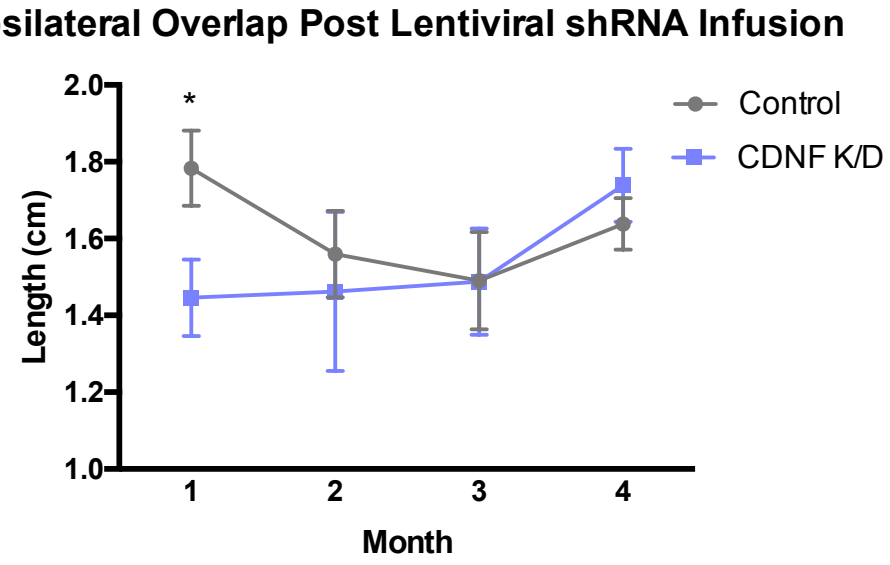
4.4.2 CDNF K/D

1, 2, 3, and 4 months after rats were unilaterally infused with lentiviral-mediated CDNF shRNA into the SN, footprint analysis revealed no significant differences compared to control rats ($P > 0.05$).

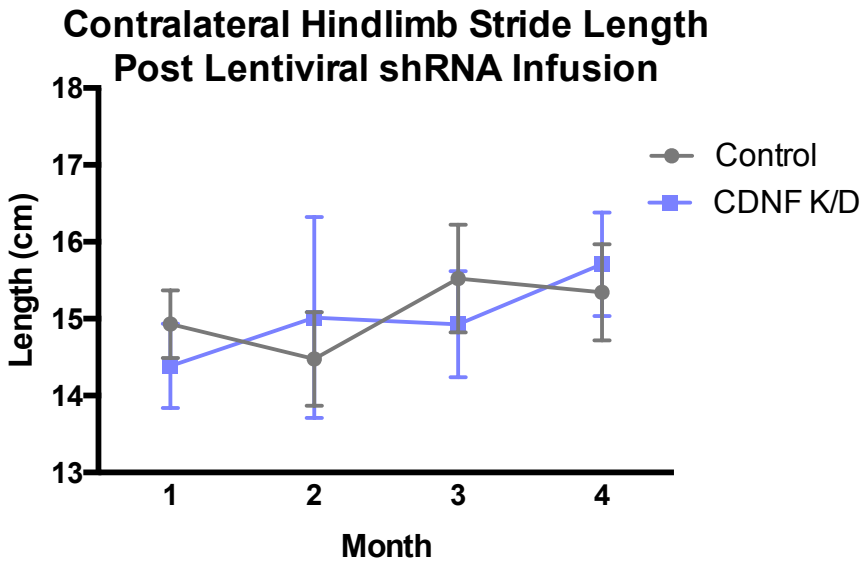
A.


Contralateral Overlap Post Lentiviral shRNA Infusion
2.0
1.8
1.6
1.4
1.2
Length (cm)
**
Control
CDNF K/D
1
2
3
4
Month

B.


Ipsilateral Overlap Post Lentiviral shRNA Infusion
2.0
1.8
1.6
1.4
1.2
1.0
Length (cm)
*
Control
CDNF K/D
1
2
3
4
Month

C.



D.

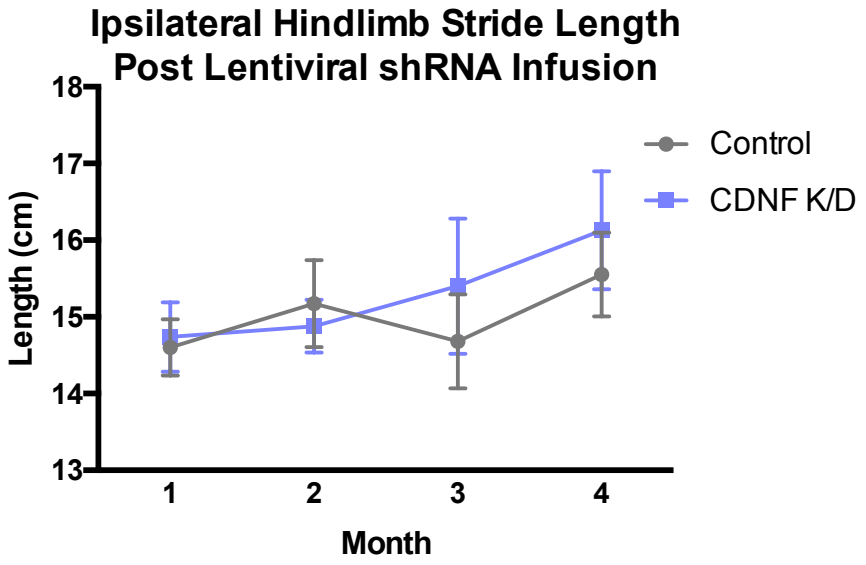
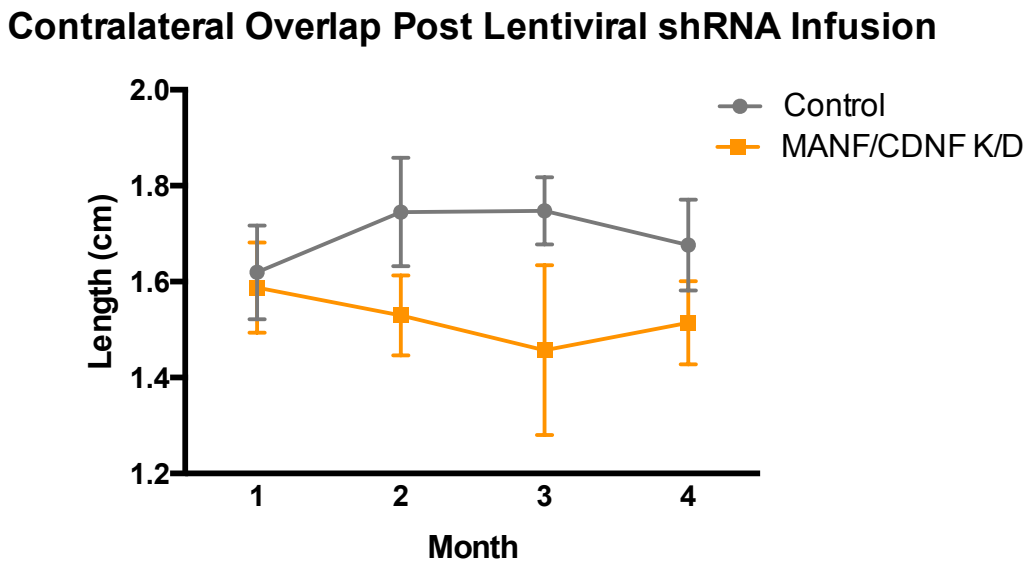


Figure 24. The effect of lentiviral-mediated CDNF shRNA on the contralateral overlap (A), ipsilateral overlap (B), contralateral hindlimb stride length (C) and ipsilateral hindlimb stride length (D) in rats 1 to 4 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter. An unpaired t-test revealed that treatment did not have a statistically significant effect compared to control rats, $P > 0.05$. Data are represented as the mean ± SEM values.

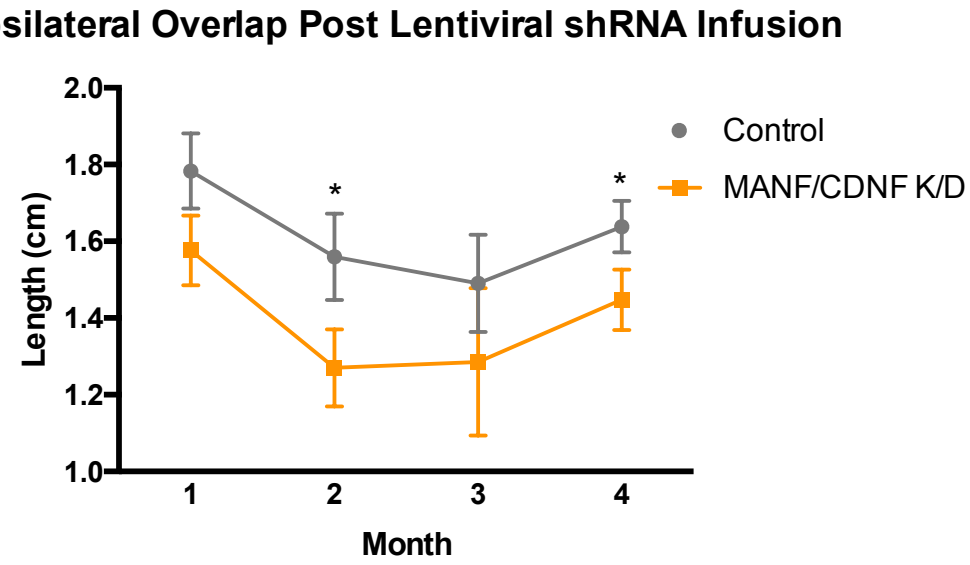
4.4.3 MANF & CDNF K/D

1, 2, 3, and 4 months after rats were unilaterally infused with lentiviral-mediated MANF and CDNF shRNA into the SN, footprint analysis revealed significant increases in ipsilateral overlap compared to control rats ($P < 0.05$). The contralateral overlap, and contralateral and ipsilateral hindlimb stride lengths did not reveal any significant changes compared to control rats ($P>0.05$).

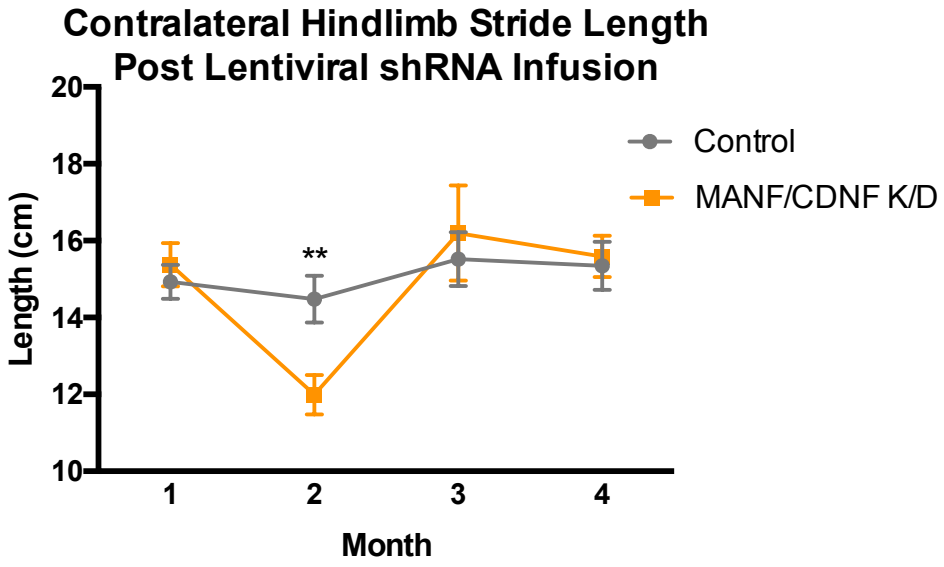
A.



B.


Ipsilateral Overlap Post Lentiviral shRNA Infusion
Length (cm)
2.0
1.8
1.6
1.4
1.2
1.0
1
2
3
4
Month
*
*
Control
MANF/CDNF K/D

C.


Contralateral Hindlimb Stride Length
Post Lentiviral shRNA Infusion
Length (cm)
20
18
16
14
12
10
1
2
3
4
Month
**
Control
MANF/CDNF K/D

D.

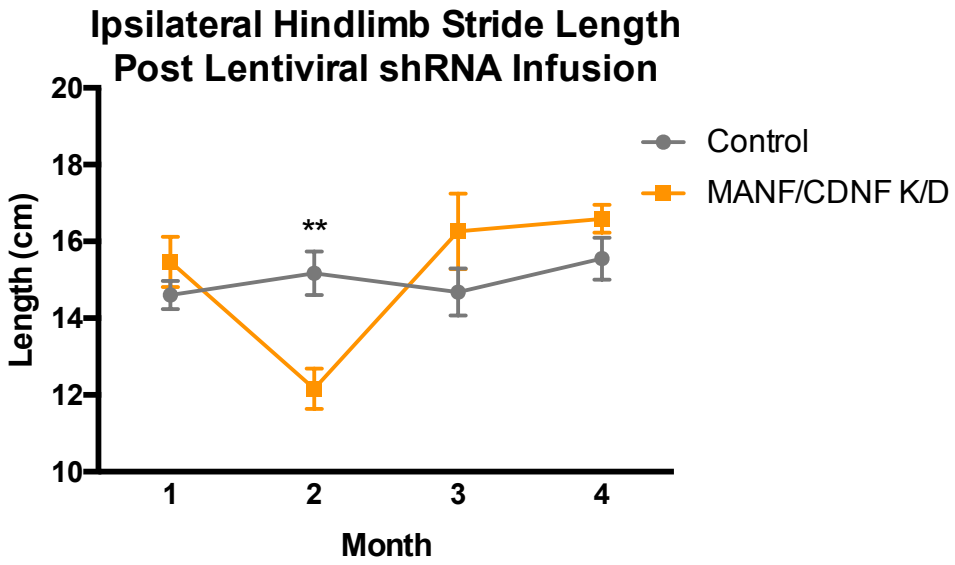


Figure 25. The effect of lentiviral-mediated MANF and CDNF shRNA on the contralateral overlap (A), ipsilateral overlap (B), contralateral hindlimb stride length (C) and ipsilateral hindlimb stride length (D) in rats 1 to 4 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter. An unpaired t-test revealed that treatment did have a statistically significant effect in the ipsilateral overlap parameter compared to control rats, $P < 0.05$. Data are represented as the mean ± SEM values.

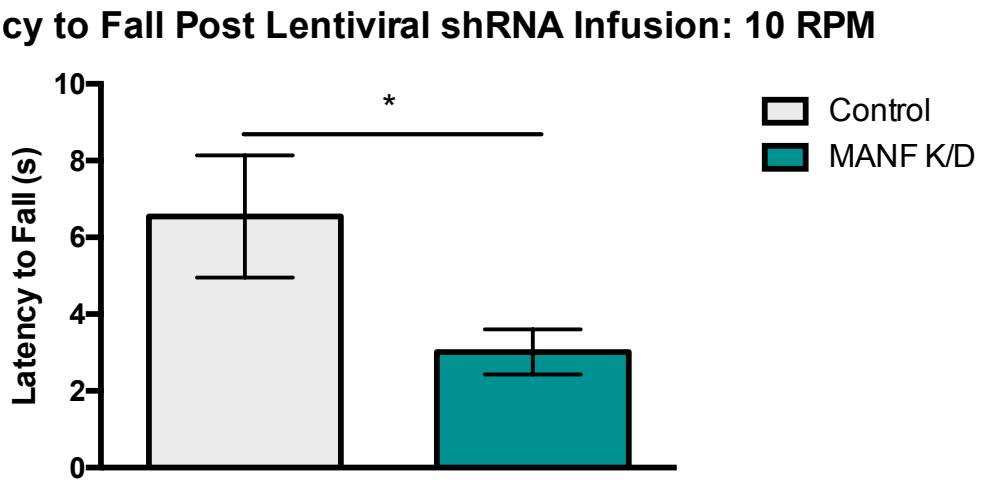
4.5 The Fixed Speed Rotarod Test

4.5.1 MANF K/D

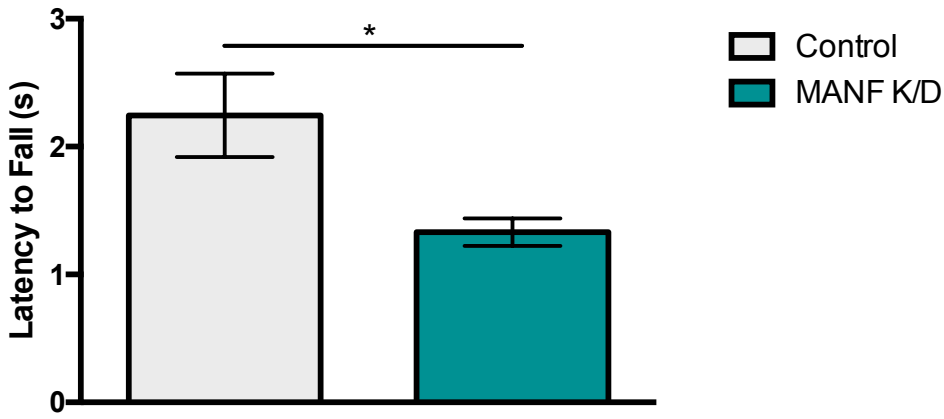
After rats were unilaterally infused with lentiviral-mediated MANF shRNA into the SN, fixed speed rotarod (FSRR) testing revealed significant declines in the latency to fall in MANF K/D rats at 10, 20 and 35 RPM compared to control rats at 4 months ($P < 0.05$), but no differences were found at 5 months ($P > 0.05$).

4 Months

A.


Latency to Fall Post Lentiviral shRNA Infusion: 10 RPM
Latency to Fall (s)
10
8
6
4
2
0
*
Control
MANF K/D

B.


Latency to Fall Post Lentiviral shRNA Infusion: 20 RPM
Latency to Fall (s)
3
2
1
0
*
Control
MANF K/D

C.

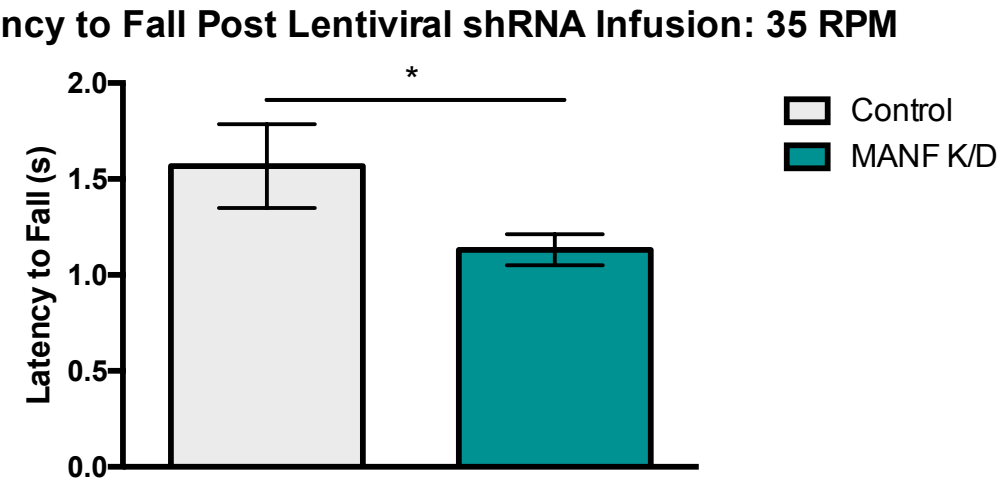
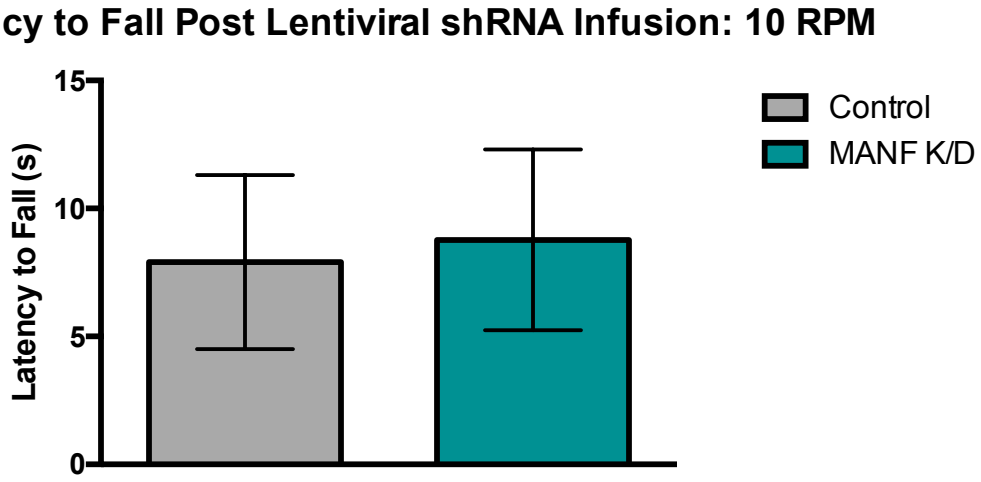


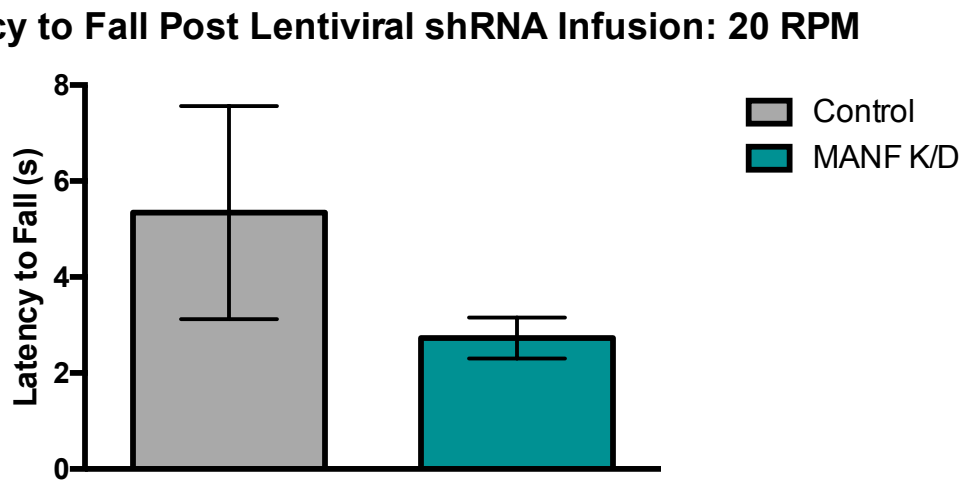
Figure 26. The effect of lentiviral-mediated MANF shRNA on the latency to fall at 10 RPM (A), 20 RPM (B), and 35 RPM (C) 4 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter and the latency to fall was measured in seconds. An unpaired *t*-test revealed that treatment did have a statistically significant effect at 10, 20 and 35 RPM compared to control rats ($P < 0.05$). Data are represented as the mean ± SEM values.

5 Months

A.



B.



C.

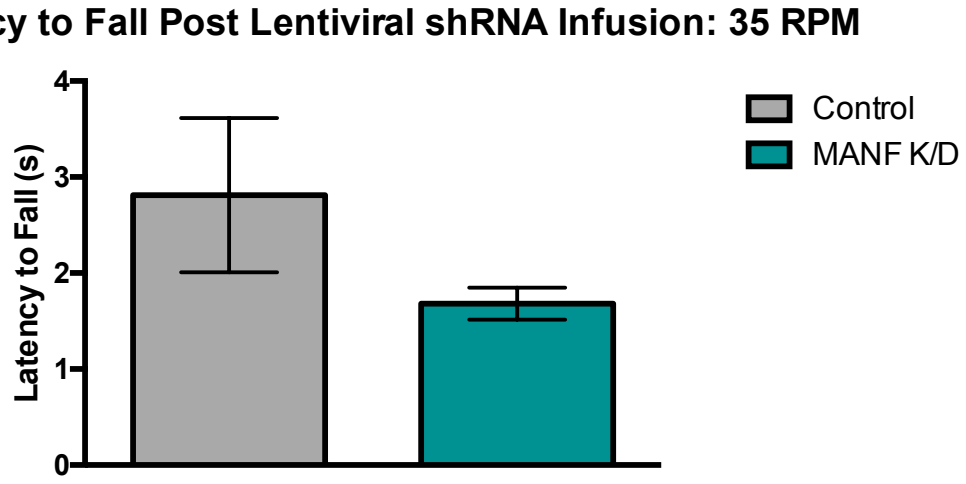


Figure 27. The effect of lentiviral-mediated MANF shRNA on the latency to fall at 10 RPM (A), 20 RPM (B), and 35 RPM (C) 5 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter and the latency to fall was measured in seconds. An unpaired *t*-test revealed that treatment did not have a statistically significant difference compared to control rats ($P > 0.05$). Data are represented as the mean ± SEM values.

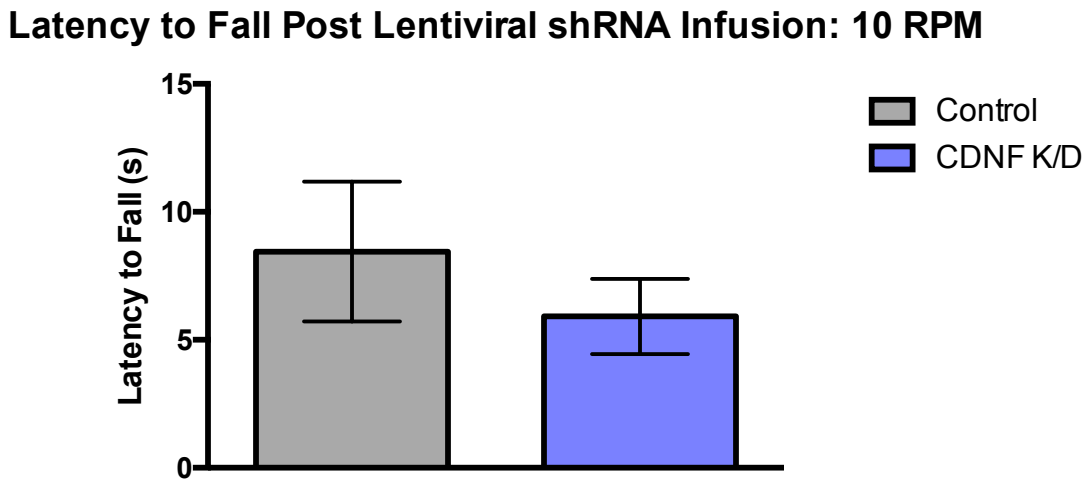
4.5.2 CDNF K/D

After rats were unilaterally infused with lentiviral-mediated CDNF shRNA into the SN, fixed speed rotarod testing (FSRT) revealed significant declines in the latency to

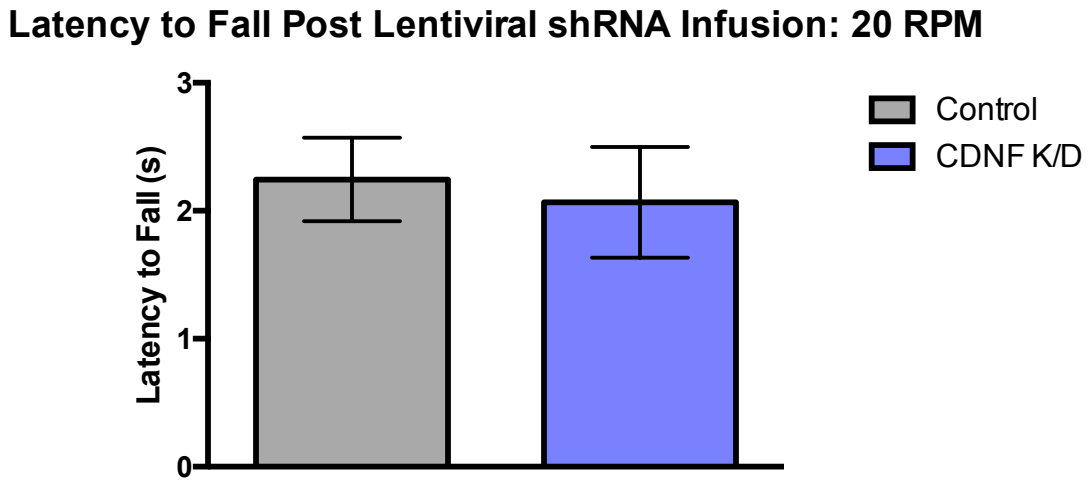
fall in MANF K/D rats at 20 and 35 RPM compared to control rats at 5 months ($P < 0.05$), but no differences were found at 4 months ($P > 0.05$).

4 Months

A.



B.



C.

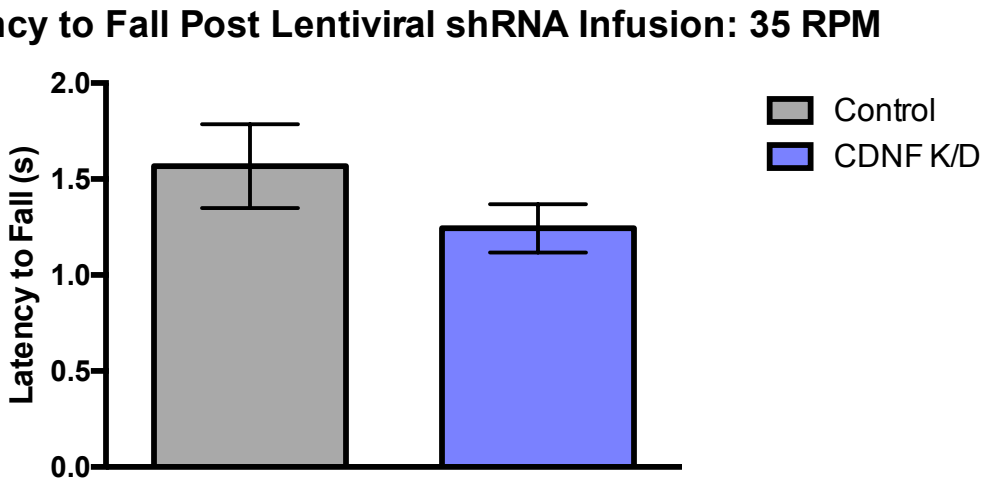
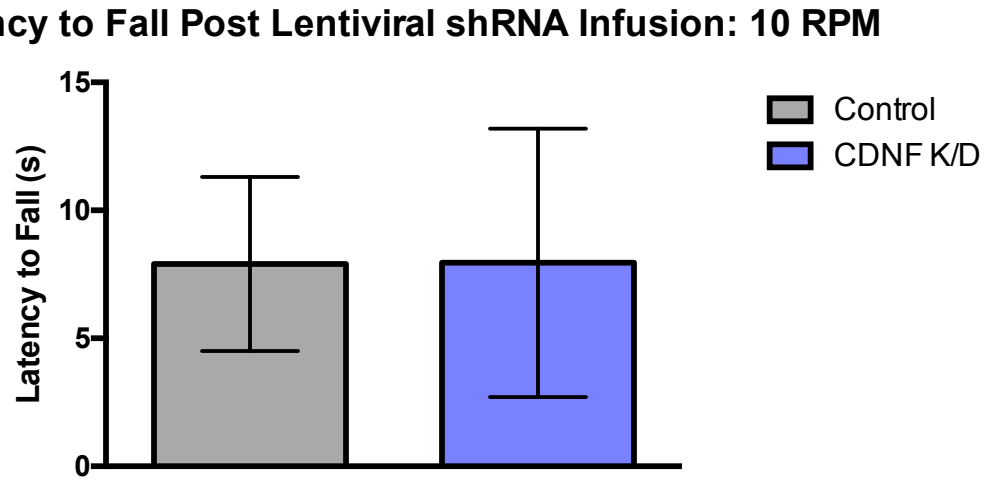


Figure 28. The effect of lentiviral-mediated CDNF shRNA on the latency to fall at 10 RPM (A), 20 RPM (B), and 35 RPM (C) 4 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter and the latency to fall was measured in seconds. An unpaired *t*-test revealed that treatment did not have a statistically significant difference compared to control rats ($P > 0.05$). Data are represented as the mean ± SEM values.

5 Months



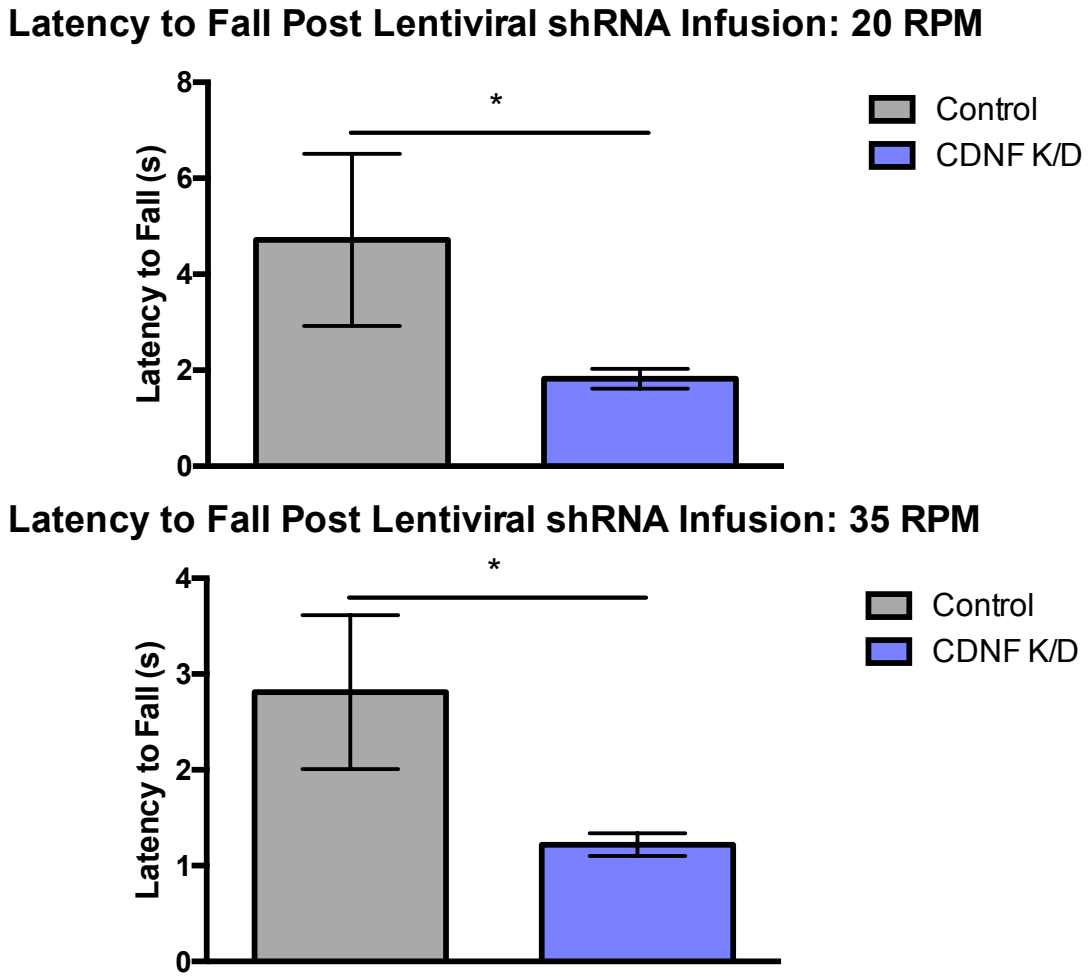


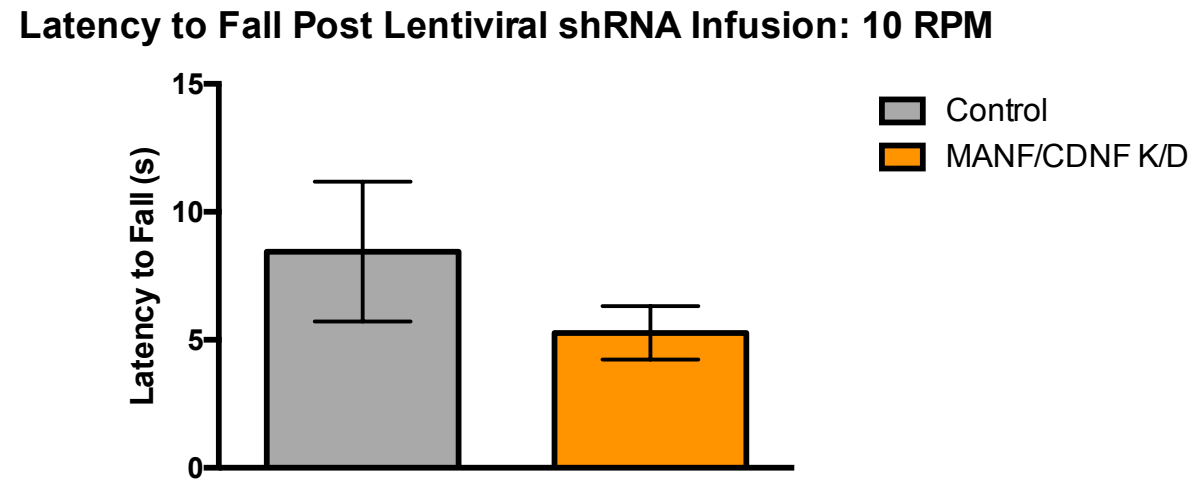
Figure 29. The effect of lentiviral-mediated CDNF shRNA on the latency to fall at 10 RPM (A), 20 RPM (B), and 35 RPM (C) 5 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter and the latency to fall was measured in seconds. An unpaired *t*-test revealed that treatment did have a statistically significant difference compared to control rats at 20 and 35 RPM ($P < 0.05$). Data are represented as the mean ± SEM values.

4.5.3 MANF & CDNF K/D

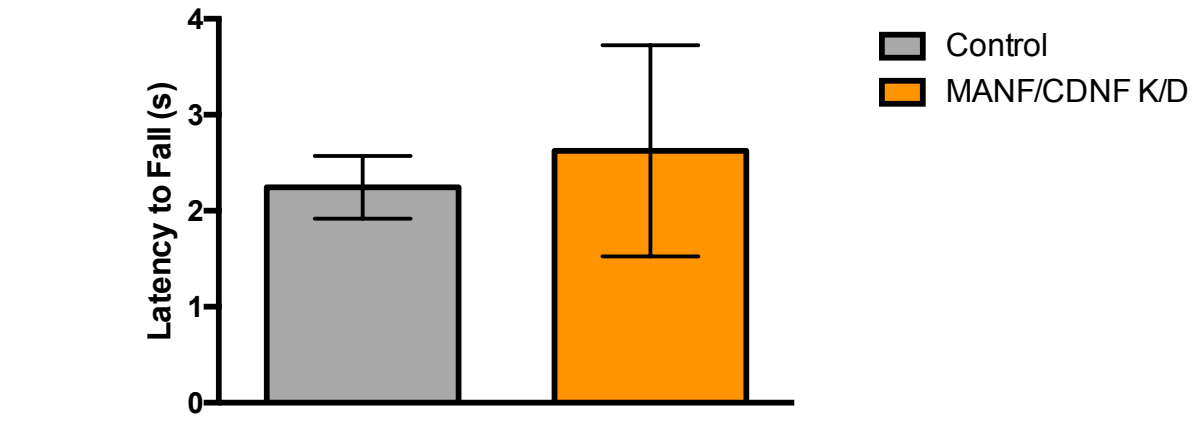
4 and 5 months after rats were unilaterally infused with lentiviral-mediated MANF and CDNF shRNA into the SN, fixed speed rotarod testing (FSRT) revealed significant declines in the latency to fall in MANF K/D rats at 35 RPM compared to control rats ($P < 0.05$).

4 Months

A.


Latency to Fall Post Lentiviral shRNA Infusion: 10 RPM
Latency to Fall (s)
15
10
5
0
Control
MANF/CDNF K/D

B.


Latency to Fall Post Lentiviral shRNA Infusion: 20 RPM
Latency to Fall (s)
4
3
2
1
0
Control
MANF/CDNF K/D

C.

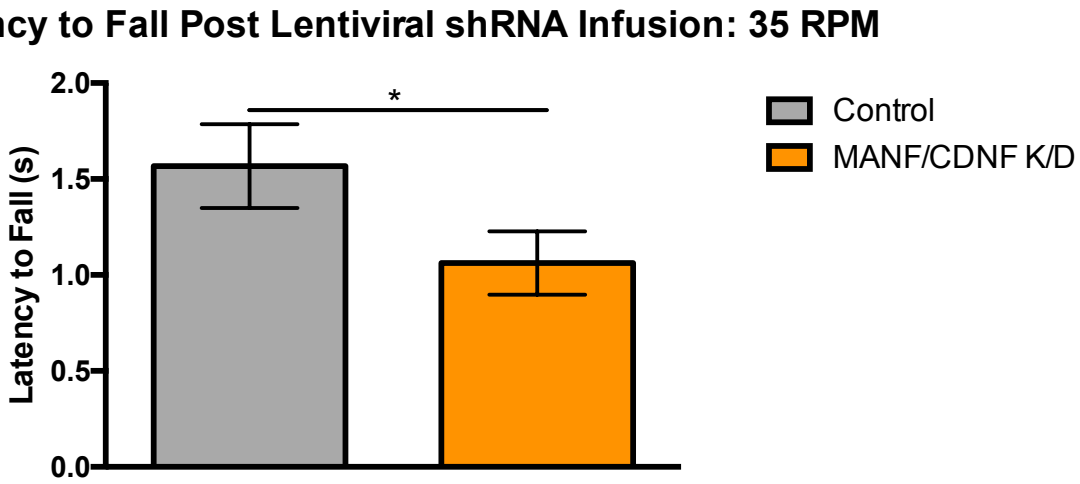
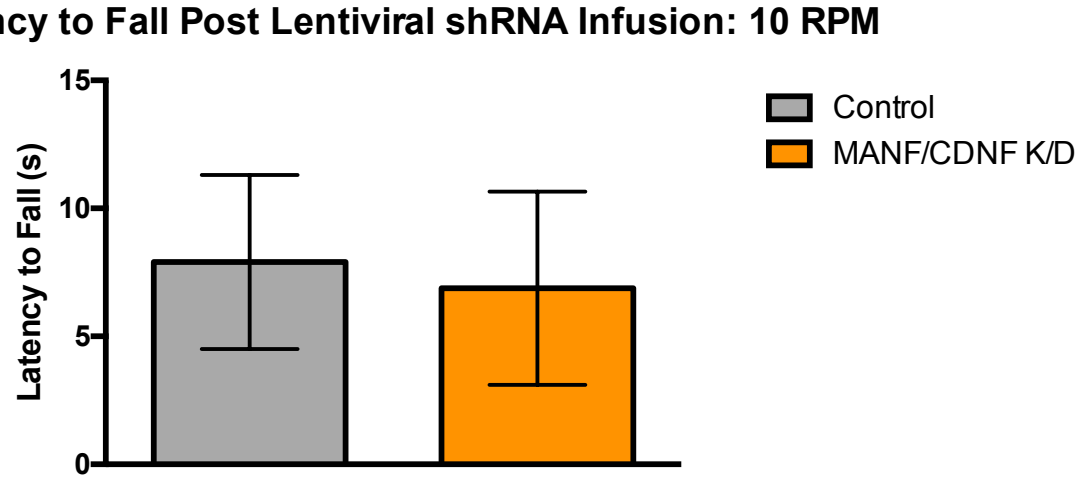


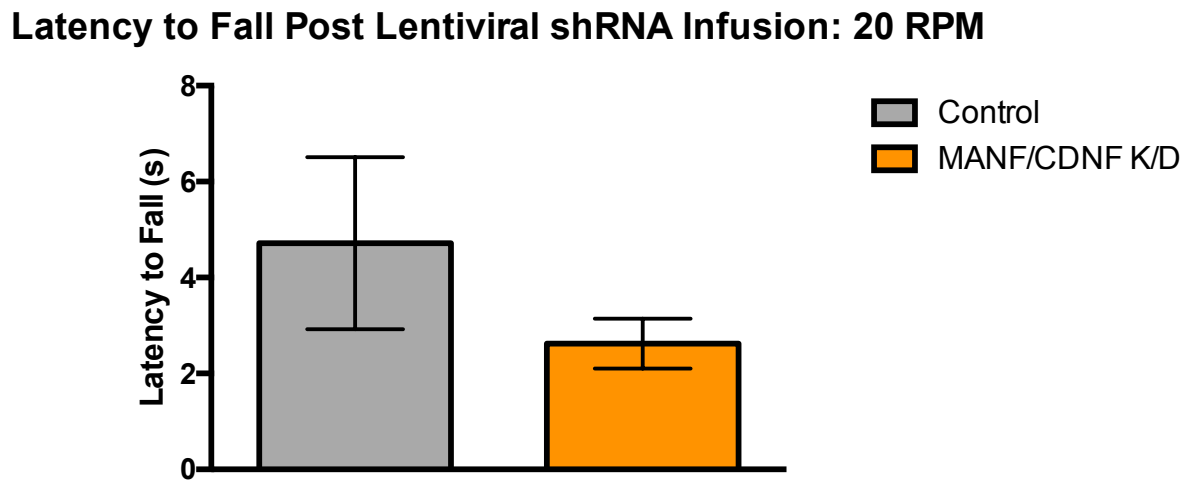
Figure 30. The effect of lentiviral-mediated MANF and CDNF shRNA on the latency to fall at 10 RPM (A), 20 RPM (B), and 35 RPM (C) 4 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter and the latency to fall was measured in seconds. An unpaired *t*-test revealed that treatment did have a statistically significant difference compared to control rats at 35 RPM ($P < 0.05$). Data are represented as the mean ± SEM values.

5 Months

A.



B.



C.

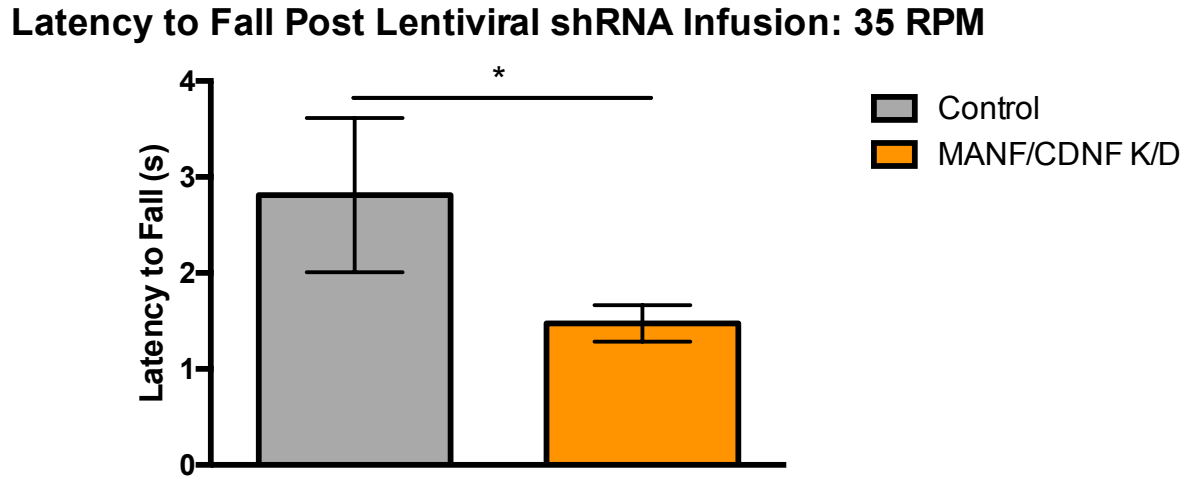


Figure 31. The effect of lentiviral-mediated MANF and CDNF shRNA on the latency to fall at 10 RPM (A), 20 RPM (B), and 35 RPM (C) 5 months post surgery. Rats (N=14) were tested and measurements were the mean of three consecutive trials for each parameter and the latency to fall was measured in seconds. An unpaired *t*-test revealed that treatment did have a statistically significant difference compared to control rats at 35 RPM ($P < 0.05$). Data are represented as the mean ± SEM values.

4.6 Cylinder Test

4.6.1 MANF K/D

4.5 and 5.5 months after rats were unilaterally infused with lentiviral-mediated MANF shRNA into the SN, the cylinder test revealed significant ipsilateral forelimb preference in MANF K/D rats compared to control ($P < 0.05$).

4.5 Months

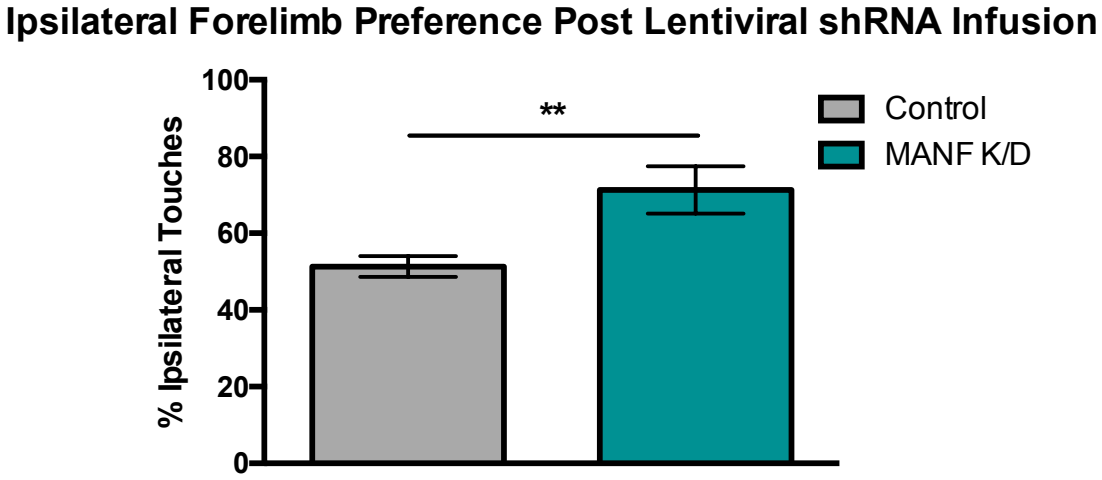


Figure 32. The effect of lentiviral-mediated MANF shRNA on ipsilateral forelimb preference 4.5 months post surgery. Rats (N=14) were tested and data are expressed as the number of ipsilateral forelimb wall contacts per 20 wall contacts. An unpaired *t*-test revealed that treatment did have a statistically significant difference compared to control rats ($P < 0.05$). Data are represented as the mean ± SEM values.

5.5 Months

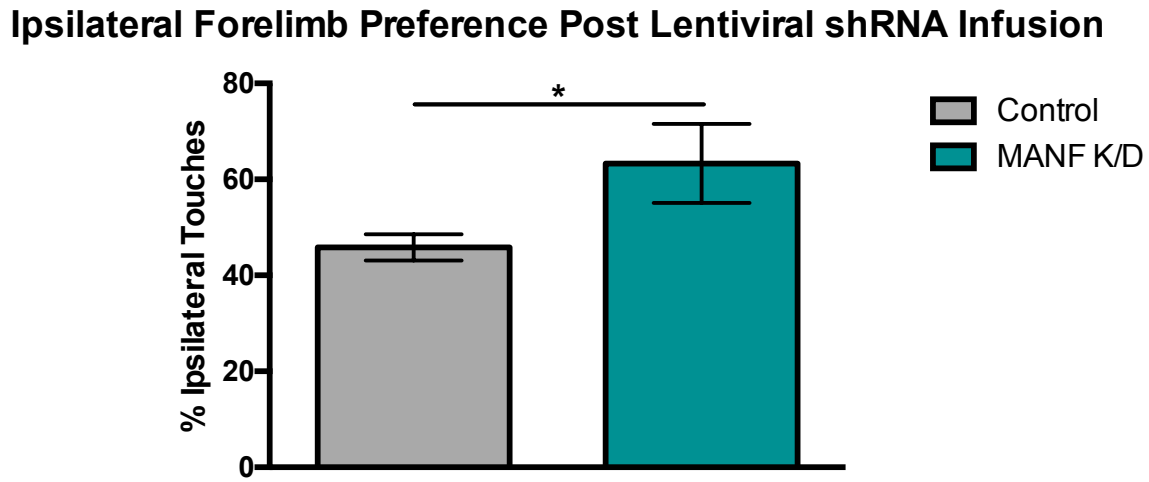


Figure 33. The effect of lentiviral-mediated MANF shRNA on ipsilateral forelimb preference 5.5 months post surgery. Rats (N=14) were tested and data are expressed as the number of ipsilateral forelimb wall contacts per 20 wall contacts. An unpaired *t*-test revealed that treatment did have a statistically significant difference compared to control rats ($P < 0.05$). Data are represented as the mean ± SEM values.

4.6.2 CDNF K/D

4.5 and 5.5 months after rats were unilaterally infused with lentiviral-mediated MANF shRNA into the SN, the cylinder test revealed no significant ipsilateral forelimb preference in CDNF K/D rats compared to control ($P > 0.05$).

4.5 Months

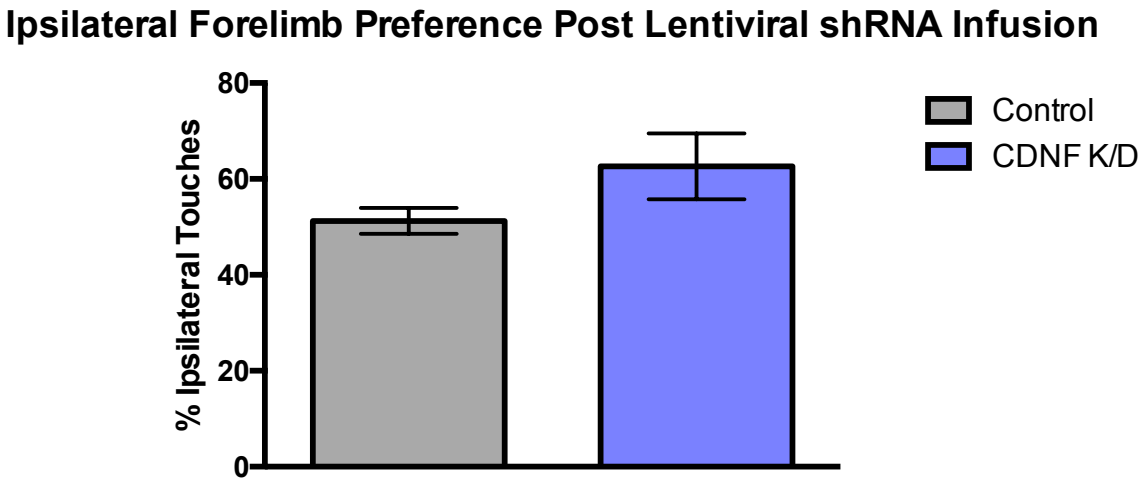


Figure 34. The effect of lentiviral-mediated CDNF shRNA on ipsilateral forelimb preference 4.5 months post surgery. Rats (N=14) were tested and data are expressed as the number of ipsilateral forelimb wall contacts per 20 wall contacts. An unpaired *t*-test revealed that treatment did not have a statistically significant difference compared to control rats ($P > 0.05$). Data are represented as the mean ± SEM values.

5.5 Months

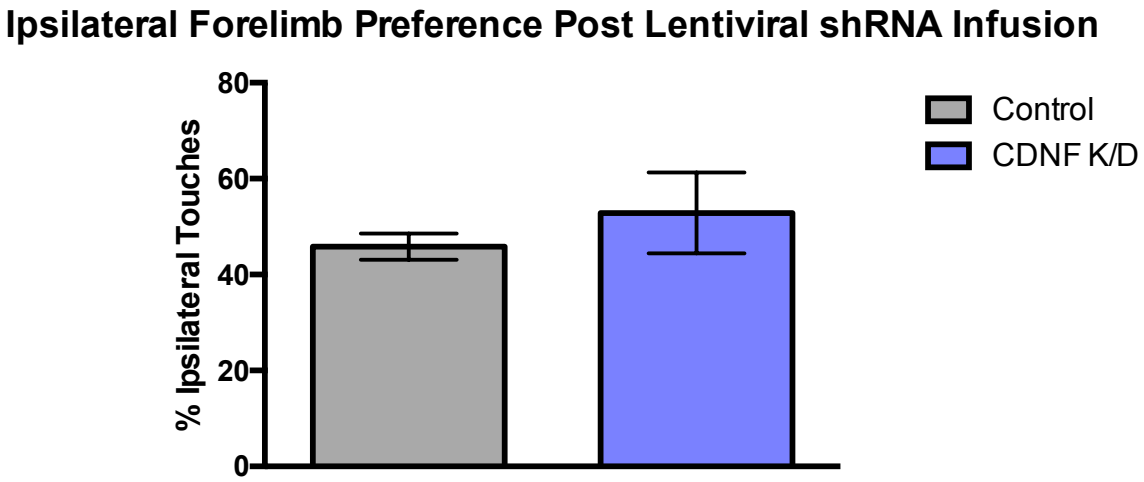


Figure 35. The effect of lentiviral-mediated CDNF shRNA on ipsilateral forelimb preference 5.5 months post surgery. Rats (N=14) were tested and data are expressed as the number of ipsilateral forelimb wall contacts per 20 wall contacts. An unpaired *t*-test revealed that treatment did not have a statistically significant difference compared to control rats ($P > 0.05$). Data are represented as the mean ± SEM values.

4.6.3 MANF & CDNF K/D

4.5 and 5.5 months after rats were unilaterally infused with lentiviral-mediated MANF and CDNF shRNA into the SN, the cylinder test revealed no significant ipsilateral forelimb preference in CDNF K/D rats compared to control ($P > 0.05$).

4.5 Months

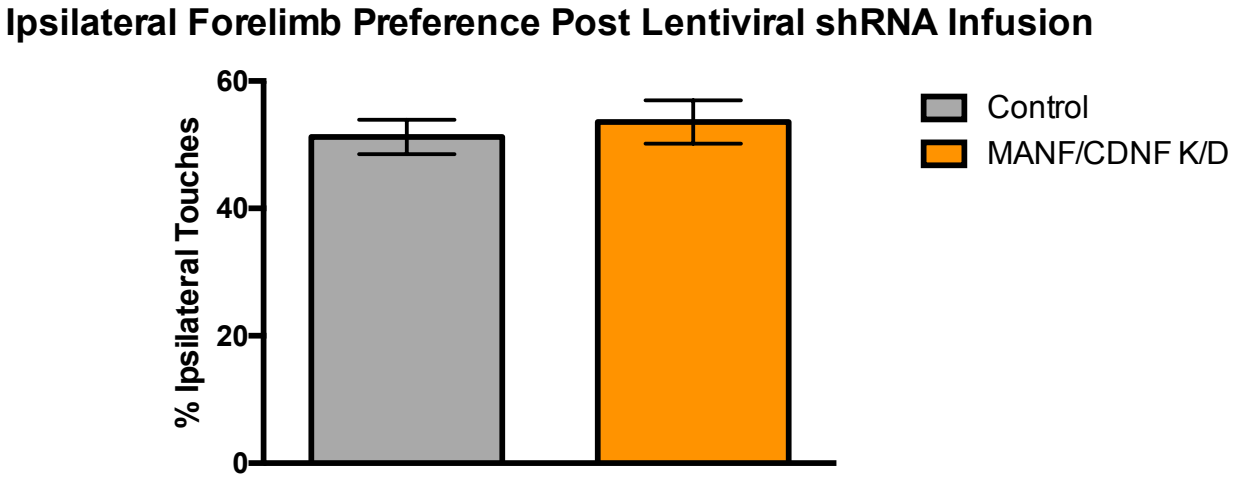


Figure 36. The effect of lentiviral-mediated MANF and CDNF shRNA on ipsilateral forelimb preference 4.5 months post surgery. Rats (N=14) were tested and data are expressed as the number of ipsilateral forelimb wall contacts per 20 wall contacts. An unpaired *t*-test revealed that treatment did not have a statistically significant difference compared to control rats ($P > 0.05$). Data are represented as the mean ± SEM values.

5.5 Months

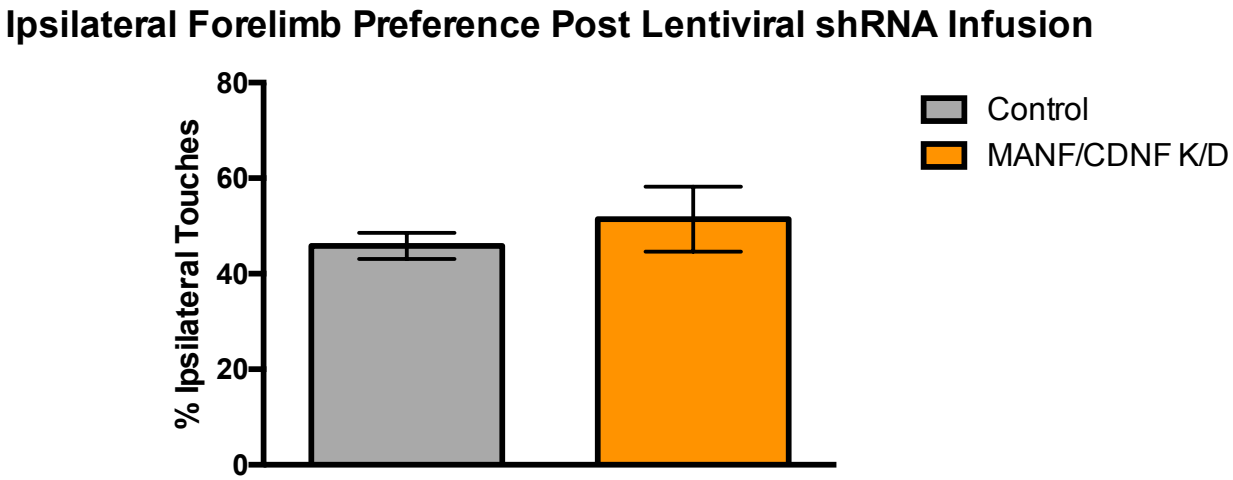
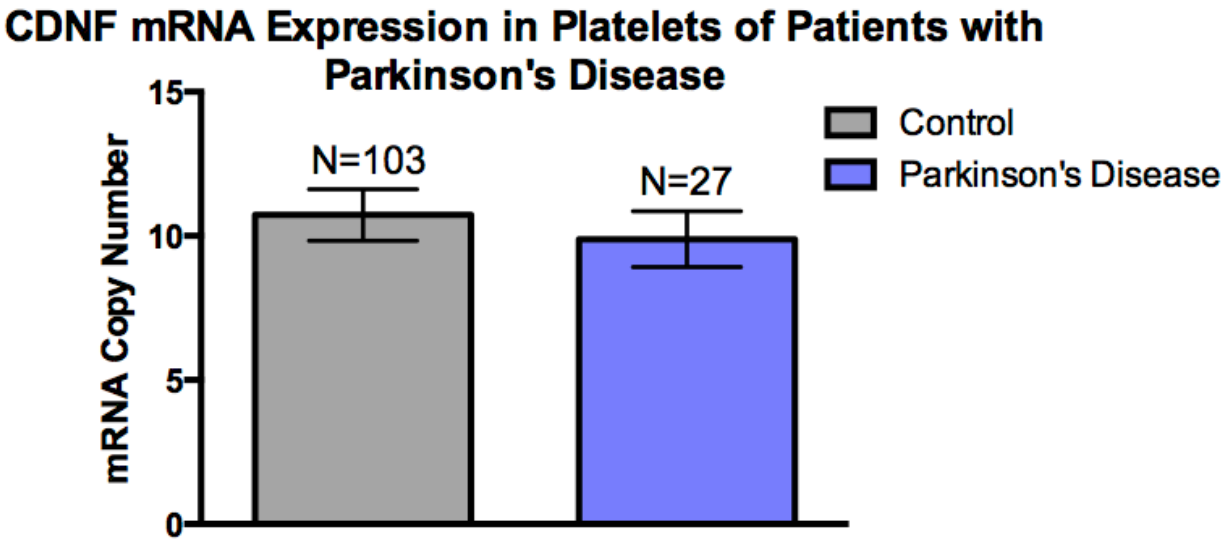


Figure 37. The effect of lentiviral-mediated MANF and CDNF shRNA on ipsilateral forelimb preference 5.5 months post surgery. Rats (N=14) were tested and data are expressed as the number of ipsilateral forelimb wall contacts per 20 wall contacts. An unpaired *t*-test revealed that treatment did not have a statistically significant difference compared to control rats ($P > 0.05$). Data are represented as the mean ± SEM values.

4.7 mRNA Expression of CDNF in Platelets of PD Patients

A.



B.

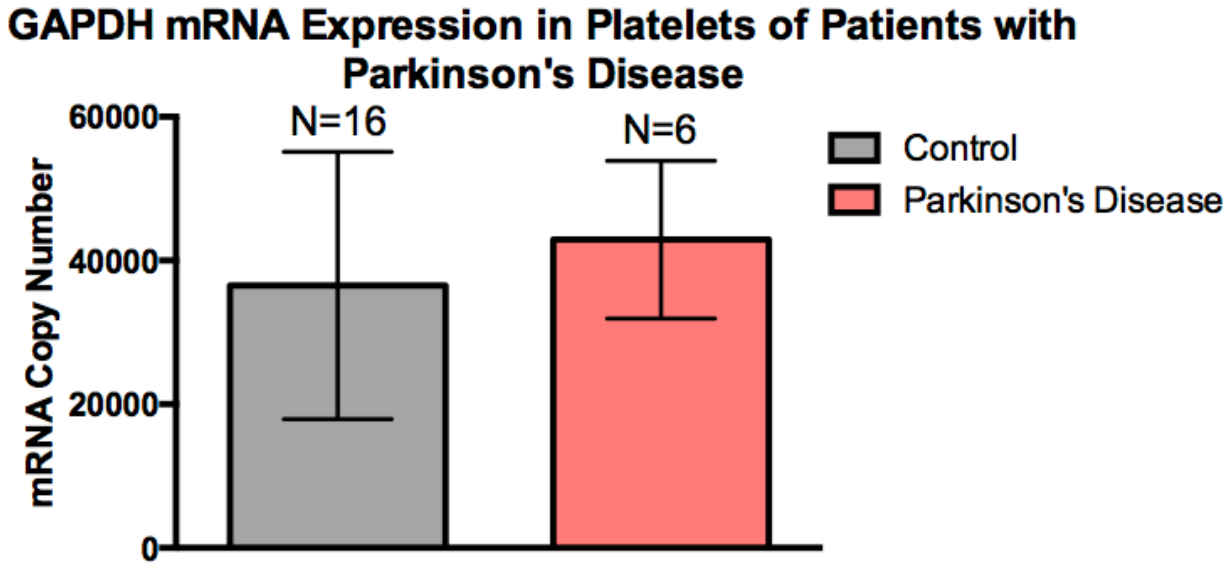


Figure 38. CDNF (A) and GAPDH (B) mRNA expression in the platelets of patients with PD. When quantifying CDNF mRNA expression, experimental groups consisted of a healthy control group aged 50 years and over (N=103; 58 Females, 45 Males) and patients with PD aged 50 years and over (N=27; 10 Females, 17 Males). An unpaired *t*-test revealed no statistically significant difference in CDNF mRNA expression in patients with PD compared to healthy controls ($P > 0.05$). When quantifying GAPDH mRNA expression, experimental groups consisted of a healthy control group aged 50 years and over (N=16) and patients with PD aged 50 years and over (N=6). An unpaired *t*-test revealed no statistically significant difference in GAPDH mRNA expression in patients with PD compared to healthy controls ($P > 0.05$). Data are represented as the mean ± SEM values.

CHAPTER 5: DISCUSSION

The potential of NTFs to provide neuroprotective and neurorestorative effects for patients with PD continues to be investigated (Cordero-Llana et al., 2015). The results from this ongoing study implicate the greater role of MANF in its ability to modulate changes in key features of parkinsonism, such as balance, gait and motor coordination.

Although BDNF was the first protein shown to directly support survival of DA neurons *in vivo*, many studies have demonstrated the neurorestorative abilities of GDNF, NRTN, MANF and CDNF in the nigrostriatal DAergic system in preclinical models of PD (Voutilainen et al., 2015). In lesion animal models of PD, BDNF did not protect and repair nigrostriatal neurons and thus, its therapeutic potential in alleviating the disease state is futile (Voutilainen et al., 2015).

Increasing evidence from studies of tissue samples from patients with PD and rodent models of PD indicated that ER stress is associated with the pathophysiology of PD (Colla et al., 2012). Current literature suggests that CDNF and MANF are involved in regulating ER stress and are upregulated *in vitro* and *in vivo* during the unfolded protein response (UPR) (Domanskyi, Saarma, & Airavaara, 2015; Hellman et al., 2011; Lindahl et al., 2014; Lindholm & Saarma, 2010). MANF-deficiency was found to lead to chronic activation of the UPR in pancreatic islets (Lindahl et al., 2014). Additionally, extracellular CDNF was demonstrated to save only the neurons that degenerated due to ER stress (Voutilainen et al., 2015). Therefore, CDNF and MANF have shown strong therapeutic potential in preclinical models of PD.

The ER is an organelle that is involved in the proper folding and processing of translated proteins directed for secretion. Under typical physiological circumstances, unfolded, misfolded or aggregated proteins are degraded. If unfolded proteins accumulate in the ER lumen, this causes ER stress and consequently activates the UPR, which is a cellular defence mechanism that counteracts ER stress. Should protein aggregation continue, this leads to chronic ER stress, which is toxic to cells and in turn leads to ER stress-induced apoptosis (Voutilainen et al., 2015). The UPR controls ER stress through three distinctive routes: (1) mRNA degradation and termination of additional protein translation; (2) the regulation of protein refolding through stimulation of ER chaperons; and (3) the activation of ER-associated degradation of unfolded proteins (Voutilainen et al., 2015). The UPR is facilitated by the ER transmembrane receptors: inositol-requiring enzyme 1 (IRE1), pancreatic ER kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Walter & Ron, 2011). After dissociating from an ER chaperone, IRE1, PERK and ATF6 are activated during periods of ER stress by dissociating from the ER chaperone GRP78. Phosphorylated PERK inhibits mRNA translation by phosphorylating eukaryotic initiation factor 2 (eIF2) α subunit. However, transcription factor ATF4 is made even though there is translational inhibition in order to restore ER homeostasis, or the transcription factor CHOP is produced after periods of chronic ER stress to induce cell death. ATF6 and IRE1 pathways control the expression of ER chaperone genes, whereby IRE1 degrades mRNA and by splicing XBP1 mRNA can induce the expression of genes that degrade proteins (Figure 6B) (Voutilainen et al., 2015).

CDNF and MANF are capable of behaving as classical NTFs by extracellularly promoting neuronal survival through the PI3K-Akt pathway, but differ from classical NTFs in that they are mainly localized in the ER, regulate ER stress and the UPR intracellularly, and can also be secreted from cells to extracellular spaces during periods of ER stress (Voutilainen et al., 2015). This can be accomplished due to the fact that MANF and CDNF possess a functional KDEL ER retention signal at their C-terminus (Glembotski et al., 2012). Studies have shown that MANF binds to KDEL receptors (Henderson, Richie, Airavaara, Wang, & Harvey, 2013) and the ER chaperone GRP78 (Glembotski et al., 2012), and can therefore operate as an ER resident protein, protecting cells intracellularly against ER stress (Apostolou et al., 2008; Glembotski et al., 2012; Henderson et al., 2013; Tadimalla et al., 2008). Interestingly, the excision of the RTDL sequence from the C-terminus of MANF upregulates its secretion and further cements the belief that CDNF–MANF proteins have two modes of action: acting intracellularly in the ER, and extracellularly through membrane receptors that are yet to be identified (Voutilainen et al., 2015). CDNF contains the sequence (KTEL), which resembles the ER retention signal (KDEL). To further support the importance of MANF and CDNF in regulating ER stress, Drosophila-null mutants of DmMANF revealed continually activated ER stress and increased PERK phosphorylation (Palgi et al., 2009). Moreover, ATF6 activation was shown to decrease ischemic damage in an *ex vivo* model of myocardial ischemia and induced genes, such as MANF (Voutilainen et al., 2015). Interestingly, recent studies have shown that CDNF protects DAergic neurons from the toxicity caused by α-synuclein aggregation, as is seen in PD (Latge et al., 2015).

A hypothesized mechanism of action of CDNF and MANF is described hereafter. Due to the fact that the majority of MANF and CDNF are held in the ER and function in a similar fashion, their most probable function and therapeutic mechanism of action within the cell lies in their regulation of ER stress and the UPR. Upon binding to GRP78, MANF can indirectly interact with the UPR receptors IRE1, PERK and ATF6 and in turn partake in their regulation. This is evidenced by MANF and CDNF's ability to ameliorate ER stress and specifically the chronic activation of IRE1, PERK and ATF6 in MANF-deficient mice (Voutilainen et al., 2015). CDNF and MANF reach the ER via: (1) retention signals at their C-terminus, KTEL and RTDL after their synthesis (Figure 2); and (2) when extracellular, CDNF and MANF may interact with injury-induced plasma membrane receptors or lipids, followed by internalization and transportation to the ER through an unknown mechanism. Moreover, a unique property of CDNF and in part MANF when compared to other NTFs is their inability to stimulate any effects in any tested cells extracellularly in the culture medium (Hellman et al., 2011; Lindholm et al., 2007; Voutilainen et al., 2011). This puzzling matter was clarified in collaboration with Kerstin Krieglstein by demonstrating that extracellular CDNF elicits its effects only after the application of ER stress or cellular damage, but not by NTF deprivation (Voutilainen et al., 2015). Although the signalling transduction pathways involved as they relate to ER-mediated survival mechanisms remain unclear, some evidence implicates the PI3K-Akt pathway (Voutilainen et al., 2015), and PKC pathways (Huang et al., 2015; Yang, Huang, Gaertig, Li, & Li, 2014).

Since patients with PD have a motor disability characterized by slowness of movement or bradykinesia, muscular rigidity, gait abnormalities with poor postural balance and akinesia (loss of voluntary movement) that is closely associated with aboulia or motivation to initiate movement, the narrow beam traversal task is a comprehensive task widely used for the characterization of PD-like motor impairments (Allbutt & Henderson, 2007; Brooks & Dunnett, 2009; Glajch, K. E., Fleming, S. M., Surmeier, D. J., & Osten, 2012; Pothakos, Kurz, & Lau, 2009). The latency duration and traversal duration evaluate akinesia and bradykinesia respectively, whereas the number of recorded slips is a marker for impairments in balance.

The deficits observed during the narrow beam traversal test may reflect *in vitro* and *in vivo* studies that have implicated MANF and CDNF in the maintenance of ER homeostasis. CDNF and MANF are believed to assist in protein folding and ameliorating ER stress, thereby preventing neuronal apoptosis and degeneration in PD and proving to be a potentially fundamental therapeutic in the maintenance of DAergic neurons in the midbrain (Lindholm & Saarma, 2010; Parkash et al., 2009). The accumulation of misfolded proteins is associated with perturbations in ER function, which is a characteristic of PD (Liu, 2014). More specifically, MANF mutants in Drosophila melanogaster led to the degeneration of DA neurons potentially through the activation of the UPR leading to cell death under irreversible ER damage (Palgi et al., 2009, 2012).

The crystal structure of MANF demonstrated a vastly disordered C-terminal domain, and thus, the solution structure of MANF via nuclear magnetic resonance (NMR) was analyzed. This structure revealed a well-defined C-terminal domain that is

homologous to the SAP (SAF-A/B, Acinus, and PIAS) domain of Ku70, which is a distinguished inhibitor of proapoptotic regulator Bcl-2-associated X (Bax) (Voutilainen et al., 2015). These data formed the basis of the hypothesis that MANF and CDNF prevent apoptosis through their direct interaction with Bax. Interestingly, intracellularly expressed CDNF and MANF was shown to counteract apoptotic pathways involving Bax in sympathetic neurons (Hellman et al., 2011).

Moreover, it was demonstrated that MANF was upregulated by ER stress in several cell lines as well as by cerebral ischemia in rats (Apostolou et al., 2008). MANF secretion is induced *in vitro* in cardiac myocytes and HeLa cells by thapsigargin treatment, which is a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase that depletes calcium from ER stores. CDNF levels are also induced by ER stress and MANF interacts with the chaperone GRP78 (or Bip, which is a member of the heat shock protein 70 kDa family, Hsp70) in a calcium-dependent manner (Glembotski et al., 2012). This posits that MANF and CDNF secretion increase in responses to pathologies that involve reduced ER calcium concentrations, such as brain and heart ischemia the accumulation of misfolded proteins (Voutilainen et al., 2015). Overexpression of MANF improved cell viability following glucose-free conditions and tunicamycin (induces the UPR) treatment. Based on these protective properties, MANF and CDNF are believed to be a novel mediator of the UPR pathway (Apostolou et al., 2008).

Additionally, studies have shown that MANF is vital for the protection of rat embryonic dopaminergic neurons *in vitro* (Petrova et al., 2003). MANF also supports the development and growth of DAergic axonal terminals (Petrova et al., 2003). Given the

numerous publications indicating the significance of MANF in the pathophysiology of PD, it is conceivable that the perturbations in motor coordination, balance and gait observed in the MANF K/D and combinational K/D of MANF and CDNF group in our study, are significantly attributable to MANF. A study by Cordero-Llana et al., (2015) revealed that MANF overexpression in the SN showed a significant protection of TH^+ neurons in the SN. However, it is important to note that behavioural recovery after the 6-OHDA insult did not occur. Interestingly, no protection to dopaminergic cell bodies was observed after CDNF overexpression in the SN (Cordero-Llana et al., 2015), which may explain the failure of the CDNF K/D group in our study to consistently recapitulate impairments in motor coordination, balance and gait attributed to degeneration of the DA system in PD across all behavioural paradigms. Thus, any impairment in the combinational MANF and CDNF K/D group may simply be a result of the MANF K/D acting alone. In contrast, CDNF overexpression showed a significant increase in DAergic innervation, which was accompanied by a significant decrease in the frequency of amphetamine-induced rotations (Cordero-Llana et al., 2015). Moreover, an individual intrastriatal infusion of CDNF and MANF showed protection of nigrostriatal DAergic neurons when administered 6 hours prior to infusion of the neurotoxin 6-OHDA (Lindholm et al., 2007; Voutilainen et al., 2009). In fact, the function of the perturbed nigrostriatal DA system was partly restored after CDNF and MANF were administered up to 4 weeks after 6-OHDA infusion (Figure 39) (Lindholm et al., 2007; Voutilainen et al., 2009). Additionally, after 14 days of uninterrupted intrastriatal administration of CDNF, the restoration of midbrain neural circuit function regulating motor control was

achieved when treatment was started 2 weeks after intrastriatal 6-OHDA. In addition, continuous infusion of CDNF protected TH-positive neurons in the SNc and fibers in striatum from 6-OHDA-induced neurodegeneration (Voutilainen et al., 2011). Interestingly, when compared with GDNF, CDNF showed greater effectiveness in protecting and restoring midbrain DA neurons in the rat 6-OHDA model of PD (Voutilainen et al., 2011). Recent literature has suggested that CDNF may support neuron repair through its suppression of neuronal inflammation via its effects on astrocytes and microglia (Nadella et al., 2014; Zhao et al., 2014, 2016). These results complicate the interpretation of our findings from behavioural tests for the CDNF K/D group that indicates CDNF does not play an important role in the nigrostriatal DA system. However, these studies further substantiate the ability of CDNF to improve the functionality of the nigrostriatal system after the 6-OHDA lesion.

Furthermore, the therapeutic potential of CDNF in MPTP model of PD in C57BL/6 mice was demonstrated when CDNF was found to be a powerful NTF that protected and restored the function of DA neurons (Airavaara et al., 2012; Lindholm et al., 2007; Voutilainen et al., 2011). When given a day prior to MPTP exposure, CDNF protected TH-positive neurites in the striatum and SN. Interestingly, this protection corresponded to behavioural and motor improvements. And upon CDNF administration one week after MPTP exposure, bilateral CDNF infusions into striatum improved locomotor behaviour and this was mirrored with increased TH immunoreactivity in the striatum and the quantity of TH+ cells in the SN (Voutilainen et al., 2015). In addition to the evidence supporting CDNF and MANF's role in protecting DAergic neurons in

preclinical models, they are stable proteins that diffuse more readily in brain tissue than all other tested NTFs in brain tissue, which may aid in their ability in restoring DAergic circuitry and protection of DAergic cell bodies in the nigrostriatal pathway (Voutilainen et al., 2009;Voutilainen et al., 2011).

Due to the vast distribution of CDNF expression reported in the CNS, especially in the striatum, (Cheng et al., 2013; Lindholm et al., 2007), it is plausible that selective K/D of CDNF in the SN in our study was counteracted by CDNF expression in the striatum. Furthermore, because CDNF was only found in solitary cells in the SN that did not express TH (an indicator for DA neurons), it is possible that lentiviral-mediated CDNF shRNA would not have had an effect on the nigrostriatal DA system (Voutilainen et al., 2015). CDNF exhibits widespread expression in human brain and non-neuronal tissues, which mirrors data from rodent studies (Voutilainen et al., 2015). Additionally, CDNF mRNA was reported in the hippocampus, thalamus, striatum, and SN. With the use of CDNF antibodies, protein was found in the adult cerebral cortex, hippocampus and striatum. High levels of CDNF protein were also found in the cerebellar Purkinje cells and the locus coeruleus area of the brain stem (Voutilainen et al., 2015).

High MANF levels were found to be widespread in the brain in the cerebral cortex, hippocampus, thalamus, hypothalamus, striatum and SN, but with the highest expression in cerebellar Purkinje cells (Lindholm et al., 2008). Because the levels of MANF are higher than those of CDNF throughout the CNS and mostly localized in neurons (Lindholm et al., 2007; Wang et al., 2014), it is conceivable that perturbations in its expression cause more pronounced adverse effects, which may, at least in part, explain

the discrepancy in motor impairments observed between the MANF versus CDNF K/D groups. Additionally, low vector transfection efficiency may explain the lack of motor impairments indicative of parkinsonism observed in the CDNF K/D group, however this is subject to verification after the quantification of CDNF mRNA expression in the SN.

The inconsistency of the behavioural results in the combinational MANF and CDNF K/D group may be attributed to the halved quantity of lentiviral mediated MANF shRNA, 1 μL versus 2 μL, that was infused into the SN, compared to the MANF K/D group that slowed the progression of the MANF gene silencing and in turn, the advancement of the disease state. With the progression of the disease, motor symptoms, especially gait impairments, such as shorter strides and shuffling are exacerbated and this is attributed to the loss of DA neurons in the SN of up to 60% and DA level depletion by up to 80% in the striatum (Meredith & Kang, 2006). Therefore, it is conceivable that these large percentile deficits of DA were not reached in the K/D groups and thus explaining why total distance travelled and footprint analyses revealed no significant changes between treatment and control groups. Moreover, a minimal loss of striatal DA levels may explain the lack of observable behavioural changes 1 month post lentiviral-mediated shRNA infusion across all K/D groups. Additionally, a major disadvantage that behavioural tests such as the cylinder, rotarod and beam traversal test share are the inevitability of practice effects, whereby animals' performance may improve simply by virtue of the fact they are becoming more and more familiar with the task with each subsequent testing session. For instance, during the FSRR test, animal gross motor function and balance is initially markedly compromised, but the animal gradually

recovers because it learns novel strategies to rely on other limbs and on tail deviation for balance and movement (Hicks, Schallert, & Jolkkonen, 2009). Additionally, although the cylinder test is a powerful test for uncovering the effects of unilateral motor impairment since lesions in a single side of the brain cause marked and lateralized loss of function in the contralateral side of the body, it is much less suitable for the purpose of measuring 'bilaterally symmetric dysfunction', which is often subtle and the form of dysfunction caused by the majority of genetic mutations leading to PD (Brooks & Dunnett, 2009). With regards to the increased rearing durations found across all K/D groups, this voluntary forelimb movement may potentially be attributed to impairments in hindlimb motor control, which can be a result of perturbations in reticulospinal neurons. Reticulospinal neurons of the reticulospinal tract (an extrapyramidal motor tract) are modulated by the nigrostriatal pathway affected in PD, which influences postural adjustments that adjust gait and provide control over hindlimb spinal motor neurons (Meredith & Kang, 2006).

Moreover, using gene therapy to target DA neurons in the SN or DA fibers in the striatum necessitates the thorough consideration of promoter, viral vector type and its serotype (Voutilainen et al., 2015). Intrastriatal injections of adeno-associated viral (AAV) vectors serotype 2 using the CMV promoter and carrying the cDNA of CDNF has been shown to protect and restore behavioural deficits caused by the 6-OHDA lesion model of PD and resulted in a significant restoration of TH immunoreactive (TH-ir) neurons in the SNc and TH-ir fiber density in the striatum (Back et al., 2013; Ren et al., 2013). In contrast, when CMV promoter, which is not specific to DAergic neurons, was

used to increase CDNF and MANF expression in the SN of rat with lentiviral vectors, such as what was done in our study, it was discovered that only the combination of both CDNF- and MANF-expressing viral vectors demonstrated protection of DA neurons (Cordero-Llana et al., 2015; Voutilainen et al., 2015).

Lastly, the use of rodent models for the study of neurodegenerative disorders presents several challenges when attempting to translate preclinical findings to humans (Cramer et al., 2011). Relative to humans, rodents have a younger relative age, more homogenous injury, a lower frequency of comorbid conditions and white matter that constitutes 14% of their total brain volume compared with 50% in humans (Cramer, 2003; Cramer et al., 2011; Goldberg & Ransom, 2003). Additionally, it is important to note that rodents are quadrupeds with a strong dependence on vibrissae, and are housed in social environments that fail to consider and replicate human social interactions (Cramer et al., 2011).

The etiology of PD remains to be elucidated and this is the first study to demonstrate that MANF K/D recapitulates key features of parkinsonism, such as impairments in balance, motor coordination, and gait. Thus, these findings coupled with the support for MANF overexpression in the SN protecting DAergic neurons that degenerate in PD, suggests the role of MANF in both the pathophysiology of and potential treatment for PD.

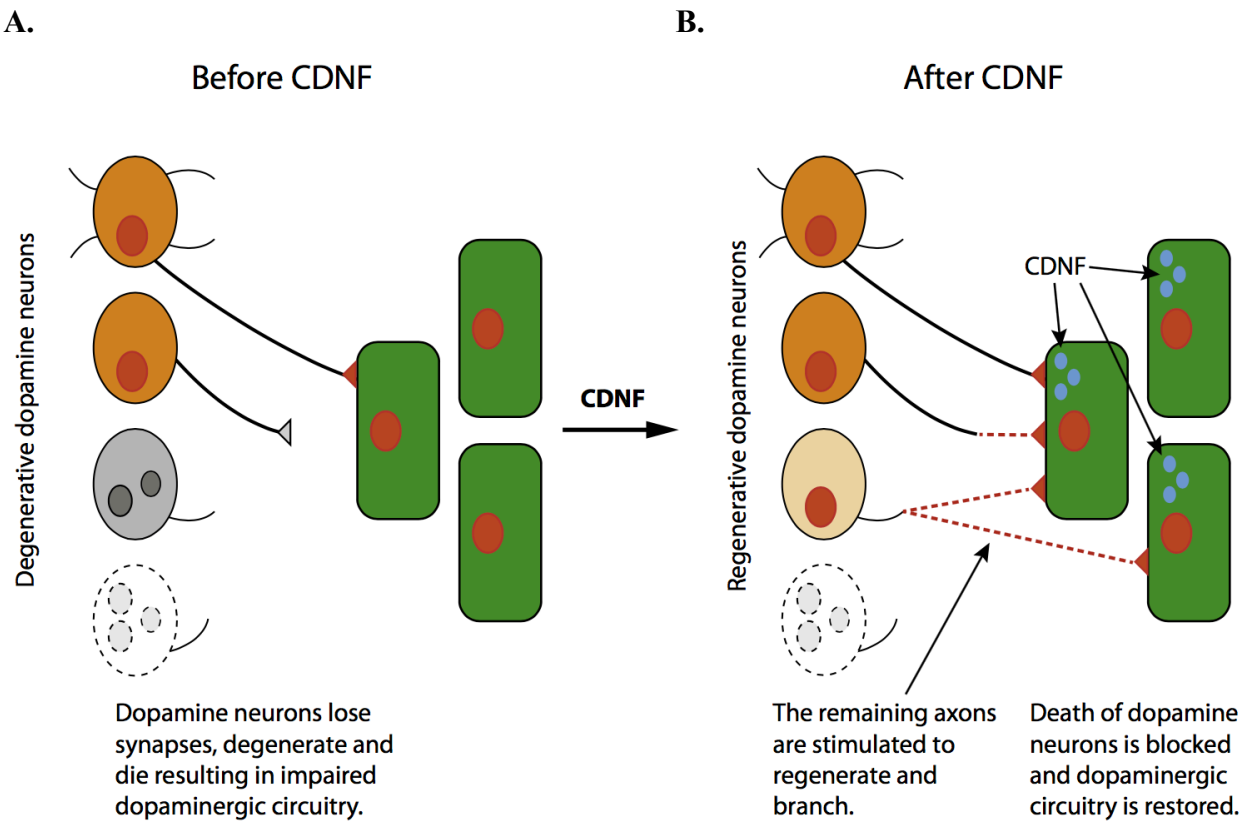


Figure 39. A schematic illustrating degenerating DA neurons and CDNF-induced regeneration. **A.** DA neurons degenerate due to 6-OHDA or MPTP. From the top left neuron: (1) DA neurons have synaptic connections to the target striatal cells. (2) DA neurons lose synaptic connections and axons degenerate. (3) DA neurons lose their DA phenotype, but maintain their cytoarchitectural structures and are alive. (4) DA neurons degenerate and die. **B.** DArgic circuitry is regenerated after CDNF treatment. From the top right neuron: (1) DA neurons have synaptic connections to target cells and CDNF protects these neurons from degeneration and death. (2) DA neurons regenerate axons and re-establish their synaptic connections. (3) DA neurons restore their DA phenotype, and restore their synaptic connections to target cells. (4) CDNF fails to restore DA neurons that have degenerated (Voutilainen et al., 2015).

5.1 Conclusion & Future Directions

CDNF and MANF are distinguishable from other NTFs by sequence, 3D structure and mechanism of action. Although they have similarities with other NTFs in that they both protect DAergic neurons, in contrast to NTFs such as GDNF and BDNF, CDNF and

MANF are not able to rescue neurons *in vitro* when added to culture medium, nor from the physiological apoptosis due to NTF deficiency, but were found to only rescue neurons during periods of ER stress (Voutilainen et al., 2015). Additionally, CDNF and MANF did not exhibit any effects on DAergic neurons when injected into the brains of healthy control rodents (Airavaara et al., 2012; Voutilainen et al., 2011). Thus, like other NTFs, CDNF and MANF protect injured neurons from degeneration through activating unidentified plasma membrane receptors and anti-apoptotic signalling pathways. Nevertheless, they are believed to mostly act intracellularly, whereby they regulate ER stress and UPR, further assisting in the maintenance, and survival of DAergic neurons in the midbrain.

The effects of the MANF, CDNF, and both MANF and CDNF shRNA vectors on MANF and CDNF mRNA and DA levels in the SN and striatum remain to be determined using Real Time qPCR. Additionally, in order to circumvent potential confounding variables associated with the use of DA agonists during behavioural testing, the drug-induced rotation paradigm will be done following the completion of other behavioural tests. Rotational behaviour has been a widely used measurement of motor asymmetry and a means to confirm unilateral lesions in rat models of PD, especially in the 6-OHDA model (Brooks & Dunnett, 2009). Following unilateral forebrain DA depletion, rodents show a postural bias ipsilateral to the lesion, which can be manipulated or amplified by pharmacological intervention into a head-to-tail turning (360 degree rotation) response. Stimulation of DA release by amphetamine causes ipsilateral rotation, whereas postsynaptic receptors on the denervated lesioned side become supersensitive and thus,

activated by low doses of the agonist apomorphine resulting in contralateral rotations (Dunnett & Lelos, 2010b). Rotational behavior is an additional parameter to evaluate the therapeutic efficacy of pharmacological interventions with dopaminergic targets of action (Brooks & Dunnett, 2009).

In the final weeks of behavioural testing, TP5, a novel peptide, will be administered (40 mg/kg) in order to investigate its ability to ameliorate impairments in balance, motor coordination, and gait caused by MANF and CDNF K/D. TP5 is a truncated, 24 amino acid peptide designed based on the structure and kinetics of the CDK5/p25 complex, which has been associated with neurotoxicity (Binukumar et al., 2015; Binukumar & Pant, 2016; Cardone, Brady, Sriram, Pant, & Hassan, 2016). TP5 selectively inhibits CDK5/p25 activity by competing with p25 binding to CDK5 and thereby reducing neurotoxicity (Amin et al., 2016; Cardone et al., 2016; Kesavapany, Zheng, Amin, & Pant, 2007). TP5, as studied *in vitro*, reduces CDK5/p25 activity independent of CDK5/p35 activity, and preliminary results from our lab have shown its protective effects in SH-SY5Y neuroblastoma cells. Subsequently, during *in vivo* studies, TP5 has attenuated dopaminergic degeneration in the MPTP mouse model of PD, while also ameliorating parkinsonism phenotypes in a 6-OHDA lesion model of PD (Binukumar et al., 2015; Binukumar & Pant, 2016). Results from the behavioural testing following TP5 administration will be compared with that of levodopa. Levodopa is the gold standard treatment for PD, which has been shown to successfully improve motor impairments by directly stimulating postsynaptic striatal DA receptors and restoring dopaminergic neurotransmission (Lewitt, 2012).

In order to establish the promising MANF K/D model as a preclinical model of PD that warrants further investigation, the scientific community has agreed on a framework that judges models on three types of validators: construct, face and predictive validity (Nestler & Hyman, 2010). Face validity assesses whether the motor and behavioural impairments seen in the preclinical model reflect the phenotypic resemblance to the human manifestation of the disease, which the MANF K/D model of PD has demonstrated in this study. Construct or etiologic validity is achieved by recreating in animals the pathophysiological processes that cause the disease state in humans and in turn also replicating neural and behavioral features of the disorder (Chadman, Yang, & Crawley, 2009). In this study, the MANF K/D model still requires the quantification of the levels of DA in the STR, the TH striatal fiber density, and the number of TH+ cells in SN in order to study the model's similarities with the pathophysiology of PD in the human condition. Additionally, the levels of α-synuclein, the primary component of Lewy bodies, will be explored using immunohistochemistry. Finally, predictive or pharmacological validity implies that animals in a proposed model respond to treatment in a way that predicts or mimics the effects of the treatments in humans. In this case, the gold standard treatment levodopa will be used to study similarities in treatment responses between the MANF K/D rats and human patients.

After the evaluation of the behavioural and biochemical effects of MANF and CDNF K/D *in vivo*, platelets and whole blood collected from patients with PD will be analyzed using Real Time qPCR for mRNA expression of CDNF and MANF. Although CDNF mRNA expression from the platelets of patients with PD did not reveal

differential expression compared to healthy controls (Figure 38), blood-based biomarkers remain attractive targets for diagnosing PD. This is due to its ease of collection and the ability of platelets to model the serotonergic and dopaminergic neuronal behaviour observed in the SN of patients with PD (Pletscher & Laubscher, 1980; Schapira et al., 1990).

As CDNF and MANF have demonstrated robust therapeutic effects in rodents, it is paramount to understand how these NTFs act on NHP models of PD and if they can rescue DAergic neurons in an α-synuclein model of PD, where GDNF fails to elicit therapeutic effects. In the future, studies evaluating whether CDNF and MANF have disease-modifying therapeutic benefits in clinical trials on patients with PD are vital. Arguably, the most important task that remains is the identification and characterization of CDNF and MANF plasma membrane receptors and clarifying their mechanism of action.

CHAPTER 6: REFERENCES

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