NEUROPROTECTION IN A ROTENONE MODEL OF PARKINSON'S DISEASE

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ABSTRACT

The pesticide/neurotoxin, rotenone, has been shown to cause systemic inhibition of mitochondrial complex I activity, with consequent degeneration of the nigrostriatal pathway, as observed in Parkinson's disease. A novel intrastriatal rotenone model of Parkinson's disease was used to examine the neuroprotective effects of valproic acid (VPA) and melatonin, both of which are known to induce neurotrophic gene expression in the central nervous system via mechanisms which may involve epigenetic modulation. In these studies, sham or lesioned rats were treated with either vehicle, VPA (4mg/mL), or melatonin (4µg/mL) in drinking water. Results from a forelimb asymmetry test indicated a significant decrease in use of the contralateral forelimb in rotenone-infused animals, in the third week post-surgery, which was abolished by VPA treatment. Apomorphine administration resulted in significantly higher ipsilateral rotation in rotenone-lesioned (12µg) animals, as compared to controls, which was attenuated by melatonin treatment. Subsequent immunohistochemical examination revealed a decrease in tyrosine hydroxylase immunoreactivity within the striatum and substantia nigra of rotenone-infused animals. VPA or melatonin treatment prevented this decrease in tyrosine hydroxylase in the striatum and substantia nigra. Stereological cell counting indicated a significant decrease in dopamine neurons within the substantia nigra of rotenone-treated animals. Importantly, this loss of dopamine neurons in rotenone-infused animals was blocked by chronic VPA or melatonin treatment. A third study explored whether rotenone infusion into the medial forebrain bundle and substantia nigra in mice could provide a model of Parkinson's disease. Densitometric analysis revealed a significant depletion of tyrosine hydroxylase immunofluorescence within the ipsilateral striatum and substantia nigra of lesioned animals, and a significant bilateral overexpression of α-synuclein in the substantia nigra, as compared to control animals. These novel findings support the use of intracranial rotenone as a Parkinsonian model, and provide a solid platform for future combinatorial therapeutic approaches with VPA and melatonin.

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ABBREVIATIONS

4HNE	4-Hydroxy-2-Nonenal
6-OHDA	6-Hydroxydopamine
AA-NAT	Arylalkylamine N-Acetyltransferase
AC	Adenylyl Cyclase
AMK	N^1-acetyl-5-Methoxykynuramine
AFMK	N^1-acetyl-N^2-Formyl-5-Methoxykynuramine
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
ATP	Adenosine Triphosphate
BDNF	Brain-Derived Neurotrophic Factor
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanylyl Cyclase Formation
CBP	CREB-Binding Protein
CDNF	Cerebral Dopamine Neurotrophic Factor
CREB	cAMP Response Element-Binding Protein
CSF	Cerebrospinal Fluid
CSPα	Cysteine-String Protein Alpha
dH_2O	Distilled Water
DJ-1	PARK7
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ERK	Extracellular Signal-Regulated Kinase

FITC	Fluorescein Isothiocyanate
GABA	Gamma-Aminobutyric Acid
GC	Guanylate Cyclase
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GSK-3β	Glycogen Synthase Kinase-3β
H_2O_2	Hydrogen Peroxide
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HOCl	Hypochlorous acid
Hsp70	Heat Shock Protein 70
JNK	c-Jun N-terminal Kinase
L-DOPA	L-3,4-Dihydroxyphenylalanine; Levodopa
LRRK2	Leucine-rich Repeat Kinase 2; Dardarin
MANF	Mesencephalic Astrocyte-Derived Neurotrophic Factor
MAPK	Mitogen-activated Protein Kinase
MFB	Medial Forebrain Bundle
MPP^+	1-Methyl-4-Phenylpyridinium
$MPDP^+$	1-Methyl-4-Phenyl-2,3-Dihydropyridinium
MPPP	1-Methyl-4-Phenyl-4-Propionoxypiperidine
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride; Saline
NADH	Nicotinamide Adenine Dinucleotide

NAS	N-Acetylserotonin
NDS	Normal Donkey Serum
NO·	Nitroxyl Radical
Nurr1	Nuclear Receptor Related 1
O_2^-	Superoxide
·OH	Hydroxyl Radical
OH^-	Hydroxyl Ion
P/CAF	p300/CBP-Associated Factor
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-Kinase
PINK1	PTEN-induced Putative Kinase 1
Pitx3	Pituitary Homeobox 3
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PTEN	Phosphatase and Tensin Homolog
RNA	Ribonucleic Acid
RO·	Alkoxyl
ROO·	Peroxyl
ROOH	Organic Hydroperoxides
SAHA	Eranilohydroxamic Acid

S.E.M.	Standard Error of the Mean
SN	Substantia Nigra
SNCA	Non A4 Component of Amyloid Precursor; Synuclein, Alpha
SNARE	Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor
SRY	Sex-determining Region Y
SUMO-1	Small Ubiquitin-Related Modifier-1
TH	Tyrosine Hydroxylase
VPA	Valproic Acid

CHAPTER 1: GENERAL INTRODUCTION

1.1: Aging, Mitochondrial Dysfunction and Oxidative Stress

Aging is a natural process that all living humans experience. It is characterized as a progressive decline of tissues, organs, and organ system function. This degeneration leads to an increased susceptibility to disease which, eventually, results in death. The progression of such age-related diseases appears to be accelerated by oxidative stress, and the generation of reactive oxygen species and the accumulation of mitochondrial deoxyribonucleic acid (DNA) mutations over time is believed to be a major contributing factor (Lin and Beal, 2006; Taylor et al., 2013). Reactive oxygen species are formed by mitochondria as a natural by-product of cellular respiration through the sequential reduction of atomic oxygen, albeit environmental stresses (i.e. ionizing radiation, photooxidation, oxidizing agents) can also increase the concentration of these oxidants, resulting in toxic levels that cause damage to DNA and cell death (Sies, 1986). The different types of reactive oxygen species that are produced include: superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), hydroxyl ion (OH^-), alkoxyl ($RO^{\cdot}$), peroxyl ($ROO^{\cdot}$), organic hydroperoxides (ROOH), hypochlorous acid (HOCl) and nitroxyl radical ($NO^{\cdot}$) (Simon et al., 2000; Apel and Hirt, 2004). These oxidants have important roles in cellular mechanisms such as homeostasis, signal transduction, and pathogen defense (Mittler, 2002). Reactive oxidants such as oxidized halogens, free radicals, and singlet oxygen, are used by phagocytes to kill invading microorganisms and mediate apoptosis, however they are also capable of oxidizing various cellular components and can lead to damage of surrounding cells (Simon et al., 2000; Apel and Hirt, 2004).

In order to prevent the accumulation of reactive oxygen species, there are many cellular antioxidant systems that remove or terminate lipid chain reactions. These mechanisms are: superoxide dismutase and catalase, glutathione peroxidase and glutathione, and vitamins E and C. Oxidative stress is the result of an imbalance between reactive oxygen species generation and metabolism, such that the concentration of reactive oxygen species exceeds the cell's ability to remove these oxidants and repair damage, resulting in extensive oxidation of biomolecules (Squier, 2001). Although controversial, many believe that mitochondrial dysfunction and the accumulation of reactive oxygen species contribute to the neurodegeneration that is associated with many age-related diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease (Golden and Melov, 2001; Barnham et al., 2004; Anderson and Maes, 2014; Indo et al., 2015).

The link between mitochondria and Parkinson's disease was first postulated in 1979, after a college student had attempted to synthesize the heroin analog 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), but accidentally contaminated the drug with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), such that when he self-administered the drug, he began to suffer from Parkinsonism-like symptoms (Davis et al., 1979). This effect was observed again in 1982, when a young group of illicit drug users developed very progressive parkinsonian symptoms following intravenous administration of MPPP that was contaminated with MPTP (Dauer and Przedborski, 2003; Toulouse and Sullivan, 2008). Research revealed that MPTP can easily cross the blood-brain barrier where it is oxidized into 1-methyl-4-phenylpyridinium (MPP^+) (Cohen et al., 1984). Due to its similarity in structure to dopamine, MPP^+ is taken up by

the dopamine transporter, leading to inhibition of complex I (nicotinamide adenine dinucleotide (NADH)-ubiquinone oxireductase) activity of the electron transport chain and, ultimately, cell death (Bové and Perier, 2012; Valadas et al., 2014). Furthermore, animal studies revealed that MPTP and other inhibitors of mitochondrial function, such as the pesticide, rotenone, produce many of the same characteristics observed in Parkinson's disease, including behavioural deficits, nigrostriatal degeneration, and protein aggregation (Vila et al., 2000a; Alam and Schmidt, 2002; Greenamyre et al., 2003; Sathiya et al., 2013).

In humans, mitochondrial dysfunction and oxidative stress appear to be important for the development of Parkinson's disease. Under normal conditions, free radical scavengers located in the inner membrane of mitochondria (i.e. vitamin E and glutathione) and enzymes (i.e. superoxide dismutase) are capable of removing the reactive oxygen species that are generated by the mitochondria (Olanow, 1992; Trushina and McMurray, 2007). However, oxidative stress can lead to excessive amounts of reactive oxygen species which can cause damage to lipid membranes, increasing lipid peroxidation. Lipid peroxidation causes concentrations of 4-hydroxy-2-nonenal (4HNE), which is normally expressed in cells (0.1-0.3 µM), including dopamine substantia nigra neurons, to increase (10µM-5mM) (Esterbauer et al., 1991; Uchida, 2003; Chen and Niki, 2006). These high concentrations of 4HNE decrease mitochondrial complex I and II function, inhibit mitochondrial aldehyde dehydrogenase catabolism of dopamine, and modify or damage DNA and ribonucleic acid (RNA) (Anderson and Maes, 2014). Ultimately, evidence suggests that disruption to mitochondrial activity and oxidative stress are factors in many degenerative diseases

including Parkinson's disease (Jenner, 2003; Winklhofer and Haass, 2010; Camilleri and Vassallo, 2014; Zaltieri et al., 2015).

1.2 Parkinson's Disease

Parkinson's disease is the most common neurodegenerative movement disorder, with age being the single most consistent risk factor, affecting 0.5-1% of the population between the ages of 65-69. The prevalence of Parkinson's disease further increases to affecting 1-3% of the population over the age of 80 (de Lau and Breteler, 2006; Toulouse and Sullivan, 2008). Parkinson's disease is characterized by the progressive loss of dopamine-producing neurons within the substantia nigra pars compacta, which in turn contributes to a depletion of dopamine within the striatum. This loss of dopamine functionality results in abnormalities in extrapyramidal function (Loonam et al., 2003; Centonze et al., 2004) and motor behavioural deficits including uncontrollable tremor, postural imbalance, rigidity (Lotharius and Brundin, 2002), and bradykinesia (Recchia et al., 2004a). In addition to the decline in motor function, patients have also exhibited insomnia, autonomic dysfunction, hyposmia, depression, and dementia (Aarsland et al., 2005; Petit et al., 2014). Onset of these clinical symptoms occur following the loss of approximately 50% of the dopaminergic neurons in the substantia nigra and 80% of the dopamine concentration within the striatum (Iancu et al., 2005; Toulouse and Sullivan, 2008). Another key biological marker is the presence of degenerating ubiquitin- or α-synuclein-positive neuronal neurites or processes (intracytoplasmic Lewy bodies and dystrophic Lewy neurites), located in all of the affected brainstem regions, particularly the dorsal motor nucleus of the vagus (Agid, 1991; Lang and Lozano, 1998; Jellinger, 2012).

In addition to age being the foremost risk factor for the development of Parkinson's disease, epidemiological studies have identified that males are twice as likely as females to suffer from the disease (Wooten et al., 2004; Taylor et al., 2007). In recent years, several studies have demonstrated the protective potential of estrogen against neurotoxin-induced dopamine depletion in animal models of Parkinson's disease (Grandbois et al., 2000; Dluzen et al., 2001; Baraka et al., 2011) , suggesting that estrogen may contribute to the decreased prevalence observed in some female populations (Taylor et al., 2007). Not only have sex hormones been associated with differences in susceptibility to Parkinson's disease, evidence suggests that sex chromosomes may also play a part in the development of this disorder. In particular, the sex-determining region Y (SRY) gene on the Y-chromosome has received attention due to its expression within dopamine-rich regions of the brain, such as the substantia nigra, and regulates dopamine synthesis and metabolism in males (Smith et al., 2005; Czech et al., 2012; Loke et al., 2015).

The specific etiology of Parkinson's disease remains unknown, however there are some genetic and environmental factors that have been linked to the development of the disease. In recent years, several causative monogenetic mutations have been discovered, including parkin, alpha-synuclein (non A4 component of amyloid precursor; SNCA), PARK7 (DJ-1), phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1), and dardarin (leucine-rich repeat kinase 2; LRRK2), and at least 12 loci have been identified (Gilks et al., 2005; de Lau and Breteler, 2006; Lev et al., 2006). However, only a small percentage of cases (5-10%) are the result of inheritable genetic mutations (Dauer and Przedborski, 2003). The remaining cases (~90%) are termed

sporadic or idiopathic in origin, although evidence suggests that exogenous toxins such as cyanide, trace metals, and organic solvents, and environmental factors, including exposure to pesticides and herbicides, increase the risk of developing Parkinson's disease (Olanow and Tatton, 1999; Drechsel and Patel, 2008; Hatcher et al., 2008; Chin-Chan et al., 2015).

1.3 Rotenone and Other Neurotoxin Models of Parkinson's Disease

The use of animals in research can provide indispensable information on the role of environmental factors in the etiology of degenerative disorders, including Parkinson's disease. In order to study Parkinson's disease, several animal models exist that produce irreversible behavioural and molecular effects, similar to those observed with the disease. The most extensively studied animal models of Parkinson's disease use 6-hydroxydopamine (6-OHDA) or MPTP. However, in recent years, pesticides such as rotenone, have been used as a model of Parkinson's disease, as they mimic many of the pathological characteristics of the disorder (Bezard and Przedborski, 2011; Blesa et al., 2012).

6-OHDA, is a hydroxylated analogue of the naturally occurring neurotransmitter, dopamine, with a high affinity for the dopamine transporter, and was the first model used to study Parkinson's disease (Schober, 2004). 6-OHDA is unable to cross the blood-brain barrier, so local injection is required in order to obtain the desired effects (Gerlach and Riederer, 1996). When 6-OHDA is injected into the substantia nigra or medial forebrain bundle, it selectively accumulates in dopamine neurons, resulting in anterograde degeneration and cell death (Fig. 1.1) (Schober, 2004; Bové and Perier, 2012). It was also discovered that when 6-OHDA is infused into the striatum, retrograde

degeneration of the nigrostriatal pathway occurs, causing a substantial loss of striatal dopamine and substantia nigra dopaminergic neurons (Bezard and Przedborski, 2011; Blandini and Armentero, 2012). Unilateral lesions of 6-OHDA also produce considerable motor deficits as a result of dopamine depletion, the extent of which can be assessed by evaluating rotational behaviour following administration of either amphetamine or apomorphine (Gerlach and Riederer, 1996; Bezard et al., 2013). Unfortunately, 6-OHDA does not induce all of pathological features of Parkinson's disease, as synucleinopathy and the formation of Lewy bodies do not occur (Beal, 2001; Schober, 2004).

As mentioned earlier, MPTP is able to cross the blood-brain barrier due to its high lipophilicity, where it is oxidized in glial cells and serotonergic neurons to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$), catalyzed by monoamine oxidase B (Winklhofer and Haass, 2010). $MPDP^+$ is very unstable and spontaneously oxidizes to MPP^+. MPP^+ is then released into the extracellular space and taken up via the dopamine transporter, where it targets mitochondria and inhibits complex I activity of the electron transport chain (Fig. 1.1). This leads to a decrease in the production of adenosine triphosphate (ATP), while increasing the generation of reactive oxygen species, resulting in neuronal cell death (Schober, 2004; Bové and Perier, 2012). MPTP is one of the most commonly used animal models for this disorder, as administration of this toxin results in motor symptoms and dopaminergic deterioration along the nigrostriatal pathway that are very similar to that observed in Parkinson's disease (Betarbet et al., 2002). The shortcomings associated with this technique include an inconsistency in the

loss of other monoaminergic neurons, such as those located in the locus coeruleus, and a lack of Lewy body formation (Beal, 2001).

Rotenone, a potent rotenoid used in insecticides, is a highly lipophilic neurotoxin that is capable of crossing the blood-brain barrier, where it can enter dopamine neurons, independent of the dopamine transporter (Dauer and Przedborski, 2003; Blandini and Armentero, 2012). Many of the characteristics present in the Parkinson's pathology are observed following treatment with rotenone, such as oxidative damage due to reactive oxygen species production, systemic mitochondrial impairment, microglial activation, α-synuclein phosphorylation, aggregation and Lewy pathology, depletion of tyrosine hydroxylase immunoreactivity, selective nigrostriatal dopaminergic degeneration, ubiquitin-proteasomal dysfunction, and L-3,4-dihydroxyphenylalanine (levodopa; L-DOPA)-responsive motor deficits (Betarbet et al., 2006; Drechsel and Patel, 2008; Cicchetti et al., 2009; Norazit et al., 2010; Bové and Perier, 2012). These effects are the result of rotenone's ability to inhibit proteasome activity and mitochondrial complex I activity, resulting in a substantial generation of reactive oxygen species (Fig. 1.1) (Blandini and Armentero, 2012). In addition to the accumulation of reactive oxygen species, rotenone has been shown to downregulate neurotrophic factors, which may potentiate the negative effects of this neurotoxin (Cho et al., 2008). Further, *in vivo* studies have demonstrated the potential of this neurotoxin for use as a Parkinsonian model. In a previous study, the researchers found that intranigral rotenone infusion caused a significant depletion of tyrosine hydroxylase immunoreactivity within the nigrostriatal system (Norazit et al., 2010). In addition to nigrostriatal degeneration, another study found that when rotenone was administered by intraperitoneal injection, it

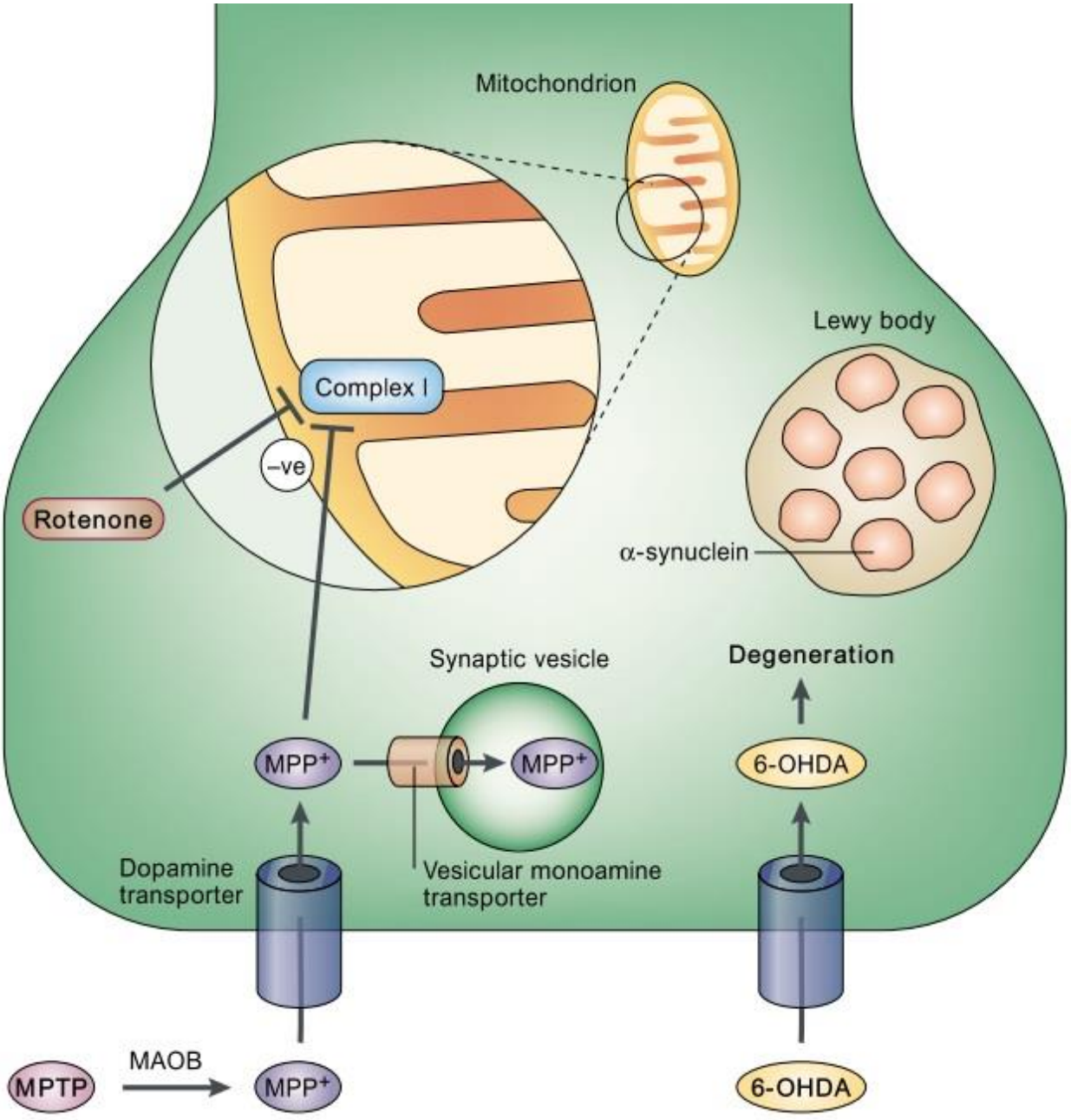

Figure 1.1: Effects of toxins used in model of Parkinson's disease on dopamine cells (Beal, 2001).

caused behavioural deficits that were responsive to the dopamine agonist, apomorphine, suggesting that the deficits observed were specific to the loss of dopamine (Cannon et al., 2009). Unfortunately, most of the foregoing studies have used systemic administration of rotenone, which has significant side effects including severe weight loss, dehydration, and high morbidity and mortality (Betarbet et al., 2000; Sherer et al., 2003; Lin et al., 2012; Tasselli et al., 2013). However, studies have revealed that intracranial infusion of rotenone does not induce these deteriorating effects, though some of the characteristics, such as protein aggregation and Lewy body formation, have yet to be found in rats (Saravanan et al., 2005; Sindhu et al., 2005, 2006; Mulcahy et al., 2011, 2012; Carriere et al., 2014). This intracranial approach is now regarded as an important Parkinsonian model for experimental purposes.

1.4: Valproic Acid

Valproic acid (2-propylvaleric acid, 2-propylpentanoic acid) is a short chain branched fatty acid that was first synthesized in 1882 by B.S. Burton as a derivative of valeric acid, which is naturally produced by valerian (*Valeriana officinalis*) (Chateauvieux et al., 2010). VPA was originally used as a solvent for organic compounds, as it was not believed to affect physiological processes, however in 1962, during a study that investigated the potential anticonvulsive activity of different molecules, VPA was found to prevent pentyleneterazol-induced convulsions in rodents (Meunier et al., 1963; Lebreton et al., 1964a, 1964b).

For clinical purposes, VPA is widely used as an anticonvulsant and mood stabilizer, and also for treatment of seizures, epilepsy, and schizophrenia (Wassef et al., 1999; Gurvich

et al., 2004; Ren et al., 2004). In humans, these effects of VPA are primarily due to its ability to potentiate the inhibitory activity of the neurotransmitter, gamma- aminobutyric acid (GABA), through several mechanisms, including decreasing turnover, inhibition of 4-aminobutyrate transaminase and GABA degradation, and increasing synthesis of GABA (Chateauvieux et al., 2010; Müschen et al., 2011). In addition to enhancing GABAergic signalling, VPA has recently been shown to inhibit classes I and IIb of histone deacetylase (HDAC) activity, through which it can target epigenetic mechanisms, altering the expression of many genes (Grayson et al., 2010; Harrison and Dexter, 2013). In light of their clinical implications, therapeutic value has been attributed to HDAC inhibitors, such as VPA, in the treatment of cancer and Parkinson's disease (Yang and Grégoire, 2005).

1.5: Metabolic Mechanisms Influenced by Valproic Acid

To date, there are no known receptors for VPA, however, its lipophilic nature allows easy entry into target cells where it is known to influence pro-survival cell behaviour through multiple mechanisms, such as the β-catenin pathway, mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase (PI3K)/Akt signalling (Monti et al., 2010). Studies suggest that VPA inhibits glycogen synthase kinase-3β (GSK-3β)-mediated phosphorylation of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), activating Wnt-dependent gene expression. It is also possible that VPA regulates Wnt signaling, thus resulting in the accumulation of the β-catenin protein, through the inhibition of HDAC activity. Evidence supports the involvement of HDAC inhibition, as VPA has been shown to affect E-cadherin expression, which has been associated with DNA

hypermethylation (Kostrouchová et al., 2007; Wang et al., 2015). VPA has also been shown, at therapeutic concentrations (0.4-1.04 mM), to stimulate the MAPK/ERK 1/2 pathway, increasing binding activity of the transcription factor, activator protein 1 (AP-1) (Yuan et al., 2001). Further, evidence demonstrates that VPA results in an increase in phosphorylation-dependent Akt activation, mediated by the PI3K pathway, which is linked to the neuroprotective effects of VPA, specifically in promoting neuronal survival (Monti et al., 2009).

1.6: Valproic Acid and Neurotrophic Factors

Neurotrophic factors are endogenous molecules that regulate migration, differentiation, and survival of neuronal precursors. Evidence also shows that they promote neuronal regeneration following injury or damage (Huang and Reichardt, 2001; Airaksinen and Saarma, 2002; Conte et al., 2003). Generally, there are at least three families of neurotrophic factors: (1) neurotrophins (i.e. brain-derived neurotrophic factor (BDNF), nerve growth factor, neurotrophin-3 and neurotrophin-4), (2) glial cell line-derived neurotrophic factor (GDNF) family ligands (i.e. GDNF, neurturin, artemin, and persephin), and (3) neuropoietic cytokines, also known as the interleukin-6 family (Lindholm and Saarma, 2010). Signalling by these families is achieved through multiple receptor types, such as transmembrane receptor tyrosine kinases and the p75 neurotrophin receptor (Soppet et al., 1991; Barbacid, 1995; Conte et al., 2003). Recently, two ligands were discovered that now make up a novel evolutionary family of conserved proteins that demonstrate neurotrophic activities. The two members of this family are: cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) (Lindholm et al., 2007; Voutilainen et al.,

2009). The signalling cascades of these ligands are still not fully understood, however, there are four mechanisms that have been proposed. The first is the consequential dimerization of CDNF or MANF following a change in pH or lipid binding. The second is the possible facilitation by the C-terminal domain of either of these neurotrophic factors in the formation of disulfide bridges on secretory proteins in the endoplasmic reticulum. Thirdly, a disulfide bond on a transmembrane receptor protein on the cell's surface may be reduced by either CDNF or MANF. And finally, the fourth possibility is that CDNF or MANF may activate transmembrane receptor proteins by interacting with the cell membrane (Lindholm and Saarma, 2010).

The previously mentioned pro-survival effects of VPA are believed to be involved in the induction of many neurotrophic factors including BDNF, GDNF, CDNF, and MANF (Chen et al., 2006; Monti et al., 2009; Niles et al., 2012), which have been implicated in neuronal survival in models of neurodegeneration including Parkinson's disease (Lindholm et al., 2007; Voutilainen et al., 2009, 2011; Duarte et al., 2012; Ren et al., 2013). Evidence has shown that VPA induces the expression of BDNF and GDNF in rat C6 glioma cells (Castro et al., 2005a), and recently VPA was shown to increase the expression of CDNF and MANF in mouse C17 neural stem cells (Almutawaa et al., 2014), rat hippocampus and striatum (Niles et al., 2012). Studies also suggest that VPA results in the induction of dopamine-preserving neurotrophic factors (Chiu et al., 2013), with consequent neuroprotection in animal models of Parkinson's disease (Monti et al., 2010).

1.7: Epigenetic Modulation, Histone Acetylation, and Inhibition of Histone Deacetylases

Every cell in an organism contains the same genetic material, however, there are variations in how these genes are regulated which affect the development and differentiation of each cell (Konsoula and Barile, 2012). The record of the combination of DNA and the modifications to post-translational histones and proteins, thereby defining the transcriptional activities of a cell, are referred to as the epigenome (Harrison and Dexter, 2013). In recent years, epigenetic research has focused on modifications in gene expression or function, without changes in DNA sequence. The major types of epigenetic changes include DNA methylation, and post-translation histone alterations (Feng et al., 2015).

Nucleosomes, an integral unit of chromatin located within the nucleus of each cell, are formed by DNA base pairs, tightly wound around an octamer of histone proteins. Each histone is comprised of an N-terminal that projects out of the nucleosome, which contains many sites that allow for potential modifications or interaction with other factors (Landgrave-Gómez et al., 2015). Alterations to these post-translational histone tails have been extensively described and include acetylation, methylation, sumoylation, ubiquitination, and phosphorylation, of which lysine residue acetylation is of particular importance (Konsoula and Barile, 2012). Histone acetylation is the addition of an acetyl group to a lysine residue, which is achieved by the enzyme, histone acetyltransferase (HAT), resulting in the activation of gene expression. On the other hand, a class of enzymes referred to as histone deacetylases (HDACs), remove acetyl groups and inhibit gene expression (Fig. 1.2) (Jenuwein and Allis, 2001; Feng et al., 2015).

In Parkinson's disease, evidence suggests that the methylation of the SNCA gene, which leads to an overexpression of α-synuclein, may prevent histone acetylation by

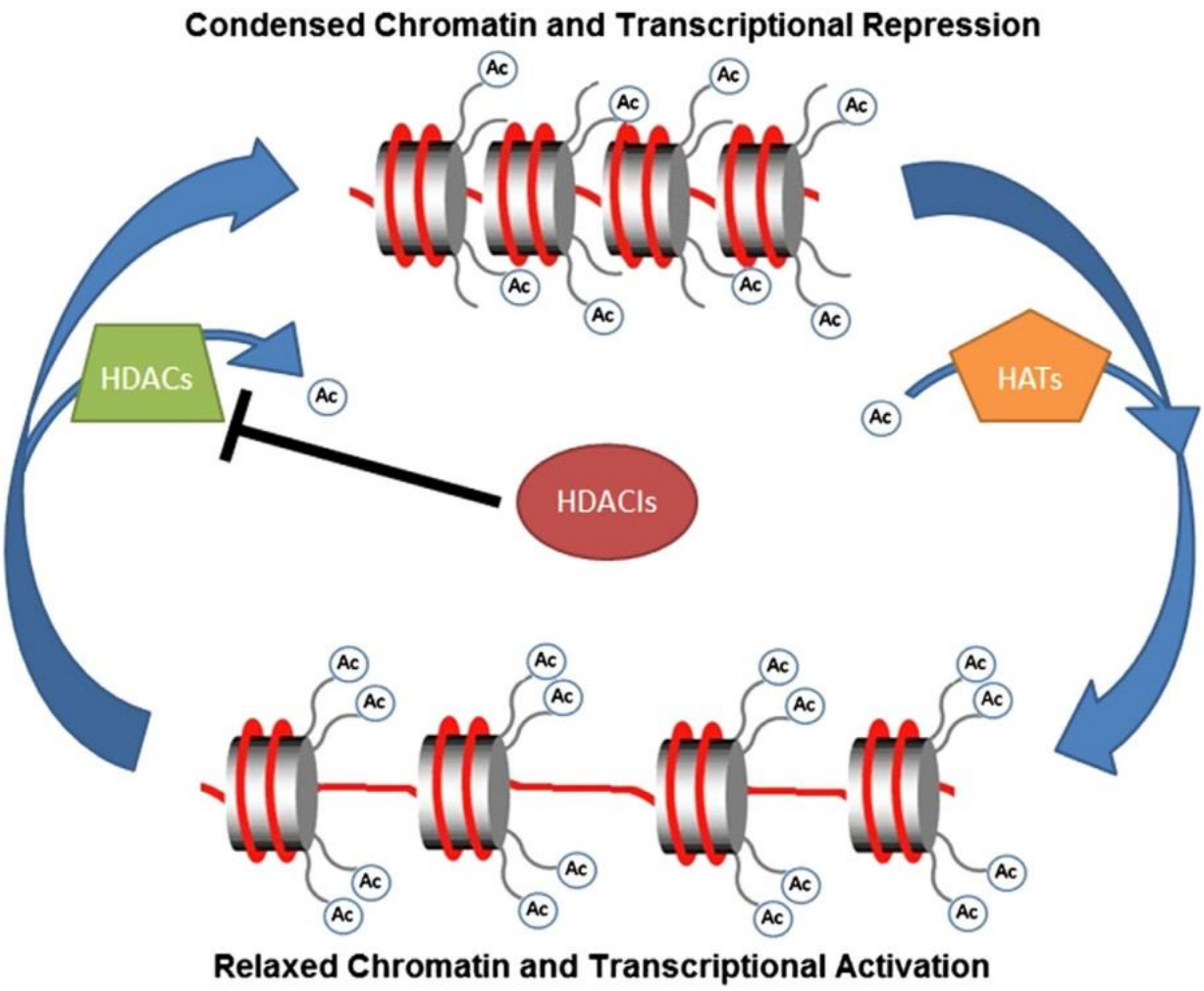

Figure 1.2: Acetylation and deacetylation of histone proteins (Harrison and Dexter, 2013).

interfering with histone acetyl transferase (CREB-binding protein (CBP), p300 and p300/CBP-associated factor (P/CAF)) activity (Outeiro et al., 2007; Konsoula and Barile, 2012; Feng et al., 2015). This obstruction of activity results in an inhibition of gene expression, which leads to mitochondrial dysfunction and eventually cell death. Using a *Drosophila* Parkinsonian model, researchers found that neuronal cell death, as a result of α-synucleinopathy, could be prevented by treatment with the HDAC inhibitors, sodium butyrate and eranilohydroxamic acid (SAHA) (Kontopoulos et al., 2006). In addition to their effects on α-synucleinopathy, HDAC inhibitors, such as VPA, have been shown to upregulate neurotrophic factors and protect dopaminergic neurons, encouraging their use in the treatment of neurodegenerative disorders, such as Parkinson's disease (Airaksinen and Saarma, 2002; Chen et al., 2006; Wu et al., 2008a).

1.8: Melatonin Synthesis, Metabolism and Biological Functions

Melatonin (*N*-acetyl-5-methoxytryptamine), an endogenous indoleamine, is a highly conserved antioxidant molecule that is mainly produced through a series of biochemical reactions within the cells of the pineal gland. Melatonin is synthesized during the dark phase, as light inhibits the biological processes that trigger melatonin biosynthesis. These light signals are conveyed from the retina to the suprachiasmatic nuclei of the hypothalamus that reaches the pineal gland after travelling through a multi-synaptic pathway to the superior cervical ganglia (Tsang et al., 2014). The production of melatonin is predominantly regulated by norepinephrine release from the sympathetic nerves, and begins with the uptake of the amino acid tryptophan, acquired through the diet, from circulation into the pineal gland. The enzyme tryptophan hydroxylase then converts tryptophan into 5-hydroxytryptophan, which is further converted into 5-

hydroxytryptamine (serotonin) by the enzyme, 5-hydroxytryptophan decarboxylase. Serotonin is then acetylated into N-acetylserotonin (NAS) by ararylalkylamine N-acetyltransferase (AANAT). The acetylation of serotonin is the rate-limiting step in the synthesis of melatonin. NAS is then O-methylated by hydroxyindole-O-methyltransferase to produce melatonin, where it is not stored in the pineal gland, but rather, immediately enters into the cerebral spinal fluid (CSF) in the third ventricle by diffusing into the capillary bed that surrounds the pinealocytes of the pineal gland (Fig. 1.3) (Tricoire et al., 2002; Barrenetxe et al., 2004; Hardeland, 2008). Synthesis of melatonin is not exclusive to the pineal gland, as it is also produced in the gastrointestinal tract, ovaries, testes, bone marrow, and eyes, though these do not significantly contribute to melatonin blood levels (Esposito and Cuzzocrea, 2010; Singhal et al., 2012). Within mammals, melatonin has been detected in essentially all biological fluids including blood, cerebrospinal fluid (CSF), bile, and saliva (Martín et al., 2000a).

Circulating melatonin has a half-life of anywhere from less than 30 minutes to 45 minutes and can be metabolized through multiple processes (Illnerova et al., 1978; Vakkuri et al., 1985; Yellon, 1996; Hardeland and Poeggeler, 2012). The liver acts as the primary site for melatonin metabolism, where the hormone is transformed into 6-hydroxymelatonin through the process of hydroxylation, and then converted into a sulfate or glucuronide (Kopin et al., 1961; Acuña-Castroviejo et al., 2014b). Other modes of melatonin metabolism include its deacetylation into 5-methoxytryptamine, which is then deaminated to 5-methoxytryptophol and 5-methoxyindoleacetic acid, which can occur in the retina and liver (Rogawski et al., 1979; Cahill and Besharse,

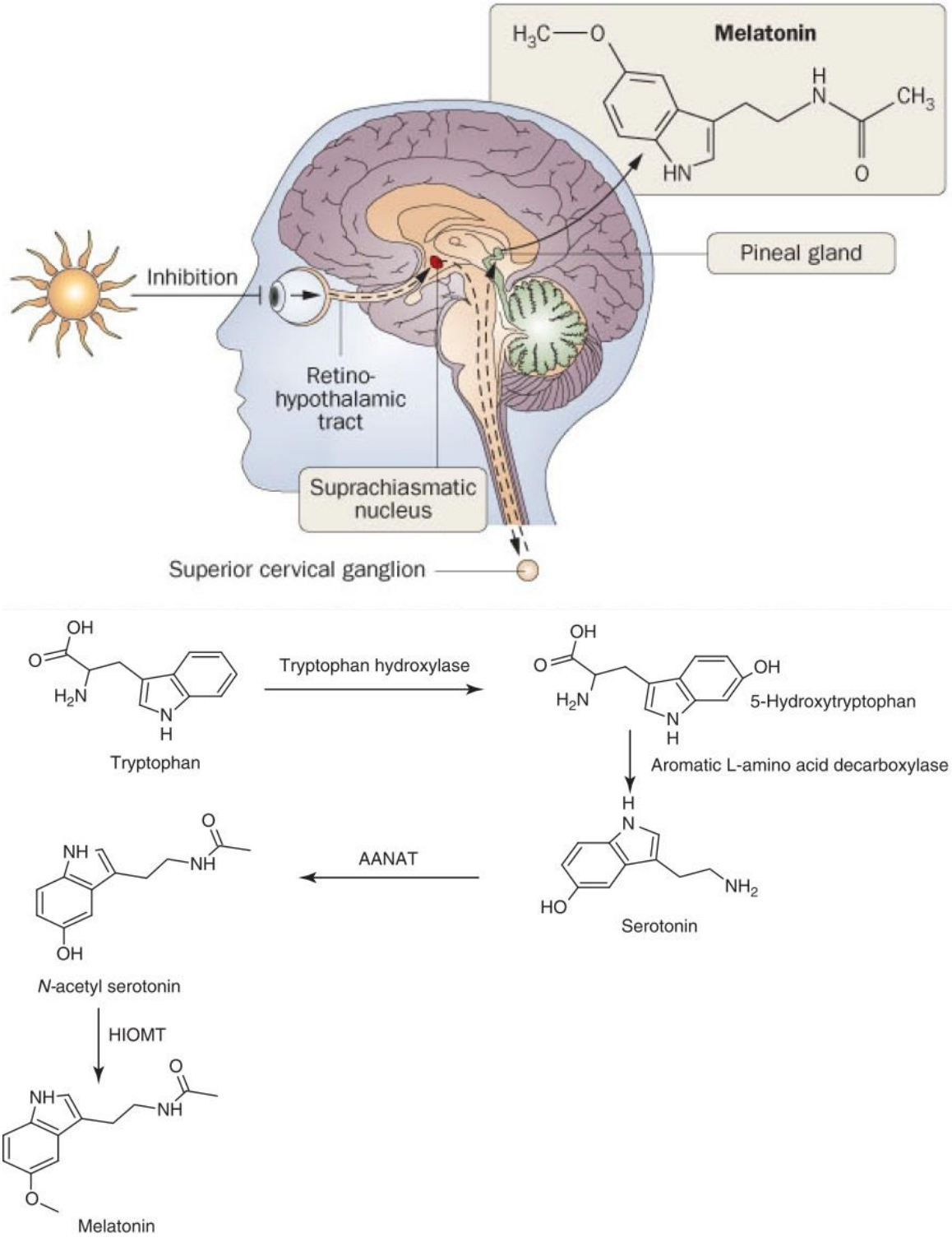

Figure 1.3: Pineal innervation and melatonin biosynthesis (Koch et al., 2009; Singh and Jadhav, 2014)

1989; Grace et al., 1991). Finally, within the brain, melatonin is metabolized into L-kynurenine by 2,3-dioxygenase, through the cleavage of the indole ring (Fujiwara et al., 1978; Vanecek, 1998). The roles of melatonin in mammals include the maintenance of circadian rhythmicity, homeostasis, energy metabolism, physiological growth, and modulation of neuroendocrine and immune function (Vanecek, 1998; von Gall et al., 2002; Macchi and Bruce, 2004; Acuña-Castroviejo et al., 2014b; Gamble et al., 2014). Beyond these roles, melatonin and its metabolites have also been implicated as having antioxidative and free-radical scavenging properties (Reiter et al., 2004; Borah and Mohanakumar, 2009; Galano et al., 2011). The various actions of melatonin have raised the possibility of its use for clinical purposes that not only include depression, seasonal affective disorder, and migraines, but also its therapeutic potential in degenerative and age-associated diseases (Luchetti et al., 2010).

1.9: Melatonin Receptors, Signalling, and Additional Binding Sites

Mediation of melatonin's effects within the mammalian system can be primarily attributed to two G protein-coupled melatonin receptor subtypes, MT_1 and MT_2, that are composed of 350 and 362 amino acids, respectively (Tan et al., 2002; von Gall et al., 2002; Hardeland, 2009). Further, the affinities of the MT_1 and MT_2 receptors for melatonin differ, with K_i values of 80.7 and 383 pM in humans, respectively (Kato et al., 2005). A third low-affinity binding site for melatonin (K_d 0.9-10 nM), previously referred to as MT_3, has been identified as the detoxifying enzyme, quinone reductase 2 (Luchetti et al., 2010). Localization of the two G-protein coupled receptor subtypes using *in situ* hybridization in rats revealed an abundance of MT_1 messenger RNA (mRNA) within the suprachiasmatic nucleus, the paraventricular thalamus, and the pars tuberalis, whereas

MT_2 mRNA was found in the suprachiasmatic nucleus and the hippocampus (von Gall et al., 2002). In addition to these locations, both receptors have been found within the nigrostriatal pathway (Uz et al., 2005). Further examination in humans has extended the distribution of receptors to the cerebellar cortex, with MT_1 mRNA located in cerebellar granule and basket-stellate cells, and MT_2 mRNA within Bergmann glia and astrocytes (Al-Ghoul et al., 1998).

Both of these G-protein coupled receptors contain seven transmembrane domains, with glycosylation sites in the N-terminal region, and palmitoylatable cysteine residues on the fourth intracellular loop (Hardeland, 2009). It was also detected that there were phosphorylation sites within the C-terminal domains for protein kinases A (PKA) and C (PKC), as well as sites for casein kinases 1 and 2 (Dubocovich and Markowska, 2005). Through both of these receptors, melatonin is capable of mediating intracellular signalling by regulating the activities of phospholipase C (PLC), guanylate cyclase (GC), and calcium and potassium channels. In addition to these signal cascades, both melatonin receptors, when coupled, can inhibit adenylyl cyclase (AC) activity through a pertussis toxin-sensitive G-protein (Carlson et al., 1989). Furthermore, both receptors allow for downstream activation of multiple signal transduction pathways.

In particular, the diversity of the response effects of the MT_1 receptor can further be attributed to its ability to couple with a variety of G-proteins, including $Gi_{\alpha 2}$, $Gi_{\alpha 3}$, $Gi_{\alpha q}$, $Gi_{\alpha s}$, $Gi_{\alpha z}$, and $Gi_{\alpha 16}$ (Brydon et al., 1999b; Ho et al., 2001; Chan et al., 2002a; Jarzynka et al., 2009). Activation of MT_1 by melatonin can also result in inhibition of the cAMP pathway (Niles and Hashemi, 1990; Morgan et al., 1991; Brydon et al., 1999a), which leads to lowered PKA activity, and decreased phosphorylation of CREB (Morgan et al.,

1994; Witt-Enderby et al., 1998). In addition to this signalling cascade, MT_1 receptors can act to stimulate PLC-dependent pathways either directly or indirectly via $G_{\beta\gamma}$ and regulate arachidonic acid formation (Godson and Reppert, 1997; Ho et al., 2001; MacKenzie et al., 2002). Beyond these cellular responses, MT_1 receptors can activate PI3K/Akt and MAPK pathways, and stimulate c-Jun N-terminal kinase (JNK) dependent signalling cascades, resulting in the stimulation of AP-1 transcription activity (Witt-Enderby et al., 2000; Chan et al., 2002b).

Similar to that observed with the MT_1 receptor, coupling of the MT_2 receptor causes inhibition of cAMP formation, stimulation of the JNK-dependent pathway, and activation of PLC (Brydon et al., 1999b; Ho et al., 2001; Chan et al., 2002b; Singh and Jadhav, 2014). In addition to these effects, the MT_2 receptor is capable of inhibiting GC and cyclic guanosine monophosphate (cGMP) (Barrenetxe et al., 2004; Acuña-Castroviejo et al., 2014a).

Not only are the actions of melatonin due to receptor-mediated events, melatonin is also able to bind to the calcium binding protein, calmodulin, whereby inhibiting enzymes that are dependent on the calcium/calmodulin complex, such as nitric oxide synthase (Bettahi et al., 1996; Luchetti et al., 2010). Further, melatonin can bind to the tyrosine kinase-like and retinoid Z orphan receptors from the superfamily of nuclear hormone receptors, including RZRα, RZRβ, RZRα2, RORα1, RORα3, and RORγ (Carlberg and Wiesenberg, 1995; Luchetti et al., 2010). The coupling of melatonin to these orphan receptors are suggested to play a role in immune modulation and upregulation of antioxidant enzymes, which may contribute to melatonin's protective effects within the central nervous system (Hardeland, 2009).

1.10: Antioxidant Properties of Melatonin

Melatonin has been reported to act as a potent antioxidant and free radical scavenger to prevent neuronal cell death that is triggered by reactive oxygen species (Akbulut et al., 2008; Acuña-Castroviejo et al., 2014a; García et al., 2014), and to protect against mitochondrial dysfunction (Martín et al., 2000b; Mayo et al., 2005; Patki and Lau, 2011). Melatonin itself can directly interact with reactive oxygen species, where it is then metabolized into several metabolites including N^1-acetyl-5-Methoxykynuramine (AMK) and N^1-acetyl-N^2-Formyl-5-Methoxykynuramine (AFMK). These metabolites have also been shown to act as scavengers, thus enhancing the ability for melatonin to reduce reactive oxygen species (Hardeland et al., 1993; Zhang et al., 1998; Mayo et al., 2005; Galano et al., 2011). Evidence from *in vivo* studies have demonstrated the antioxidant effects of melatonin, resulting in protection within the central nervous system, through the prevention of the accumulation of damage to proteins, lipids and nuclear and mitochondrial DNA, increasing brain glutathione and superoxide dismutase levels and regulating pro- and anti-apoptotic protein levels (Baydas et al., 2005; Akbulut et al., 2008; Esposito et al., 2009). Such mechanisms have all been implicated in promoting neuronal survival in neurodegenerative diseases including Parkinson's disease (Ma et al., 2009; Tapias et al., 2009).

1.11: Melatonin and Neurotrophic Factors

As previously mentioned, GDNF is a neurotrophic factor that has been shown to exhibit protective effects within the central nervous system, particularly among dopaminergic neurons (Sun et al., 2005). *In vitro* and *in vivo* studies have demonstrated that GDNF treatment can prevent toxin-induced cell death and behavioural impairments, promote

nuclear receptor related 1 protein (Nurr1) and pituitary homeobox 3 (Pitx3) expression, increase tyrosine hydroxylase levels along the nigrostriatal pathway, and restore dopamine levels and function (Tomac et al., 1995; Akerud et al., 2001; Gill et al., 2003; Peng et al., 2007; Lei et al., 2011; Stahl et al., 2011). These effects of GDNF have led to investigations into possible therapies for neurodegenerative disorders, including Parkinson's disease.

Both *in vitro* and *in vivo* studies have revealed that melatonin can regulate neurotrophic factors. Melatonin has been found to upregulate GDNF in the neurons of the nigrostriatal and mesolimbic dopaminergic pathways following administration into the striatum (Tang et al., 1998). Intraperitoneal injection of melatonin has also shown to increase GDNF levels within the locus coeruleus, with protective effects against iron-induced oxidative stress (Chen et al., 2003). In addition to these studies, systemic pretreatment of melatonin before kainic acid injection was able to elevate hippocampal levels of GDNF within hours of the injection (Martin et al., 2006). Furthermore, melatonin-induced increases in GDNF mRNA expression have been demonstrated within C6 glioma cells (Armstrong and Niles, 2002) and C17.2 neural stem cells (Niles et al., 2004). These results advocate a protective role for melatonin in degenerative disorders, which could in part be due to its ability to mediate neurotrophic factor expression.

1.12: Rationale and Objectives

Study 1: Chronic VPA treatment in an intrastriatal rotenone model of Parkinson's disease

Evidence from previous studies suggest that VPA is protective and promotes neuronal cell viability, both *in vitro* and *in vivo*. Recent reports have indicated that VPA is neuroprotective in an MPTP mouse model of Parkinson's disease, when co-administered with the neurotoxin for five days and continued for an additional two weeks (Kidd and Schneider, 2011). Another study showed that pretreatment with VPA in human neuroblastoma SH-SY5Y cells that were treated with rotenone, enhanced cell viability, decreased nuclear fragmentation and apoptosis, and moderated reactive oxygen species generation (Xiong et al., 2011). Moreover, in a recent *in vivo* study, VPA was able to protect dopamine neurons in a systemic rotenone model of Parkinson's disease (Monti et al., 2010), in which rats were pretreated with VPA (in standard chow) for four weeks and, while continuing VPA treatment, received rotenone subcutaneously via osmotic minipumps for one week before sacrifice. However, as previously stated, systemic administration of rotenone has deleterious effects in animals including high morbidity and mortality (Cannon et al., 2009; Monti et al., 2010). In contrast, when rotenone is injected into the striatum, the general health of the animals is not affected, though nigrostriatal degeneration and contralateral motor dysfunction are observed (Mulcahy et al., 2011).

Given these findings, and evidence that VPA upregulates antioxidant levels, including glutathione (Cui et al., 2007), and neuroprotective gene expression, as

mentioned earlier, my first study used a slight modification of this intracranial model to assess if the chronic treatment of VPA could protect against the dopaminergic degeneration caused by rotenone. It is important to note that our animals were not pretreated but instead received VPA in their drinking water immediately upon their recovery, following stereotaxic infusion of rotenone into the striatum. The first objective of this study was to examine if intrastriatal rotenone infusion would result in contralateral motor deficits, as well as depletion of tyrosine hydroxylase immunoreactivity within the striatum and dopamine cell loss within the substantia nigra, using a stereological approach. The second objective was to determine if chronic VPA treatment was protective against motor dysfunction and degeneration along the nigrostriatal pathway.

Study 2: Chronic low-dose melatonin treatment in an intrastriatal rotenone model of Parkinson's disease

Studies have revealed that pharmacological doses (e.g. 10-30 mg/kg) of melatonin are neuroprotective in the 6-OHDA (Joo et al., 1998; Sharma et al., 2006; Borah and Mohanakumar, 2009), MPTP (Acuña-Castroviejo et al., 1997; Thomas and Mohanakumar, 2004), and rotenone models of Parkinson's disease (Saravanan et al., 2007; Bassani et al., 2014). However, earlier studies have shown that such pharmacological (micromolar) concentrations of melatonin can bind to both central-type and peripheral-type benzodiazepine receptors, which are linked to GABAergic and mitochondrial function, respectively (Tenn and Niles, 2002). Moreover, both of these benzodiazepine receptors are involved in mediating the acute inhibition of nigrostriatal dopaminergic function in rats, following treatment with a pharmacological dose (10

mg/kg) of melatonin (Tenn and Niles, 1995, 1997). In order to avoid potential side-effects associated with chronic high doses of melatonin, it was administered at a low dose within its endogenous physiological range, which can protect against decreased mitochondrial complex I activity (Dabbeni-Sala et al., 2001) and tyrosine hydroxylase depletion following 6-OHDA lesioning (Sharma et al., 2006). Researchers have postulated that the neuroprotective effects of melatonin may be due to its ability to act as an antioxidant and free radical scavenger (Mayo et al., 2005; Vega-Naredo et al., 2005; Lin et al., 2008), as well as its ability to increase the activity of mitochondrial complexes I and IV (Martín et al., 2000b). Moreover, as discussed earlier, the melatonin receptors, MT_1 and MT_2, are distributed along the nigrostriatal pathway (Singh and Jadhav, 2014) and are able to significantly increase GDNF levels (Mayo et al., 2005), enhance tyrosine hydroxylase activity (Venero et al., 2002), and increase affinities of dopamine receptors (Hamdi, 1998; Sumaya et al., 2004; Lin et al., 2008). Based on these findings, the objective of the second study was to determine the effectiveness of chronic melatonin treatment, at physiological doses in drinking water, as a neuroprotective agent in an intrastriatal rotenone model of Parkinson's disease. For this study, we increased the dose of rotenone to 12 µg, as compared with 6 µg used in the above VPA study, assuming that a higher dose would result in the development of additional behavioural or other characteristics of Parkinson's disease. The first objective of this study was to determine if low-dose melatonin would be protective against motor dysfunction and tyrosine hydroxylase depletion following infusion of rotenone into the striatum, which has yet to be reported. The second objective was to determine if, at a higher dose, unilateral infusion of rotenone into the striatum could

result in changes to α-synuclein expression and apomorphine-induced behavioural changes.

Study 3: Intracranial rotenone infusion in mice

To date, there are no animal models of Parkinson's disease that encompass the important characteristics of the disease without being detrimental to the health of the animals. Recently, one report found that unilaterally lesioning the medial forebrain bundle in mice results in an overexpression of α-synuclein and motor dysfunction (Weetman et al., 2013). However, the authors provided no evidence of dopaminergic degeneration along the nigrostriatal pathway and did not provide any quantified results of motor deficits. For this third study, the objective was to investigate if the infusion of rotenone into both the medial forebrain bundle and the substantia nigra would result in contralateral forelimb dysfunction, changes in α-synuclein expression, and most importantly, if this would result in a reduction of tyrosine hydroxylase immunoreactivity within the striatum and substantia nigra. As the study by Weetman et al. (2013) did not report any changes in tyrosine hydroxylase activity, which could potentially be due to a lack of susceptibility to the toxin, we decided to lesion two sites, in the hopes of inducing a more advanced stage Parkinsonian model, which would mimic the depletion of tyrosine hydroxylase immunoreactivity observed in rat rotenone models of Parkinson's disease.

1.13: Hypotheses

Based on the neuroprotective effects of VPA or melatonin in other models of Parkinson's disease, it is anticipated that for the first two studies, chronic treatment with either VPA or melatonin following the infusion of rotenone into the striatum, will protect

dopaminergic neurons in the rat striatum and substantia nigra.

For the third study, we predict that infusion of rotenone into both the medial forebrain bundle and substantia nigra in mice will result in contralateral motor dysfunction, depletion of tyrosine hydroxylase immunoreactivity in the striatum and the substantia nigra, and α-synucleinopathy in the mouse brain.

CHAPTER 2: NEUROPROTECTION BY VALPROIC ACID IN AN INTRASTRIATAL ROTENONE MODEL OF PARKINSON'S DISEASE

Candace H. Carriere, Na Hyea Kang, and Lennard P. Niles. Neuroprotection by valproic acid in an intrastriatal model of Parkinson's disease. *Neuroscience* 2014, 267C:114-121.

Contributions of Authors:

LPN: Conceived and planned the study and edited the manuscript

CHC: Assisted in planning the study, performed the surgeries, conducted behavioural testing, performed the immunohistochemical studies, and wrote the manuscript

NHK: Assisted with the surgeries and behavioural testing

Abstract

Rotenone, which is used as a pesticide and insecticide, has been shown to cause systemic inhibition of mitochondrial complex I activity, with consequent degeneration of dopaminergic neurons within the substantia nigra and striatum, as observed in Parkinson's disease. A novel intrastriatal rotenone model of Parkinson's disease was used to examine the neuroprotective effects of valproic acid (VPA), which is known to upregulate neurotrophic factors and other protective proteins in the brain. Sham or lesioned rats were treated with either vehicle or VPA at a dose of 4 mg/mL in drinking water. The right striatum was lesioned by infusion of rotenone at three sites (2 µg/site) along its rostrocaudal axis. A forelimb asymmetry (cylinder) test indicated a significant ($p < 0.01$) decrease in use of the contralateral forelimb in rotenone-lesioned animals, in the third week post-lesioning, which was abolished by VPA treatment. Similarly, a significant ($p< 0.01$) and persistent increase in use of the ipsilateral forelimb in lesioned animals over the 4 weeks of testing, was not seen in animals treated with VPA. Results of the asymmetry test illustrate that intrastriatal infusion of rotenone causes contralateral motor dysfunction, which is blocked by VPA. The significant increase in ipsilateral forelimb use has not been documented previously, and presumably represents a compensatory response in lesioned animals. Six weeks post-surgery, animals were sacrificed by transcardial perfusion. Subsequent immunohistochemical examination revealed a decrease in tyrosine hydroxylase immunoreactivity within the striatum and substantia nigra of rotenone-lesioned animals. VPA treatment attenuated the decrease in tyrosine hydroxylase in the striatum and abolished it in the substantia nigra. Stereological cell counting indicated a significant ($p < 0.05$) decrease in tyrosine

hydroxylase-positive dopamine neurons in the substantia nigra of rotenone-lesioned animals, which was confirmed by Nissl staining. Importantly, this loss of dopamine neurons in rotenone-lesioned animals, was blocked by chronic VPA treatment. These findings strongly support the therapeutic potential of VPA in Parkinson's disease.
Key words: Parkinson's disease, rotenone, intrastriatal, valproic acid, neuroprotection.

INTRODUCTION

Regarded as the second most common age-related neurodegenerative disorder after Alzheimer's disease (Dauer and Przedborski 2003), Parkinson's disease is characterized by the loss of dopamine-producing neurons within the substantia nigra pars compacta, which in turn contributes to a depletion of postsynaptic dopamine within the striatum. This loss of dopamine functionality results in motor behavioral deficits including uncontrollable tremor, postural imbalance, rigidity and bradykinesia (Lotharius and Brundin 2002). The severity of these motor symptoms seems to be due to the loss of tyrosine hydroxylase-positive dopaminergic neurons in the substantia nigra (Iancu et al. 2005).

Administration of rotenone, a potent rotenoid used in insecticides (Dauer and Przedborski 2003), has been shown to result in the systemic inhibition of mitochondrial complex I activity, which leads to the degeneration of dopaminergic neurons within the substantia nigra and striatum (Alam and Schmidt 2002; Yang et al. 2006). Intraperitoneal administration of rotenone has been used as a model of Parkinson's disease, but its deleterious effects including high morbidity and mortality (Cannon et al. 2009; Monti et al. 2010), make it unsuitable for extended studies of neuroprotection. An earlier study showed that an acute intranigral injection of rotenone produces

progressive Parkinsonian symptoms over several weeks (Saravanan et al. 2005). Recently, an intrastriatal rotenone infusion approach was found to produce a useful Parkinsonian model, as shown by behavioral, immunohistochemical and biochemical analyses of nigrostriatal function (Mulcahy et al. 2011). There is growing support for the beneficial effects of valproic acid (VPA), a widely used anticonvulsant and antidepressant, which can inhibit histone deacetylase activity (Göttlicher et al. 2002) with consequent changes in neurotrophic or neuroprotective gene expression (Monti et al. 2009; Chiu et al. 2013). Therefore, we have examined for the first time, the neuroprotective effects of chronic treatment with VPA, in an intrastriatal rotenone model of Parkinson's disease.

EXPERIMENTAL PROCEDURES

Experimental design

All experimental procedures were carried out in accordance with the guidelines set by the Canadian Council for Animal Care and approved by the McMaster University Animal Research Ethics Board. Male Sprague–Dawley rats weighing 225–267 g at the start of testing, were used. Baseline performance in behavioral tests (forelimb asymmetry and postural instability) was determined 12 days pre-operatively. Rats were individually housed and randomly divided into sham or lesioned groups, and further divided into one of two treatment conditions. The treatments were a VPA vehicle (drinking water) or VPA (4 mg/mL in drinking water; Sigma–Aldrich Inc., St. Louis, MO, USA). Animals received fresh water with or without VPA every Tuesday and Friday for six weeks, at which times body weight and water consumption were recorded. Behavioral testing was conducted for up to 4 weeks, starting in the third week post-

surgery. Six weeks after lesioning, rats were sacrificed by transcardial fixation and brains were harvested for quantitative tyrosine hydroxylase immunohistochemistry.

Surgery

Unilateral lesioning of the right striatum was conducted under isoflurane anesthesia (5% in O_2 for induction and 2.5% in O_2 for maintenance) in a stereotaxic apparatus with the nose bar set at -2.5mm. Lesioned rats received rotenone (Sigma-Aldrich Inc.) that was dissolved in a vehicle of DMSO, Cremophor® (Sigma-Aldrich Inc.) and saline (1:1:18) and sham-lesioned rats received an equal volume of vehicle (Mulcahy et al. 2011). The striatum was lesioned by infusion (0.3 µl min^{-1} for 6-7 min with 3 min for diffusion) at three points along its rostro-caudal axis at the stereotaxic coordinates AP +1.0, ML -3.0; AP -0.1, ML -3.7; AP -1.2, ML -4.5 (from bregma) and DV -5.0 below dura (Kirik, Rosenblad, and Björklund 1998). All rats received a total of 6 µl infusion (over three sites) of either vehicle (2.0 µl/site) or rotenone (2.0 µg in 2.0 µl/site). Treatment with VPA began on the same day as the stereotaxic surgery, as rats recovered and were returned to their cages.

VPA blood levels

Chronic treatment with VPA (4 mg/mL in drinking water) was started immediately after surgery. Earlier studies indicate that chronic treatment with 3.3 or 6.6 mg/mL of VPA in drinking water produces day and night blood VPA concentrations of 0.15–0.4 and 0.25–0.67 mM, respectively in rats (Frisch et al. 2009). Therefore, the dose of 4 mg/mL of VPA in drinking water, administered in this study, is well within the lower end of the therapeutic range of about 0.4–1 mM VPA blood levels (Yuan et al. 2001).

Behavioral testing

A postural instability test was used to measure forelimb akinesia (Woodlee et al. 2008). Animals were handled daily to acclimate them for the postural instability test. The rat's hind limbs and one forelimb were carefully restrained, with the torso positioned above a table surface. The length of the adjusting step taken by the free forelimb, when the rat experienced a change in its center of gravity, was measured manually using a ruler which was taped to the stepping board. One examiner conducted the test while another recorded the distance. A forelimb use asymmetry test was used to evaluate motor performance in all treatment groups. Animals were acclimated in a transparent Plexiglas cylinder (22 cm diameter and 30 cm height) for 5 min once prior to testing. Subsequently, forelimb use during explorative behavior was videotaped for 5 min. This behavior was quantified based on the independent use of either the left or right forelimb for contacting the wall during a full rear (Fleming, Delville, and Schallert 2005). All behavioral measurements were blind, with a coding system used to identify animals only after data compilation.

Tyrosine hydroxylase immunohistochemistry

Animals were deeply anaesthetized and sacrificed by transcardial perfusion with a pre-wash of saline (0.9% NaCl) followed by 4% paraformaldehyde fixative (PFA; Caledon Canada Ltd., Georgetown, Ont.). Brains were harvested and post-fixed in 4% PFA overnight and then transferred into PBS with 0.06M sodium azide and stored at 4°C until slicing. Brains were cryoprotected in a 30% sucrose solution 72 hours before cryosectioning. The striatum and substantia nigra were identified in the prepared tissue using the Paxinos and Watson (2007) rat brain atlas. The striatum coordinates

were: 2.20 mm from Bregma to -3.80 mm from Bregma, with one slice collected for every four taken. The substantia nigra coordinates were: -4.52 mm from Bregma to -6.30 mm from Bregma, with one slice collected for every six taken. Tissue was sectioned at 35 μm and placed in individual tissue wells filled with PBS and 0.06M sodium azide solution and stored at 4°C until processed. The primary antibody was an anti-tyrosine hydroxylase rabbit IgG antibody (Millipore, Temecula, CA), diluted 1:1000 in 1.5% normal donkey serum (NDS) in phosphate buffered saline (PBS). The secondary antibody was a fluorescein (FITC)-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch, Inc., West Grove, PA), diluted 1:200 in 1.5% NDS in PBS. Tissue sections were rinsed with PBS-Triton X (0.5% concentration) three times for 10 minutes and then placed in 1.5% NDS in PBS for 1 hour. Tissue sections were incubated in the primary antibody solution for 72 hours at 4°C. Following incubation, tissue was rinsed three times for 10 minutes in PBS-Triton X and placed in the secondary antibody solution for 2 hours at room temperature. Again, tissue sections were rinsed three times for 10 minutes with PBS-Triton X and mounted and cover-slipped using Dako Mounting Medium (Dako, Burlington, ON). Separate sections from the substantia nigra were mounted overnight to gelatin-coated slides in preparation for Nissl staining. Samples were delipidized in ethanol/xylene, then rehydrated through a series of ethanol/dH2O washes. Tissue was stained with 0.13% Cresyl Violet (Sigma–Aldrich Inc.), rinsed in dH2O, and dehydrated in ethanol/xylene (Wu et al. 2012). Nissl-stained cell counts were obtained from these samples, using the same stereological procedure described below for tyrosine hydroxylase cell counts from FITC-stained substantia nigra sections, in order to validate treatment-induced

changes in dopamine cell number. To control for any variability that may occur throughout processing, full sets of tissue samples, one brain from each treatment, were sliced, stained and imaged at the same time. The laser and program settings also remained constant across all samples.

Stereological analysis of dopamine neurons in substantia nigra

Samples from the substantia nigra were imaged using a confocal microscope (LSM 510, Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) equipped with a Plan-Apochromatic 63x 1.4 Oil DIC, with excitation using a 488-nm Argon laser for high resolution imaging. Every sixth section throughout the substantia nigra was collected with the first section being randomly selected, allowing for an unbiased series of sections. Optical stacks of images were acquired from the substantia nigra at 63x magnification for stereological procedures. LSM and Zen software were used for data analysis. Estimation of volume was performed using the Cavalieri principle (Mayhew and Gundersen 1996). Cell counts were collected from z-stack images of 2 µm thickness, for a total thickness of 40 µm. A guard zone of 5 µm at each superficial portion of the tissue was used to prevent bias due to super-saturation of cells or artefacts resulting from the cutting process (M J West 1999). These images were saved at 512 pixels by 512 pixels for analysis. Each cell was identified and counted using unbiased design-based stereological procedures. Using the Optical Fractionator method (Mayhew and Gundersen 1996), estimated total cell numbers were calculated. Densities were calculated by taking the total estimated number of cells and dividing by the substantia nigra estimated volume, and converting each number to cells per mm^3.

Statistical analyses were run using total cell counts rather than densities to avoid potential bias (M J West 1999).

Statistics

Behavioral data were analyzed by a two-way analysis of variance (ANOVA) (treatment x time) and significant group differences (with $p<0.05$ taken as the level of significance) were determined by a Bonferroni test (Graphpad Prism version 4.0), when appropriate. Total counts of tyrosine hydroxylase-positive neurons in the substantia nigra were converted to percentage values and analyzed by a one-way ANOVA followed by a Newman–Keuls test. Data shown are expressed as Means ± S.E.M.

RESULTS

Unilateral intrastriatal infusion of rotenone does not affect rat health

Throughout the study, a 100% survival rate was maintained in both lesioned and control rats. In addition, there were no significant differences in either body weight or water consumption between any of the treatment groups (Fig 1).

VPA abolishes motor dysfunction induced by unilateral intrastriatal infusion of rotenone

Two-way ANOVA indicated a significant treatment effect ($F_{3,60} = 3.18$; $p<0.03$; $n = 5–7$) for use of the contralateral forelimb in the postural instability test. The distance for sham-lesioned VPA-treated animals to make an adjustment step with their contralateral forelimb was significantly ($p<0.05$) greater than for the control group at 5 weeks post-lesioning (Fig. 2A). This finding may be due to the tranquilizing/sedative effect of VPA, particularly as the postural instability test was conducted during the daytime, when rats are least active. There were no significant differences observed for the ipsilateral forelimb adjustment distance between any of the groups for the duration of testing (Fig.

2B). Analysis of contralateral forelimb use asymmetry data revealed significant treatment ($F_{3,78}$ = 6.91; p<0.0003; n = 5–7) and time ($F_{3,78}$ = 4.64; p<0.0049; n = 5–7) effects, but there was no interaction. Post hoc (Bonferroni) analysis indicated that in the third week post-surgery, rotenone-lesioned animals showed a significant (p<0.05 or p<0.01) decrease in the use of their contralateral forelimb, as compared to all other groups (Fig. 3A). The contralateral forelimb use of rotenone-lesioned VPA-treated animals did not differ from that of sham-lesioned animals, suggesting a neuroprotective effect for VPA. There were no significant differences in the use of the contralateral forelimb between any of the groups over the subsequent 3 weeks of testing (Fig. 3A). The apparent decrease in contralateral forelimb use in most groups at 4 weeks is not significant, as assessed by a two-way ANOVA. Analysis of ipsilateral forelimb use asymmetry data revealed a significant treatment effect ($F_{3,74}$ = 20.58; p<0.0001; n = 5–7), but there were no time or interaction effects. Post hoc analysis confirmed a significant (p<0.05–0.001) increase in the use of the ipsilateral forelimb in the rotenone-lesioned animals versus all other groups over the 4 weeks of testing. It is noteworthy that this abnormality was completely blocked in rotenone-lesioned animals treated with VPA (Fig 3B).

Unilateral intrastriatal infusion of rotenone induces nigrostriatal degeneration that is blocked by VPA treatment

Using tyrosine hydroxylase immunohistochemical staining, it was observed that unilateral intrastriatal rotenone infusion depleted tyrosine hydroxylase immunoreactivity in the striatum and substantia nigra. This loss of tyrosine hydroxylase was attenuated in the striatum (Fig. 4) and in the substantia nigra (Fig. 5) of lesioned animals treated

with VPA. In accordance with the above, a one-way ANOVA indicated a significant rotenone effect ($F_{3,11} = 8.91$; $p<0.0063$; $n = 3$) on tyrosine hydroxylase-positive (dopamine) neurons in the substantia nigra. Dopamine cell counts in the lesioned (ipsilateral) substantia nigra, as a percentage of counts from the intact (contralateral) side or from sham controls, were decreased significantly as compared with sham-vehicle ($p<0.05$) and sham-VPA treated ($p<0.01$) groups (Fig. 6). VPA treatment blocked the rotenone-induced loss of dopamine neurons, as there was no difference between cell counts of the rotenone-lesioned VPA-treated animals and sham-lesioned animals (Fig. 6). Interestingly, chronic VPA treatment increased the number of tyrosine hydroxylase-positive cells in the substantia nigra by about 25–30%, although the low n = 3 value precluded significance. This is in keeping with recent evidence that chronic VPA treatment significantly increases tyrosine hydroxylase protein levels in the rat substantia nigra (Monti et al. 2010), and earlier reports that VPA influences the expression of this enzyme in the rat brain (Sands, Guerra, and Morilak 2000) and cultured PC12 cells (D'Souza et al. 2009).

Nissl staining confirmed that rotenone infusion results in the loss of cells within the substantia nigra, such that lesioned animals exhibited ipsilateral cell counts which were 34.6% of that on the intact (contralateral) side. The percentages of lesioned side to intact side for vehicle, VPA, and rotenone–VPA-treated animals were 110.7%, 99.7%, and 88.0%, respectively, demonstrating that VPA is neuroprotective against cell death resulting from rotenone infusion.

DISCUSSION

Rotenone models of Parkinson's disease, based on systemic administration of this pesticide, have been shown to produce Parkinsonian symptoms such as α-synucleinopathy (Uversky, Li, and Fink 2001), dopaminergic degeneration and motor dysfunction in rodents (Alam and Schmidt 2002; Sherer et al. 2003; Cannon et al. 2009). Unfortunately, the high morbidity and mortality rates associated with systemic rotenone treatment (Cannon et al. 2009) have been problematic. In contrast, stereotaxic intracranial rotenone models appear to resolve these health issues, while inducing dopaminergic degeneration and motor abnormalities (Sindhu et al. 2005; Xiong et al. 2009; Norazit et al. 2010), although α-synucleinopathy has not been observed (Mulcahy et al. 2011). An important advantage of the stereotaxic model is its suitability for long-term studies of potential therapeutic agents. Therefore, an intrastriatal rotenone rat model of Parkinson's disease was used, in order to determine whether the neuroprotective drug, VPA, can maintain dopaminergic integrity in this model. The normal body weights and 100% survival rate observed in lesioned animals, encourages intrastriatal infusion of rotenone as a useful model of dopaminergic neurodegeneration.

In assessing possible motor dysfunction, we used an asymmetry (cylinder) test, which revealed a significant decrease in left (contralateral) forelimb use but an increase in right (ipsilateral) forelimb use by rotenone-lesioned animals, as compared to all other groups for the first week. This finding presumably represents a contralateral motor deficit inflicted by rotenone lesioning, as all animals were lesioned in the right striatal hemisphere. This is in accordance with evidence that intrastriatal infusion of rotenone

causes contralateral motor dysfunction (Mulcahy et al. 2011). However, our observation of a significant increase in ipsilateral forelimb use has not been documented previously in this model, and may be due to a compensatory response in lesioned animals, as a result of contralateral motor impairment. Interestingly, these forelimb motor abnormalities were not seen in rotenone-lesioned animals treated chronically with VPA, indicating its neuroprotective ability in this Parkinsonian model. Moreover, immunohistochemical examination revealed a partial or complete preservation of tyrosine hydroxylase immunoreactivity in the striatum and substantia nigra, respectively, of lesioned animals treated with VPA, as shown in Figs. 4 and 5. Using unbiased stereological cell counting, we show for the first time that intrastriatal injection of rotenone causes a significant decrease in tyrosine hydroxylase-positive neurons in the substantia nigra on the infusion side, as compared to the contralateral side of the same animal or either side of sham-lesioned animals (Fig. 6). This finding confirms that retrograde degeneration occurs along the nigrostriatal pathway in this intrastriatal model. Importantly, treatment with VPA prevented the rotenone-induced loss of dopamine neurons in the substantia nigra (Fig. 6), strongly supporting a protective role for VPA in Parkinson's disease.

Previously, VPA was reported to protect dopamine neurons following rotenone administration (Monti et al. 2010), but there are notable differences between that study and this one. In the earlier study, rats were pretreated with VPA (in standard chow) for 4 weeks and, while continuing VPA treatment, they received rotenone subcutaneously via osmotic mini-pumps for 1 week before sacrifice. In the present study, animals were not pretreated but instead received VPA in their drinking water immediately upon their

recovery, following stereotaxic infusion of rotenone into the striatum. Given the expected immediate toxicity associated with intrastriatal injection of this lipophilic neurotoxin, the ability of relatively late treatment with VPA to preserve nigrostriatal integrity in this Parkinsonian model, indicates the therapeutic potential of this drug. In agreement with the foregoing, recent evidence indicates that VPA is neuroprotective in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease, when co-administered with the neurotoxin for 5 days and continued for an additional 2 weeks (Kidd and Schneider 2011).

In assessing the neuroprotective potential of various agents, it is ultimately essential to understand the underlying mechanisms in order to optimize therapeutic approaches. Although there are no known receptors for VPA, its lipophilic nature allows easy entry into target cells where it is known to interact with pro-survival protein kinases via multiple signaling cascades, including the phosphatidylinositol 3-kinase/Akt, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), and the Wnt/b-catenin pathways (Monti et al. 2009). In addition, VPA is thought to bind directly to the catalytic core of Class I histone deacetylases to inhibit their activity (Göttlicher et al. 2002), with consequent histone hyperacetylation and induction of gene transcription (Phiel et al. 2001). There is evidence that histone deacetylase inhibition by VPA plays a role in its neuroprotective effects in models of neurodegeneration, both in vitro (Leng et al. 2008; Marinova et al. 2009) and in vivo (Ren et al. 2004; Monti et al. 2009; Chiu et al. 2013). The above effects of VPA may be involved in its induction of diverse neurotrophic or protective proteins including brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), cerebral dopamine

neurotrophic factor (CDNF), mesencephalic astrocyte-derived neurotrophic factor (MANF), and heat shock protein 70 (Hsp70) (Chen et al. 2006; Monti et al. 2009; Marinova et al. 2009; Niles et al. 2012), which have been implicated in neuronal survival in models of neurodegeneration including Parkinson's disease (Lindholm et al. 2007; Voutilainen et al. 2009, 2011; Duarte et al. 2012; Ren et al. 2013).

In summary, we have presented behavioral and neuropathological evidence of the neurodegenerative effects of intrastriatal infusion of rotenone in a rat model of Parkinson's disease, which did not affect the overall health of animals over the 6-week duration of the study. Treatment with VPA abolished forelimb motor dysfunction and retrograde degeneration of the nigrostriatal pathway, with preservation of tyrosine hydroxylase immunoreactivity in the striatum and tyrosine hydroxylase-positive neurons in the substantia nigra, indicating its therapeutic potential in Parkinson's disease.

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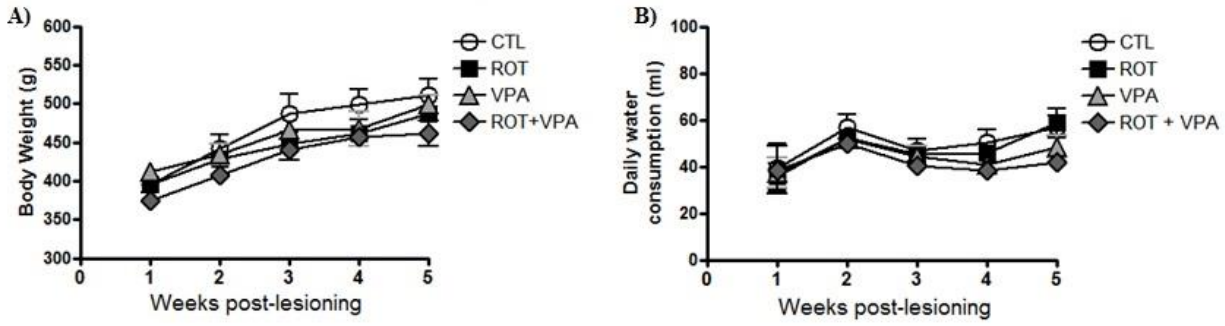

Fig. 2.1. Unilateral intrastriatal infusion of rotenone does not affect body weight or water consumption in rats. (A) There were no significant differences in the weight gain of sham vs. lesioned animals. (B) There were no significant differences in the water consumption of sham vs. lesioned animals.

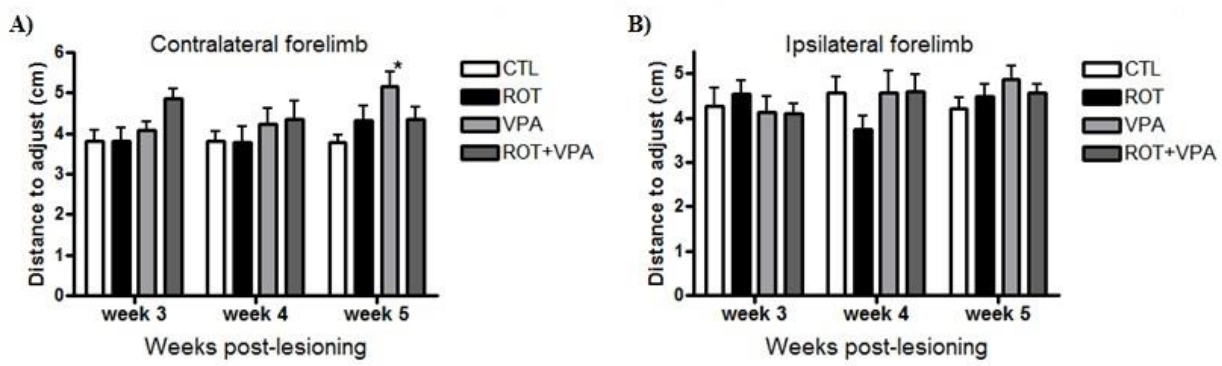

Fig. 2.2. Unilateral intrastriatal infusion of rotenone does not affect postural stability in rats. (A) A postural instability test of the left (contralateral) forelimb revealed no differences between groups except for the VPA group, which showed a significant increase in the distance required to make an adjusting step in the fifth week after lesioning. (B) There were no significant postural differences for the right (ipsilateral) forelimb. Data shown are means ± SEM. *$p<0.05$ vs. control. Two-way ANOVA and Bonferroni post-tests.

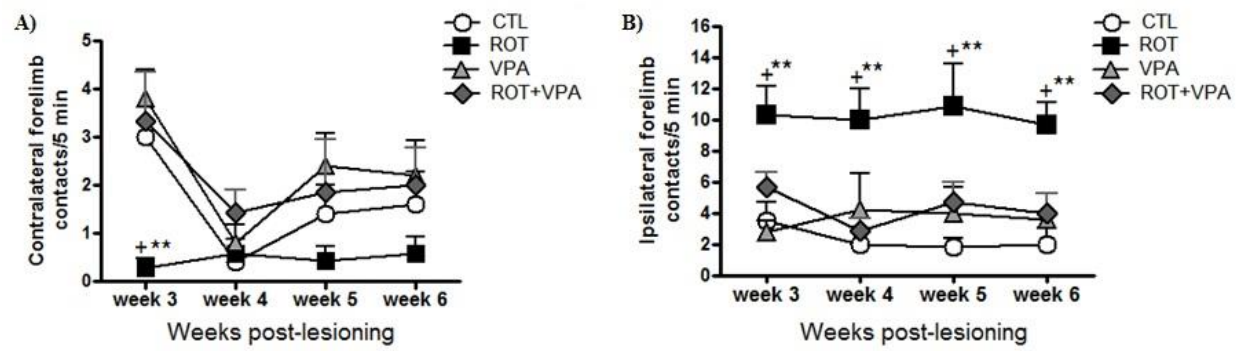

Fig. 2.3. VPA abolishes forelimb motor dysfunction induced by unilateral intrastriatal infusion of rotenone. (A) Number of left (contralateral) forelimb contacts on the cylinder wall during the asymmetry test over 4 weeks. +p<0.05 vs. control, **p<0.01 vs. VPA and ROT+VPA groups at 3 weeks post-lesioning. (B) Number of right (ipsilateral) contacts on the cylinder wall during the asymmetry test over 4 weeks. +p<0.05 vs. control, **p<0.01 vs. VPA (week 3); +p<0.05 vs. VPA, **p<0.01 vs. control and ROT +VPA (week 4); +p<0.05 vs. VPA and ROT+VPA, **p<0.001 vs. control (week 5); +p<0.05 vs. VPA and ROT+VPA, **p<0.01 vs. control (week 6). Two-way ANOVA and Bonferroni posttests.

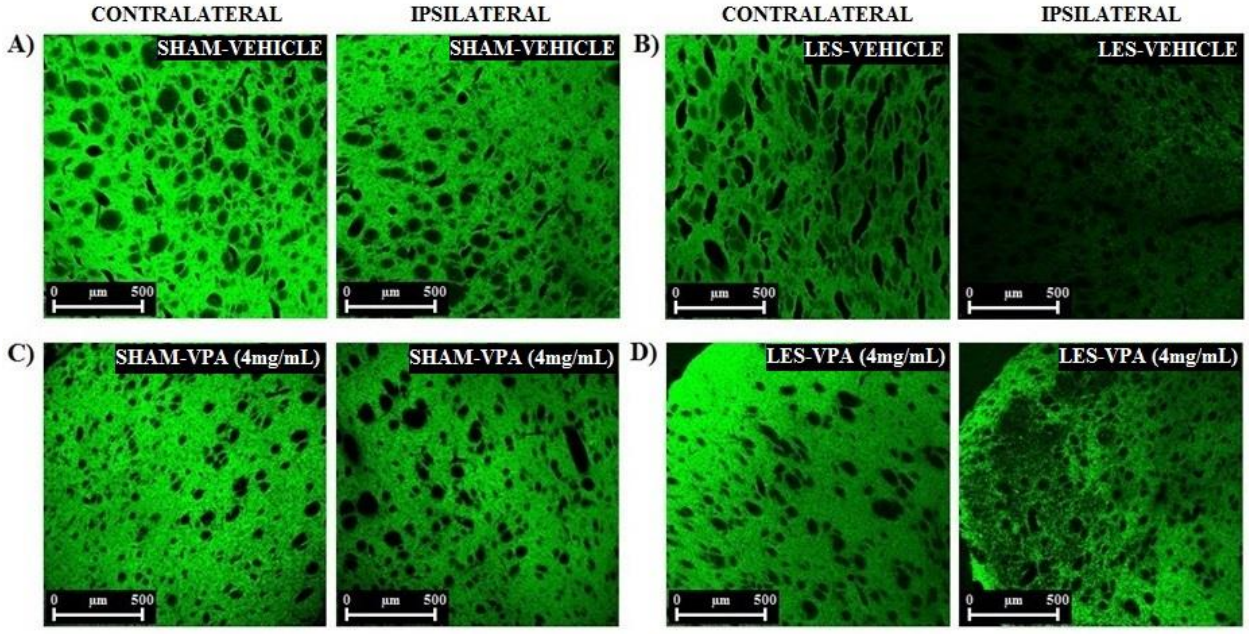

Fig. 2.4. VPA attenuates the loss of tyrosine hydroxylase immunoreactivity in the striatum of rotenone-lesioned rats. Contralateral and ipsilateral images of FITC-stained tyrosine hydroxylase in the striatum at 10x magnification are shown for: (A) Sham animals treated with vehicle; (B) rotenone-lesioned rats treated with vehicle; (C) VPA-treated rats (4 mg/mL in drinking water); (D) rotenone + VPA-treated rats.

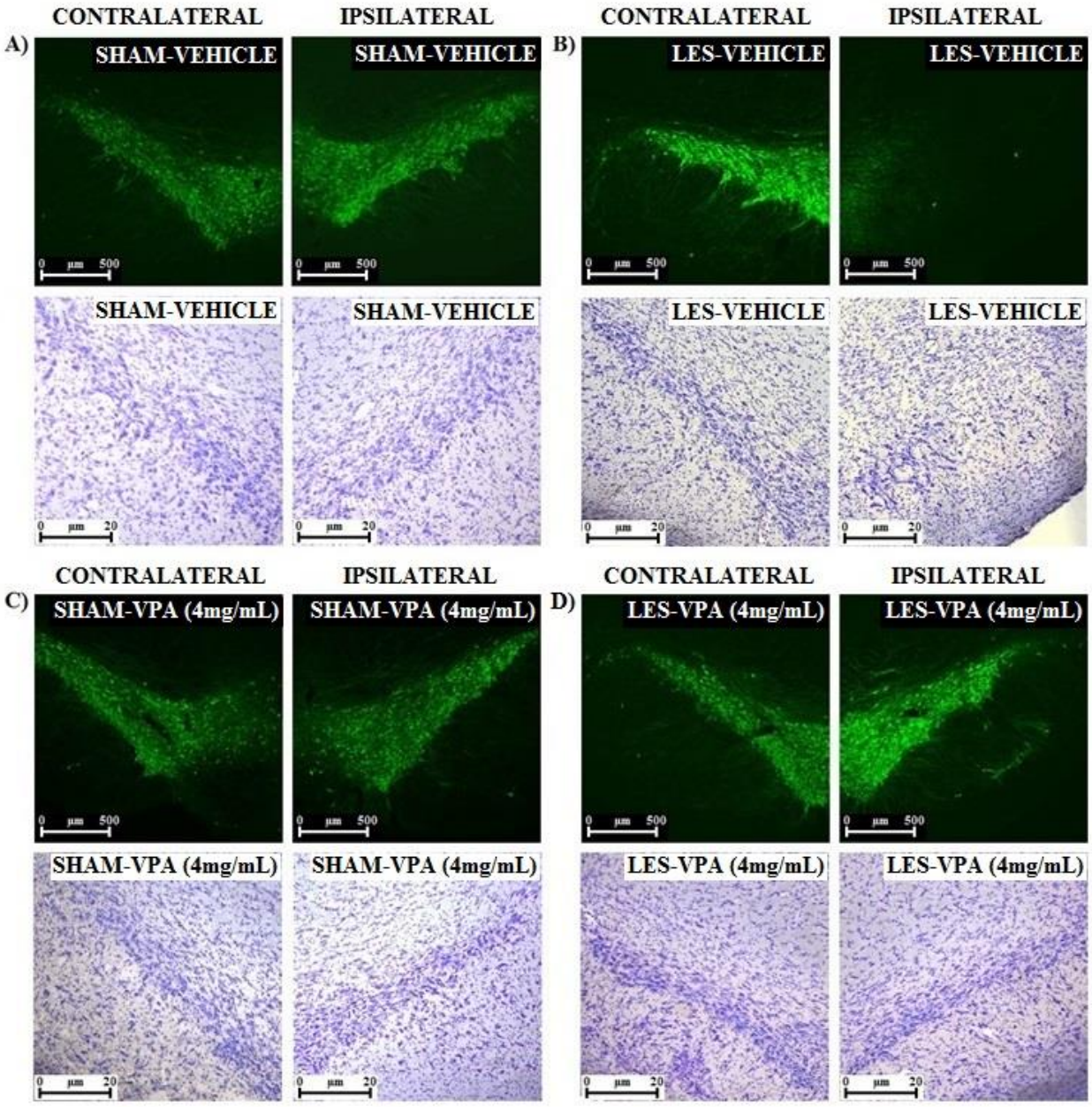

Fig. 2.5. VPA preserves tyrosine hydroxylase immunoreactivity in the substantia nigra of rotenone-lesioned rats. Contralateral and ipsilateral images of FITC-stained tyrosine hydroxylase in the substantia nigra at 5x magnification, are shown for each group. Nissl-stained images are shown immediately below corresponding tyrosine hydroxylase images for each treatment group. (A) Sham animals treated with vehicle; (B) rotenone-lesioned rats treated with vehicle; (C) VPA-treated (4 mg/mL in drinking water) rats; (D) rotenone + VPA-treated rats.

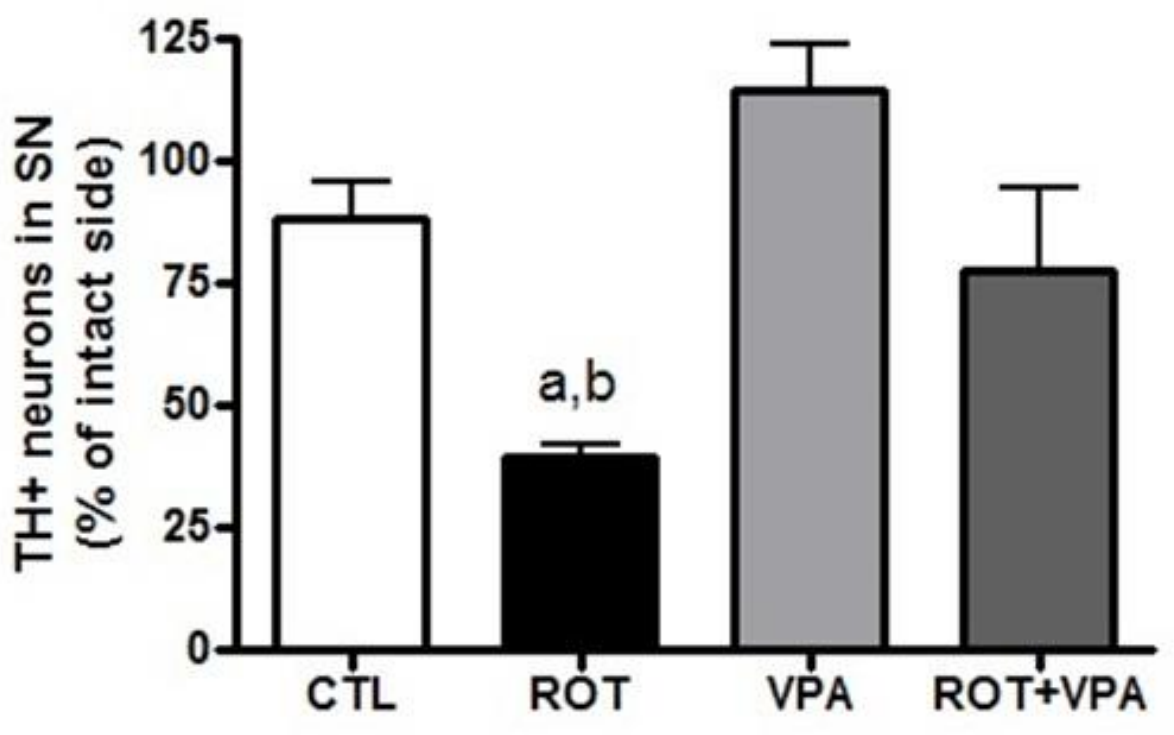

Fig. 2.6. VPA prevents the loss of tyrosine hydroxylase-positive neurons in the substantia nigra of rotenone-lesioned rats. One-way ANOVA revealed a significant ($p<0.006$) decrease in the number of tyrosine hydroxylase-positive neurons in the substantia nigra (SN) of rotenone-lesioned animals. [a]$p<0.05$ vs. sham-lesioned, vehicle treated animals; [b]$p<0.01$ vs. sham-lesioned, VPA-treated animals (Newman–Keuls analysis).

CHAPTER 3: CHRONIC LOW-DOSE MELATONIN TREATMENT MAINTAINS NIGROSTRIATAL INTEGRITY IN AN INTRASTRIATAL ROTENONE MODEL OF PARKINSON'S DISEASE

Candace H. Carriere, Na Hyea Kang, and Lennard P. Niles. Chronic low-dose melatonin treatment maintains nigrostriatal integrity in an intrastriatal rotenone model of Parkinson's disease. *Brain Research* 2015 (submitted).

Contributions of Authors:

LPN: Conceived and planned the study and edited the manuscript

CHC: Assisted in planning the study, performed the surgeries, conducted behavioural testing, performed the immunohistochemical studies, and wrote the manuscript

NHK: Assisted with the surgeries and behavioural testing

ABSTRACT

Parkinson's disease is a major neurodegenerative disorder which primarily involves the loss of dopaminergic neurons in the substantia nigra and related projections in the striatum. The pesticide/neurotoxin, rotenone, has been shown to cause systemic inhibition of mitochondrial complex I activity in nigral dopaminergic neurons, with consequent degeneration of the nigrostriatal pathway, as observed in Parkinson's disease. A novel intrastriatal rotenone model of Parkinson's disease was used for the first time, to examine the neuroprotective effects of chronic low-dose treatment with the antioxidant indoleamine, melatonin, which can upregulate neurotrophic factors and other protective proteins in the brain. Sham or lesioned rats were treated with either vehicle (0.04% ethanol in drinking water) or melatonin at a dose of 4 µg/mL in drinking water. The right striatum was lesioned by stereotactic injection of rotenone at three sites (4 µg/site) along its rostrocaudal axis. Apomorphine administration to lesioned animals resulted in a significant increase in ipsilateral rotations, which was suppressed by melatonin. Nine weeks post-surgery, animals were sacrificed by transcardial perfusion. Subsequent immunohistochemical examination revealed a decrease in tyrosine hydroxylase immunoreactivity within the striatum and substantia nigra of rotenone-lesioned animals. Melatonin treatment attenuated the decrease in tyrosine hydroxylase in the striatum and abolished it in the substantia nigra. Stereological cell counting indicated a significant decrease in tyrosine hydroxylase-positive dopamine neurons in the substantia nigra of rotenone-lesioned animals, which was confirmed by Nissl staining. Importantly, chronic melatonin treatment blocked the loss of dopamine

neurons in rotenone-lesioned animals. These findings strongly support the therapeutic potential of long-term and low-dose melatonin treatment in Parkinson's disease.

Keywords: Parkinson's disease; low-dose melatonin; neuroprotection; tyrosine hydroxylase; rotenone; intrastriatal.

1. INTRODUCTION[1]

Parkinson's disease is the most common neurodegenerative movement disorder and it is characterized by a marked loss of dopamine-producing neurons mainly within the substantia nigra pars compacta, which leads to a depletion of postsynaptic dopamine within the striatum (Shimohama et al., 2003). This deficit in dopaminergic function results in motor behavioral abnormalities including postural imbalance, rigidity, uncontrollable tremor and bradykinesia (Lotharius and Brundin, 2002). The severity of these motor symptoms has been linked to the loss of tyrosine hydroxylase (TH)-positive dopaminergic neurons in the substantia nigra (Grealish et al., 2010; Iancu et al., 2005). Oxidative stress is thought to be a major contributing factor to neurodegeneration including Parkinson's disease pathogenesis, as dopamine neurons are particularly

[1] Abbreviations:
6-OHDA: 6-Hydroxydopamine
AMK: N^1-acetyl-5-Methoxykynuramine
AFMK: N^1-acetyl-N^2-Formyl-5-Methoxykynuramine
ANOVA: Analysis of Variance
DMSO: Dimethyl Sulfoxide
DNA: Deoxyribonucleic Acid
ERK: Extracellular Signal-regulated Kinase
FITC: Fluorescein Isothiocyanate
GDNF: Glial Cell Line-Derived Neurotrophic Factor
JNK: c-Jun N-terminal Kinase
MAPK: Mitogen-activated Protein Kinase
MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
NDS: Normal Donkey Serum
PBS: Phosphate Buffered Saline
PI3K: Phosphatidylinositol 3-Kinase
TH: Tyrosine Hydroxylase

prone to an imbalance between the generation of free radicals versus antioxidant defense activity (Coyle and Puttfarcken, 1993; Jenner, 1992; Taylor et al., 2013). This involves the continuous production of reactive oxygen species via autoxidation and monoamine oxidase-mediated metabolism of dopamine, and the presence of increased iron and lower total glutathione levels in the substantia nigra, as compared with other brain regions (Berg et al., 2004; Jenner, 2003).

Melatonin (*N*-acetyl-5-methoxytryptamine), an endogenous indoleamine, is a highly conserved antioxidant molecule that is primarily secreted b the pineal gland, but it is also produced in other organs including the gastrointestinal tract, ovaries, testes, bone marrow, and eyes (Esposito and Cuzzocrea, 2010; Singhal et al., 2012). Melatonin has been detected in essentially all biological fluids such as blood, cerebrospinal fluid, bile, and saliva (Martín et al., 2000a). There are two high-affinity G-protein coupled melatonin receptor subtypes, MT_1 and MT_2, which can couple to multiple signal transduction cascades (Hardeland et al., 2011; von Gall et al., 2002). A third low-affinity binding site for melatonin, referred to as MT_3, has been identified as the detoxifying enzyme, quinone reductase 2 (Luchetti et al., 2010). Beyond its role in maintaining circadian rhythmicity, homeostasis, and modulation of neuroendocrine and immune function (Acuña-Castroviejo et al., 2014; Hardeland et al., 2011; Macchi and Bruce, 2004), melatonin and its metabolites have antioxidative and free-radical scavenging properties (Borah and Mohanakumar, 2009; Galano et al., 2011; Reiter et al., 2004) and they are protective against mitochondrial disease (Martín et al., 2002; Reiter et al., 2008; Srinivasan et al., 2011). Previous studies have demonstrated that melatonin is neuroprotective in the 6-hydroxydopamine (6-OHDA) (Borah and Mohanakumar, 2009;

Sharma et al., 2006), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Acuña-Castroviejo et al., 1997; Thomas and Mohanakumar, 2004), and systemic rotenone models of Parkinson's disease (Bassani et al., 2014). Researchers have postulated that the neuroprotective effects of melatonin may be due to its actions as an antioxidant and free radical scavenger (Lin et al., 2008; Mayo et al., 2005), as well as its ability to increase the activity of mitochondrial complexes I and IV (Martín et al., 2000b). When melatonin is administered at low doses within its endogenous physiological range, it can protect against decreased mitochondrial complex I activity (Dabbeni-Sala et al., 2001) and TH depletion following 6-OHDA lesioning (Sharma et al., 2006).

Several studies have reported that environmental risk factors such as pesticide exposure may be a major cause of Parkinson's disease. Interestingly, many pesticides can inhibit the mitochondrial electron transport chain, and this dysfunction is thought to be one of the key factors in the development of various neurodegenerative disorders (Berg et al., 2004; von Bohlen und Halbach et al., 2004; Wallace and Starkov, 2000). Rotenone, which is used as a pesticide and insecticide, has been shown to reproduce many of the behavioral and pathological features of Parkinson's disease by inhibiting mitochondrial complex I activity, with consequent degeneration of dopaminergic neurons within the substantia nigra and striatum (Alam and Schmidt, 2002; Yang et al., 2006). Systemic administration of rotenone has been used as a model of Parkinson's disease, but its deleterious effects including high morbidity and mortality (Betarbet et al., 2000; Cannon et al., 2009; Monti et al., 2010), make it unsuitable for extended studies of neuroprotection. In contrast, the infusion of rotenone into the striatum was found to produce a healthy and useful parkinsonian model, as shown by behavioral,

immunohistochemical and biochemical analyses of nigrostriatal function (Carriere et al., 2014; Mulcahy et al., 2011). In the present study, we have examined for the first time, the neuroprotective effects of chronic low-dose melatonin treatment in this intrastriatal rotenone model of Parkinson's disease.

2. RESULTS

2.1 Unilateral intrastriatal injection of rotenone is not harmful in rats

Throughout the duration of this study, a 100% survival rate was observed in both lesioned and control animals. Furthermore, there were no significant differences in either body weight (Fig. 1A) or water consumption (Fig. 1B) between treatment groups.

2.2 Unilateral intrastriatal injection of rotenone affects contralateral postural stability

Two-way ANOVA of data assessing the distance it took animals to make an adjustment step with their contralateral forelimb revealed significant treatment ($F_{3,95}$ = 17.04; p<0.0001; n = 5–7) and time ($F_{4,95}$ = 5.995; p<0.0002; n = 5–7) effects, but there was no interaction. Post hoc analysis indicated that in the sixth and seventh week post-surgery, rotenone-lesioned animals treated with vehicle showed a significant (p<0.05 or p<0.001) increase in the distance required to make an adjustment with their contralateral forelimb, as compared to sham lesioned animals treated with melatonin (Fig. 2A). It was also found that animals lesioned with rotenone and treated with melatonin had a significant (p<0.05 or p<0.01) increase in the distance required to make an adjustment step with their contralateral forelimb in the first two weeks of testing, as compared to sham lesioned animals treated with vehicle, and in the last week of testing (p<0.01), as compared to sham lesioned animals treated with melatonin (Fig. 2A). Analysis of the distance it took animals to make an adjustment step using their

ipsilateral forelimb revealed a significant treatment effect ($F_{3,95}$ = 2.869; p<0.05; n = 5–7), but there were no time or interaction effects, and post hoc analysis did reveal any significant differences between groups (Fig. 2B).

2.2 Apomorphine affects forelimb use and rearing behaviour in rats that are unilaterally lesioned with rotenone in the striatum

One-way ANOVA of the total amount of time animals were in a rearing/wall climbing position found a significant treatment effect ($F_{3,19}$ = 5.379; p<0.007; n = 5-7). Post hoc analysis indicated that animals lesioned with rotenone and treated with vehicle had a significantly (p<0.05) lower total amount of time spent on their hind limbs, as compared to all other groups (Fig. 3A). It is worth noting that rotenone-lesioned animals treated with melatonin did not significantly differ from controls. Analysis of the average amount of time animals were in a rearing/wall climbing position (total time divided by the total number of rears/wall climbs) also showed a significant treatment effect ($F_{3,18}$ = 14.96; p<0.0001; n = 5-7). Post hoc analysis indicated that animals lesioned with rotenone and treated with vehicle, spent a significantly lower amount of time on their hind limbs, as compared to sham lesioned animals treated with vehicle (p<0.01), sham animals treated with melatonin, and rotenone-lesioned animals treated with melatonin (p<0.001) (Fig. 3B). Again, rotenone-lesioned animals treated with melatonin did not differ from controls.

Analysis of the total number of full rears/wall climbs revealed a significant treatment effect ($F_{3,19}$ = 27.55; p<0.0001; n = 5-7). Post hoc analysis showed that rotenone-lesioned animals treated with vehicle had significantly fewer full rears/wall climbs, as compared to controls and melatonin treated animals (p<0.001) (Fig. 3C). It was also

found that sham-animals that were treated with melatonin had a significantly reduced number of rears/wall climbs as compared to sham animals treated with vehicle (p<0.01), and lesioned animals that were treated with melatonin also had a significantly lower number of rears/wall climbs as compared to sham-lesioned animals treated with vehicle (p<0.001) or melatonin (p<0.01) (Fig. 3C).

2.3 Apomorphine stimulates ipsilateral rotation in animals unilaterally lesioned with rotenone

Analysis of rotational behaviour following apomorphine administration revealed a significant treatment effect ($F_{7,38}$ = 23.14; p<0.0001; n = 5-7). Newman Keuls post hoc analysis found a significant (p<0.001) increase in the number of ipsilateral rotations completed by animals unilaterally lesioned with rotenone in the striatum (Fig. 4), as compared to contralateral rotations in the same animals and to all other groups. It was also discovered that rotenone-lesioned animals that were treated with melatonin had a significantly higher number of ipsilateral rotations as compared to contralateral rotations in the same animals (p<0.05); however, the number of rotations in these animals did not differ significantly from controls (Fig. 4).

2.4 Unilateral intrastriatal injection of rotenone induces nigrostriatal degeneration that is blocked by melatonin treatment

Unilateral intrastriatal lesioning with rotenone depleted TH immunoreactivity in the striatum and substantia nigra. This loss of TH was attenuated in the striatum (Fig. 5) and in the substantia nigra (Fig. 6) of lesioned animals treated with melatonin. In accordance with the above, two-way ANOVA of TH immunofluorescence in the striatum indicated significant interaction ($F_{3,118}$ = 59.26; p<0.0001), hemispheric ($F_{1,118}$ = 128.6;

$p<0.0001$) and treatment ($F_{3,118} = 107.7$; $p<0.0001$) effects. Post hoc analysis revealed that TH immunofluorescence was significantly ($p<0.001$) lower in the ipsilateral striata of lesioned rats as compared with the contralateral hemisphere of the same animals and both sides of all other groups (Fig. 5E). Although melatonin blocked the effect of rotenone, TH immunofluorescence was lower ($p<0.01$) on the ipsilateral side of the rotenone plus melatonin group versus sham controls and the melatonin group. TH immunofluorescence levels in the melatonin group were higher ($p<0.01$) in comparison with controls and the lesioned side of the rotenone plus melatonin group (Fig. 5E). One-way ANOVA indicated a significant ($F_{3,11} = 57.32$; $p<0.0001$) rotenone effect on TH-positive (dopamine) neurons in the substantia nigra. Dopamine cell counts from the lesioned (ipsilateral) substantia nigra, as a percentage of counts from the intact (contralateral) side, were decreased significantly ($p<0.001$) as compared with vehicle, melatonin and rotenone plus melatonin groups (Fig. 7). Melatonin treatment blocked the rotenone-induced loss of dopamine neurons, as there was no significant difference between cell counts of the sham vehicle-treated animals and rotenone lesioned animals treated with melatonin (Fig. 7). There was a trend towards increased TH-labelled neurons in the melatonin group, whose dopamine cell counts were significantly ($p<0.05$) greater than that in the rotenone plus melatonin group (Fig. 7).

Nissl staining confirmed that intrastriatal rotenone injection results in the loss of cells within the substantia nigra, such that lesioned animals exhibited ipsilateral cell counts which were 43.1% of that on the intact (contralateral) side. The percentages of ipsilateral to contralateral side for vehicle, melatonin, and rotenone plus melatonin-treated animals were 105.1%, 92.7%, and 79.2%, respectively, demonstrating that

melatonin is neuroprotective against dopaminergic cell death resulting from rotenone treatment (Fig. 6).

3. DISCUSSION

The present study describes the neuroprotective effects of the indoleamine antioxidant, melatonin, against rotenone-induced neurotoxicity. The most important observations made herein are the attenuation of apomorphine-induced motor dysfunction, the maintenance of tyrosine hydroxylase (TH) immunoreactivity in the striatum and the preservation of TH-positive neurons in the substantia nigra of lesioned animals, by chronic low-dose melatonin ingestion, for nine weeks in drinking water. In contrast to the apomorphine-induced contralateral rotations typically seen following intrastriatal or intranigral lesions with 6-OHDA (Da Cunha et al., 2008), a significant increase in ipsilateral rotations was observed in animals injected intrastriatally with rotenone. Ipsilateral rotations following apomorphine treatment, have been documented in animals unilaterally lesioned with rotenone or MPP^+ in the striatum or substantia nigra (Da Cunha et al., 2008; Dombrowski et al., 2010). Moreover, contralateral rotations were observed, following injection of MPP^+ or rotenone into the medial forebrain bundle, at 16 and 30 days post-lesioning, respectively (Sindhu et al., 2006). Thus, rotational behavior is influenced by the neurotoxin and dose used and the site of injection (Da Cunha et al., 2008; Dombrowski et al., 2010). Importantly, melatonin caused a significant inhibition of rotational behavior in lesioned animals supporting the neuroprotective properties of this hormone. Our novel observations, during an apomorphine challenge, that rotenone-lesioned animals spend both overall and on average, significantly less time in a rear/wall climb, and that these animals complete significantly fewer full rears/wall climbs

as compared to controls, indicate the motor impairment of these animals and support the use of this parkinsonian model. In addition, rotenone was shown to significantly affect postural stability, specific to the contralateral forelimb, indicating that the unilateral infusion of rotenone into the striatum results in motor dysfunction. The inability of melatonin to ameliorate this effect may involve an enhancement of its sedative or tranquilizing effect (Golombek et al., 1996; Pandi-Perumal et al., 2008), when administered in combination with rotenone, which has been reported to alter sleep (Yi et al., 2007).

In the present study and in accordance with our earlier work (Carriere et al., 2014), a significant reduction in TH immunoreactive protein levels was observed within the striatum and substantia nigra following infusion of rotenone into the striatum. This decrease in TH levels is indicative of degeneration of dopaminergic neurons along the nigrostriatal pathway. Using unbiased stereological cell counting, we showed that injection of rotenone into the striatum causes a significant decrease in TH-positive (dopamine) neurons in the substantia nigra. This finding confirms that retrograde degeneration occurs along the nigrostriatal pathway in this intrastriatal model. Importantly, chronic treatment with melatonin prevented the rotenone-induced decrease of TH immunofluorescence in the striatum (Fig. 5), as well as the loss of dopamine neurons in the substantia nigra (Fig. 6 & 7), strongly supporting the neuroprotective potential of melatonin in Parkinson's disease.

Previous studies have shown that melatonin is able to protect dopamine neurons following 6-OHDA-induced insult (Dabbeni-Sala et al., 2001; Joo et al., 1998; Sharma et al., 2006; Singhal et al., 2012) and to be neuroprotective in MPTP (Acuña-Castroviejo et

al., 1997; Thomas and Mohanakumar, 2004) and other rotenone models of Parkinson's disease (Bassani et al., 2014; Lin et al., 2008; Saravanan et al., 2007). However, this is the first time that the effects of melatonin have been investigated in the intrastriatal rotenone model. In addition, while several studies have examined the effects of pharmacological doses (e.g.10 mg/kg or higher) of melatonin in systemic (Bassani et al., 2014; Lin et al., 2008) and intranigral rotenone models (Saravanan et al., 2007), this is the first study to examine the protective effects of melatonin when administered at a low (physiological) dose over several weeks, and in this model. Our findings indicate that low-dose melatonin is neuroprotective in this intrastriatal rotenone rat model of Parkinson's disease, as reflected by its ability to significantly attenuate the degeneration of TH-positive neurons along the nigrostriatal pathway.

While the mechanisms underlying neuroprotection by melatonin await clarification, studies have shown that it interacts with pro-survival proteins via multiple signaling cascades, including phosphatidylinositol 3-kinase (PI3K)/Akt, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), and the Wnt/β-catenin pathways (Kilic et al., 2005; Koh, 2008; Tang et al., 2014). Melatonin has also been found to upregulate glial cell line-derived neurotrophic factor (GDNF) (Armstrong and Niles, 2002; Tang et al., 1998), which has been specifically linked to the protection of dopaminergic neurons in various animal models of Parkinson's disease (d'Anglemont de Tassigny et al., 2015). Furthermore, melatonin is able to act as a potent antioxidant to prevent neuronal cell death that is triggered by reactive oxygen species (Luchetti et al., 2010), and to protect against the loss of mitochondrial complex I activity (Martín et al., 2000b). It stimulates the expression of antioxidant and detoxification genes (Acuña-

Castroviejo et al., 1997; Luchetti et al., 2010), and suppresses the degradation of Nrf2, a transcription factor for antioxidant enzymes, as well as enhancing its nuclear translocation (Vriend and Reiter, 2015). Evidence from *in vivo* studies has demonstrated the neuroprotective benefits of melatonin in preventing damage to proteins, lipids and nuclear and mitochondrial DNA, while increasing brain glutathione levels and regulating pro- and anti-apoptotic protein levels (Akbulut et al., 2008; Baydas et al., 2005; Esposito et al., 2009), all of which have been implicated in neuronal survival in neurodegenerative diseases such as Parkinson's disease (Ma et al., 2009; Tapias et al., 2009).

There is increasing awareness that drug-induced epigenetic changes in gene transcription can exert neuroprotective effects in neurodegenerative conditions including Parkinson's disease (Harrison and Dexter, 2013). Interestingly, physiological doses of melatonin, similar to that used in the present study, can significantly alter chromatin configuration via histone acetylation in the brain and other target cells (Niles et al., 2013; Pan and Niles, 2015; Sharma et al., 2008). Evidence of an association between histone acetylation and neuroprotective gene expression (Feng et al., 2015; Harrison and Dexter, 2013; Konsoula and Barile, 2012) suggests involvement of this epigenetic mechanism in the neuroprotective effects of melatonin.

In summary, we have presented neuropathological evidence of the neurodegenerative effects of intrastriatal infusion of rotenone in a rat model of Parkinson's disease, which did not affect the health of animals over the duration of the study. We have demonstrated that chronic administration of melatonin, at a physiological dose, is capable of ameliorating apomorphine-induced behavioral disturbances and abolishing

retrograde degeneration of the nigrostriatal pathway, in this rotenone model. Specifically, we showed that melatonin treatment is neuroprotective against rotenone in being able to preserve TH immunoreactivity in the striatum and TH-positive (dopamine) neurons in the substantia nigra, indicating its therapeutic potential in Parkinson's disease.

4. Experimental Procedure

All experimental procedures were carried out in accordance with the guidelines set by the Canadian Council for Animal Care and approved by the McMaster University Animal Research Ethics Board. Male Sprague–Dawley rats weighing 289–364 g at the start of testing, were used. Rats were individually housed and randomly divided into sham or lesioned groups, and further divided into one of two treatment conditions. The treatments were a melatonin vehicle (0.04% ethanol in drinking water) or melatonin (4 µg/mL in drinking water; Sigma–Aldrich Inc.). Animals received fresh water with or without melatonin every Tuesday and Friday for nine weeks, at which times water consumption was recorded. Body weights were also recorded on a weekly basis to monitor the health of the animals. Nine weeks after lesioning, rats were sacrificed by transcardial fixation and brains were harvested for quantitative TH immunohistochemistry.

4.1 Surgery

Unilateral lesioning of the right striatum was conducted under isoflurane anesthesia (5% in O2 for induction and 2.5% in O2 for maintenance) in a stereotaxic apparatus with the nose bar set at −2.5 mm. Lesioned rats received rotenone (Sigma–Aldrich Inc.) that was dissolved in a vehicle of DMSO, Cremophor® (Sigma–Aldrich Inc.) and saline (1:1:18)

and sham-lesioned rats received an equal volume of vehicle (Carriere et al., 2014; Mulcahy et al., 2011). The striatum was lesioned by infusion (0.3 μL min^{-1} for 6–7 min with 3 min for diffusion) at three points along its rostro-caudal axis at the stereotaxic coordinates AP +1.0, ML −3.0; AP −0.1, ML −3.7; AP −1.2, ML −4.5 (from bregma) and DV −5.0 below dura (Kirik et al., 1998). All rats received a total of 6 μL infusion (over three sites) of either vehicle (2.0 μL/site) or rotenone (4.0 μg in 2.0 μL/site). Treatment with melatonin began one week before neurosurgery and continued for an additional nine weeks.

4.2 Behavioural Testing

A postural instability test was used to measure forelimb akinesia (Woodlee et al., 2008). Animals were handled daily to acclimate them for the postural instability test. The rat's hind limbs and one forelimb were carefully restrained, with the torso positioned above a table surface. The length of the adjusting step taken by the free forelimb, when the rat experienced a change in its center of gravity, was measured manually using a ruler which was taped to the stepping board. One examiner conducted the test while another recorded the distance. Two weeks following surgery, animals were injected with apomorphine (0.25 mg/kg, sc; Sigma-Aldrich; Oakville, ON), during their dark phase. After a 5 minute waiting period, each animal was placed into a Plexiglas cylinder and explorative behaviour was videotaped for 10 minutes (Sharma et al., 2006). The total amount of time animals spent rearing/wall climbing, the time per rear/wall climb, the total number of rears/wall climbs, and the number of rotations were quantified. All behavioral measurements were blind, with a coding system used to identify animals only after data compilation.

4.3 Immunohistochemistry

Tyrosine hydroxylase immunohistochemistry was performed as described previously (Carriere et al., 2014). In brief, animals were deeply anaesthetized and sacrificed by transcardial perfusion. Brains were harvested and cryoprotected in a 30% sucrose solution 72 hours before cryosectioning. The striatum and substantia nigra were identified in the prepared tissue using a rat brain atlas (Paxinos and Watson, 2007), and slices were collected at 35 µm thickness. The primary antibody used was a rabbit anti-tyrosine hydroxylase (TH) antibody (AB152; Millipore, Temecula, CA), diluted 1:1000 in 1.5% normal donkey serum (NDS) in phosphate buffered saline (PBS), and the secondary antibody used was a fluorescein (FITC)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, Inc., West Grove, PA), diluted 1:200 in 1.5% NDS in PBS. Samples from the striatum and the substantia nigra were imaged using a confocal microscope (Leica TCS SP5 and LSM 510, Carl Zeiss MicroImaging, Inc.) with excitation using a 488 nm Argon laser for high resolution imaging. Also, positive and negative controls for antibody staining were completed, where tissue slices were incubated in either the primary antibody only (secondary antibody was replaced with the serum blocker) or incubated in the secondary antibody only (primary antibody was replaced with the serum blocker during this step of the staining process). The positive and negative controls did not show any fluorescence. Separate sections from the substantia nigra were mounted overnight to gelatin-coated slides in preparation for Nissl staining. Samples were delipidized in ethanol/xylene, then rehydrated through a series of ethanol/dH_2O washes. Tissue was stained with 0.13% Cresyl Violet (Sigma–Aldrich Inc.), rinsed in dH_2O, and dehydrated in ethanol/xylene (Wu et al., 2012). Nissl-stained

cell counts were obtained from these samples, using the same stereological procedure described below for TH cell counts from FITC-stained substantia nigra sections, in order to validate treatment-induced changes in dopamine cell number. To control for any variability that may occur throughout processing, full sets of tissue samples, one brain from each treatment group, were sliced, stained and imaged at the same time. The laser and program settings also remained constant across all samples.

4.4 Densitometric analysis

Four images (one from each quadrant) were acquired from four sections from separate animals, for a total of 16 images (TH) per treatment group (Sharma et al., 2006). The striatum was analyzed for any luminosity changes in TH fluorescence. The parameters used for image acquisition were identical for all treatment groups. Images from equivalent regions were processed using ImageJ and the mean luminosity values were recorded.

4.5 Stereological analysis of dopamine neurons in substantia nigra

Substantia nigral sections were imaged using a confocal microscope (LSM 510, Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) equipped with a Plan-Apochromatic 63× 1.4 Oil DIC, with excitation using a 488-nm Argon laser for high resolution imaging. Every sixth section throughout the substantia nigra was collected with the first section being randomly selected, allowing for an unbiased series of sections. Optical stacks of images were acquired from the substantia nigra at 63x magnification for stereological procedures and LSM and Zen software were used for data analysis. Volumes were estimated using the Cavalieri principle (Mayhew and Gundersen, 1996). Cell counts were generated from *z*-stack images that were collected using 2 µm intervals, resulting

in a total thickness of approximately 39 μm per *z*-stack. A guard zone of 5 μm at each superficial portion of the tissue was used to prevent bias due to supersaturation of cells or artefacts resulting from the cutting process (West, 1999). Images were saved at 512 pixels by 512 pixels for analysis. Each cell was identified and counted using unbiased design-based stereological procedures. Using the Optical Fractionator method (Mayhew and Gundersen, 1996), estimated total cell numbers were calculated. Densities were calculated by taking the total estimated number of cells and dividing by the substantia nigra estimated volume, and converting each number to cells per mm^3. Statistical analyses were run using total cell counts rather than densities to avoid potential bias (West, 1999). Cell counts were completed blind to the treatment conditions, with a coding system used to identify animals only after data compilation.

4.6 Statistics

Behavioral data were analyzed by a one- or two-way analysis of variance (ANOVA) (treatment × time) and significant group differences (with $p < 0.05$ taken as the level of significance) were determined by a Bonferroni or Newman Keuls test (Graphpad Prism version 4.0), when appropriate. Similarly, contralateral and ipsilateral values (arbitrary units) of TH immunofluorescence were analyzed by two-way ANOVA followed by Bonferroni analysis where appropriate. Ipsilateral and contralateral luminosity values were separately analyzed using one-way ANOVA and post-hoc analysis. Total counts of tyrosine hydroxylase-positive (dopamine) neurons in the substantia nigra were converted to percentage values and analyzed by a one-way ANOVA followed by a Newman–Keuls test. Data shown are expressed as means ± S.E.M.

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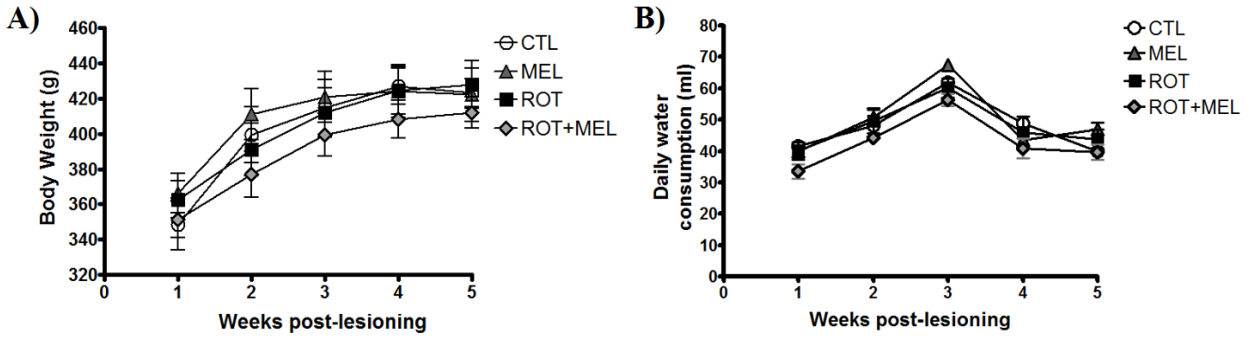

Figure 3.1. Unilateral intrastriatal injection of rotenone does not affect rat health. There were no significant differences in body weight (A) or water consumption (B) between treatment groups.

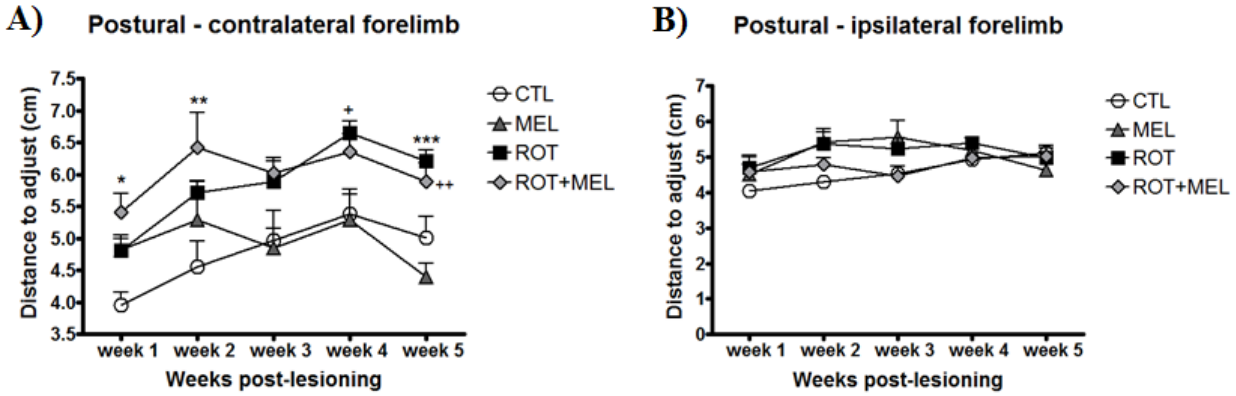

Figure 3.2. Rotenone lesioning affects postural stability in rats. (A) In the sixth and seventh week post-surgery, rotenone-lesioned animals treated with vehicle showed a significant increase in the distance to make an adjustment with their contralateral forelimb, as compared to sham lesioned animals treated with melatonin. Postural stability was altered in animals lesioned with rotenone and treated with melatonin, as shown. (B) There were no group differences in the distance required for an ipsilateral forelimb adjustment. Data shown are means ± SEM. +p<0.05 vs. sham lesioned animals treated with melatonin; ***p<0.001 vs. shams treated with melatonin; *p<0.05 vs. shams treated with vehicle; **p<0.01 vs. shams treated with vehicle; ++p<0.01 vs. shams treated with melatonin.

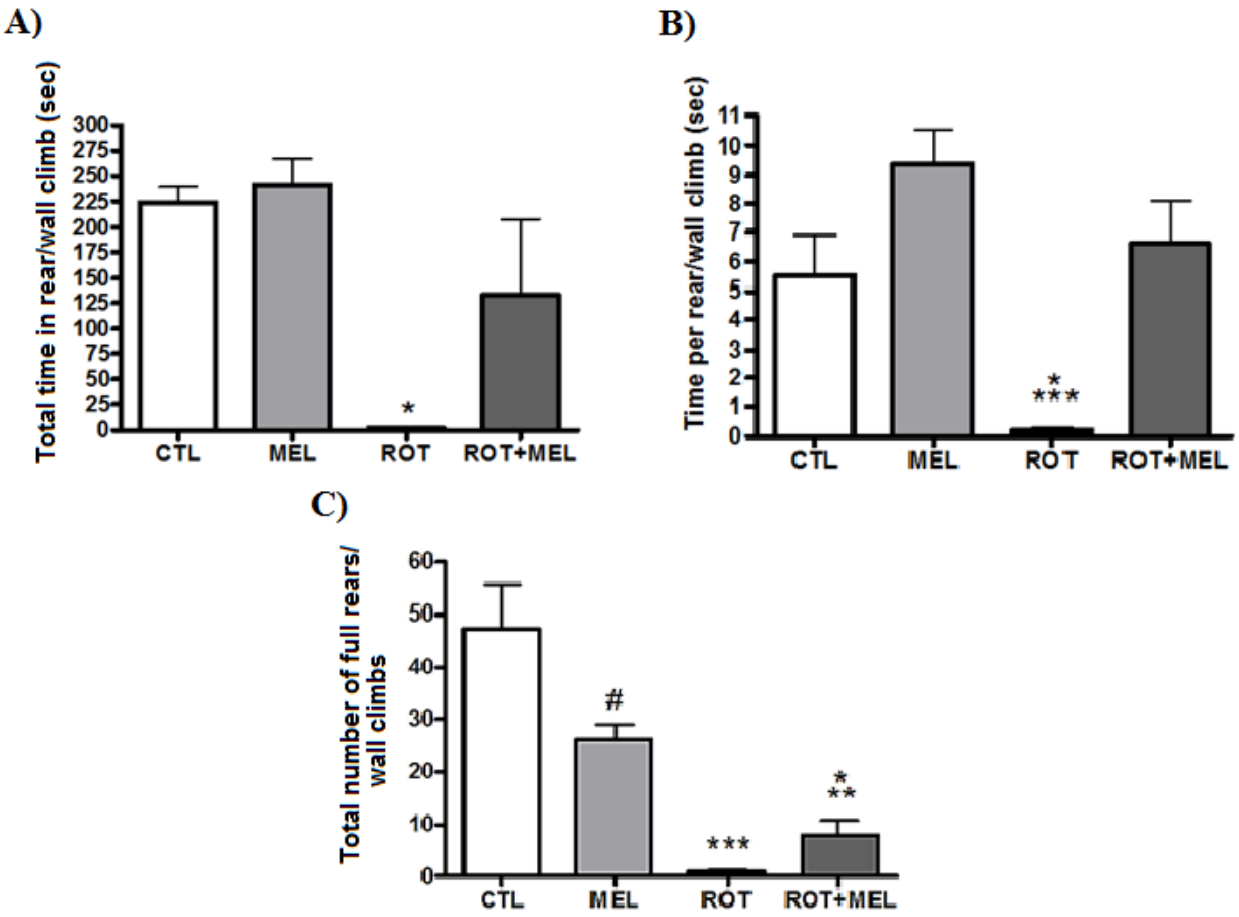

Figure 3.3. Rotenone lesioning suppresses apomorphine-induced rearing behaviour. (A) The total amount of time rotenone-lesioned animals spend rearing/wall climbing following apomorphine treatment is significantly less than that of all other groups. *p<0.05 vs. control groups and rotenone-lesioned animals treated with melatonin. (B) The average amount of time rotenone-lesioned animals spend rearing/wall climbing following apomorphine treatment is significantly less than that of all other groups. *p<0.01 vs. sham lesioned animals treated with vehicle; ***p<0.001 vs. sham lesioned animals treated with melatonin and rotenone-lesioned animals treated with melatonin. (C) Rotenone-lesioned animals had a significantly lower total number of full rears/wall climbs as compared to shams treated with vehicle or melatonin. Sham-animals treated with melatonin had a significantly reduced number of rears/wall climbs as compared to sham+vehicle, and lesioned+melatonin animals had a significantly lower number of rears/wall climbs as compared to shams treated with vehicle or melatonin. ***p<0.001 vs. sham controls; *p<0.01 vs. sham+melatonin; **p<0.001 vs. sham+vehicle; #p<0.01 vs. sham+vehicle. Data shown are means ± SEM.

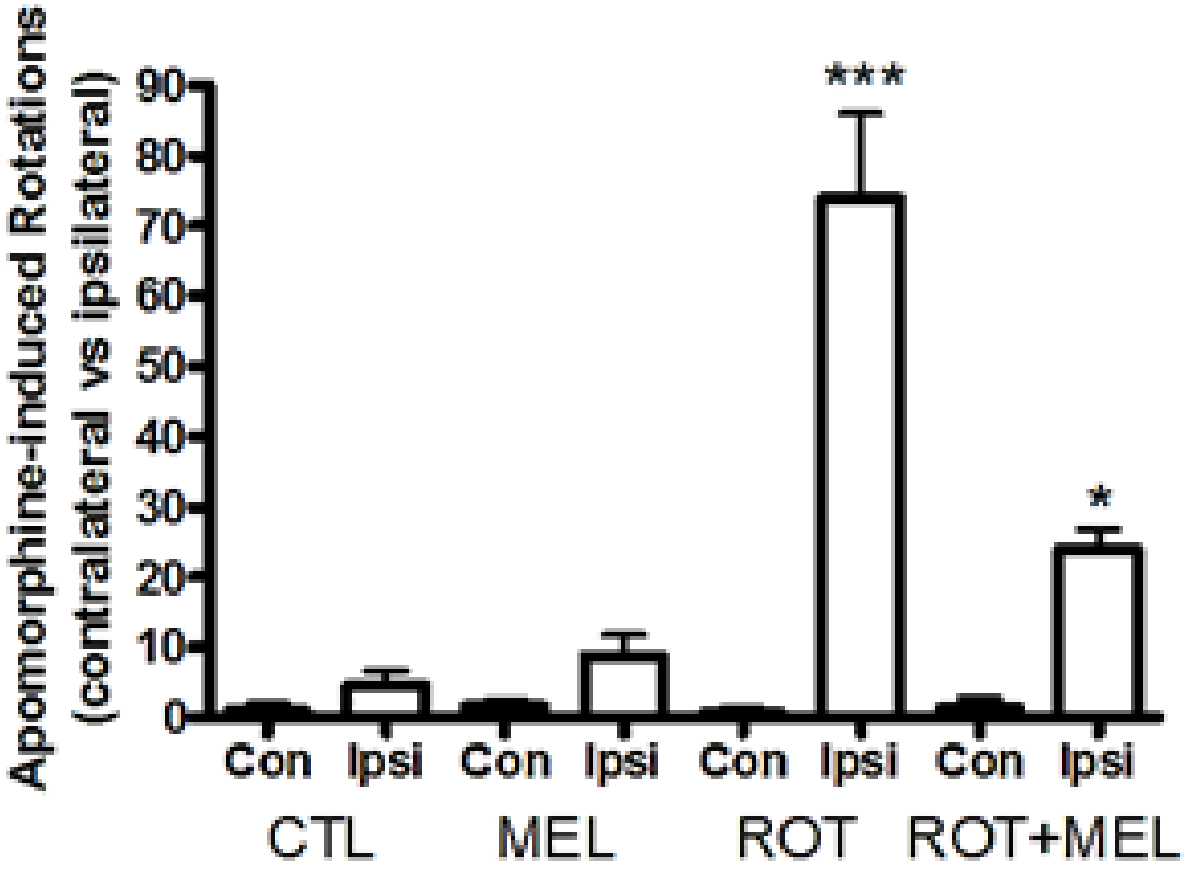

Figure 3.4. Apomorphine induces ipsilateral rotations in animals unilaterally lesioned with rotenone. Rotenone-lesioned animals had a significantly higher number of apomorphine-induced ipsilateral rotations as compared to contralateral rotations in the same animals and to either ipsilateral or contralateral rotation in all other groups. Lesioned animals which were treated with melatonin also showed a significant increase in the number of apomorphine-induced ipsilateral rotations, as compared to contralateral rotations in the same animals. ***$p<0.001$ vs. contralateral rotations of the same group and all other groups; *$p<0.05$ vs. contralateral rotations of the same group. Data shown are means ± SEM.

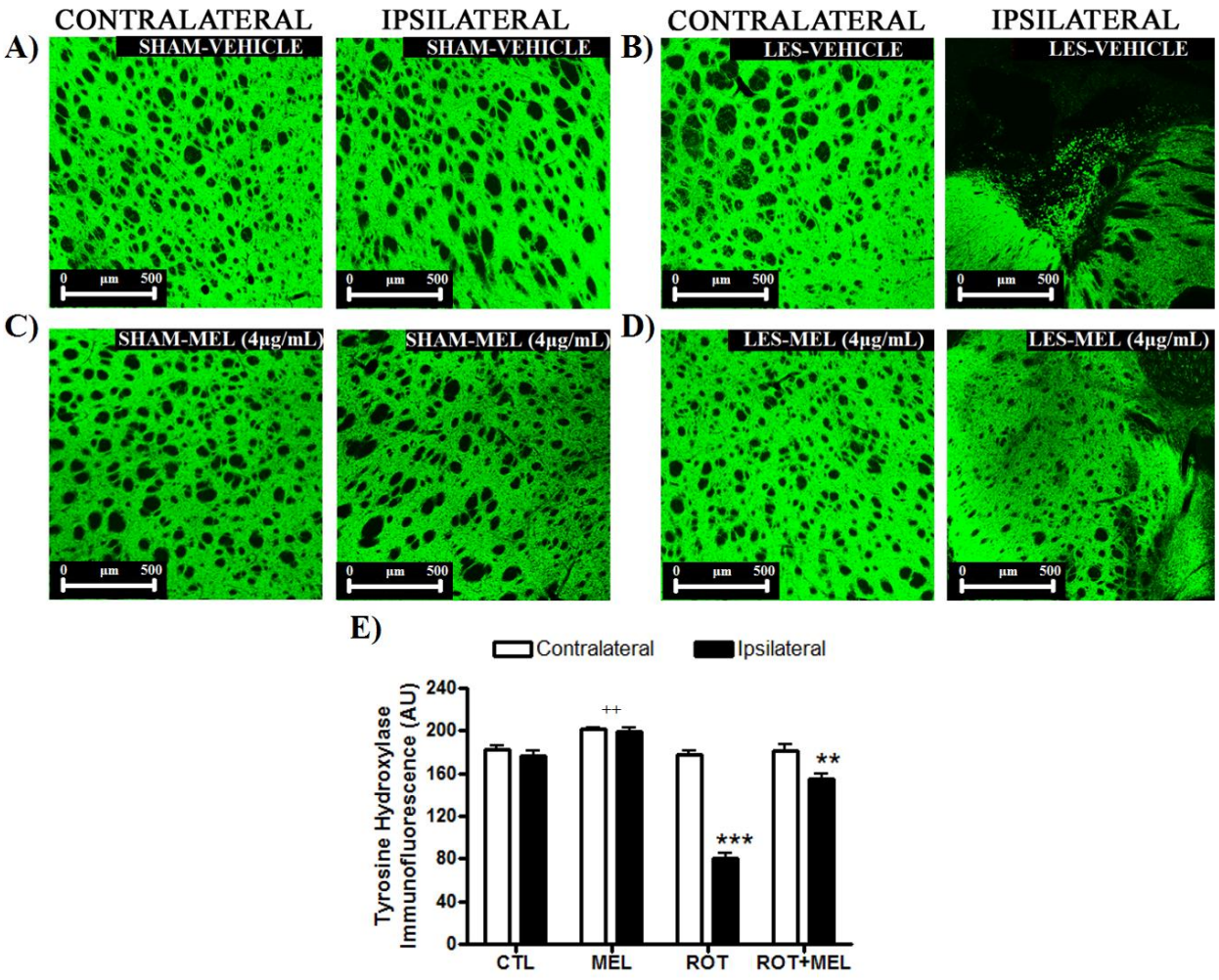

Figure 3.5. Melatonin attenuates the loss of tyrosine hydroxylase immunoreactivity in the striatum of rotenone lesioned rats. Contralateral and ipsilateral images of FITC-stained tyrosine hydroxylase in the striatum at 5x magnification are shown. (A) Sham treated with vehicle; (B) Rotenone lesioned treated with vehicle; (C) Sham treated with melatonin (4 µg/mL in drinking water); (D) Rotenone **+** Melatonin treated. (E) Densitometric analysis revealed a significant (***p<0.001) decrease in tyrosine-hydroxylase immunofluorescence in the ipsilateral striata of lesioned animals, as compared to the contralateral hemispheres of the same animals, and both sides of all other groups. Tyrosine hydroxylase immunofluorescence was also significantly lower (**p<0.01) on the ipsilateral side of the rotenone plus melatonin group versus sham controls and the melatonin group; and significantly higher in the melatonin group (**++**p<0.01) versus controls and the lesioned side of the rotenone plus melatonin group. AU = arbitrary units.

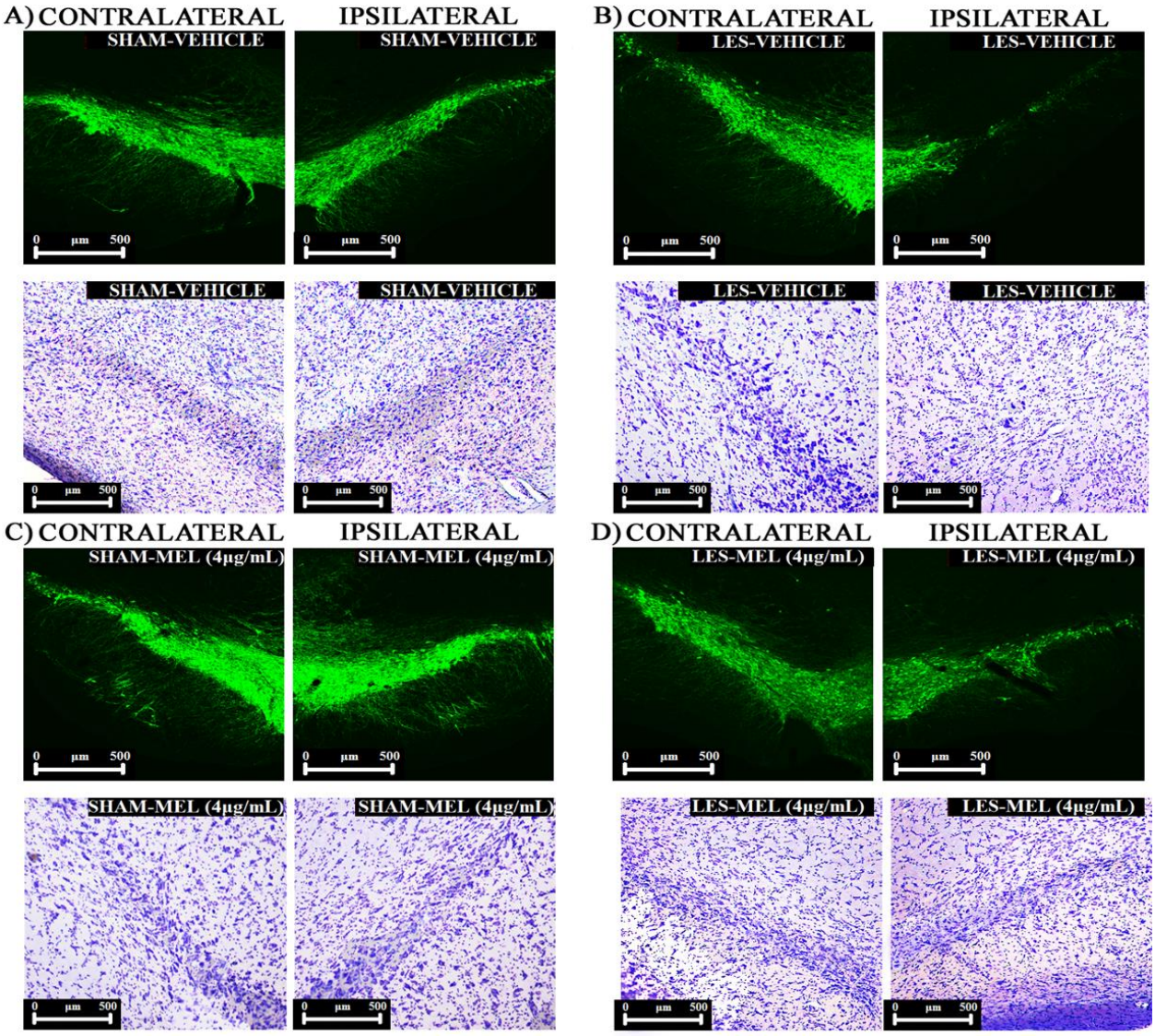

Figure 3.6. Melatonin preserves tyrosine hydroxylase immunoreactivity in the substantia nigra of rotenone lesioned rats. Contralateral and ipsilateral images of FITC-stained tyrosine hydroxylase in the substantia nigra at 5x magnification, are shown. Nissl stained images are shown immediately below corresponding tyrosine hydroxylase images for each treatment group. (A) Sham animals treated with vehicle; (B) Rotenone lesioned treated with vehicle; (C) Shams treated with melatonin (4 µg/mL in drinking water); (D) Rotenone plus melatonin treated.

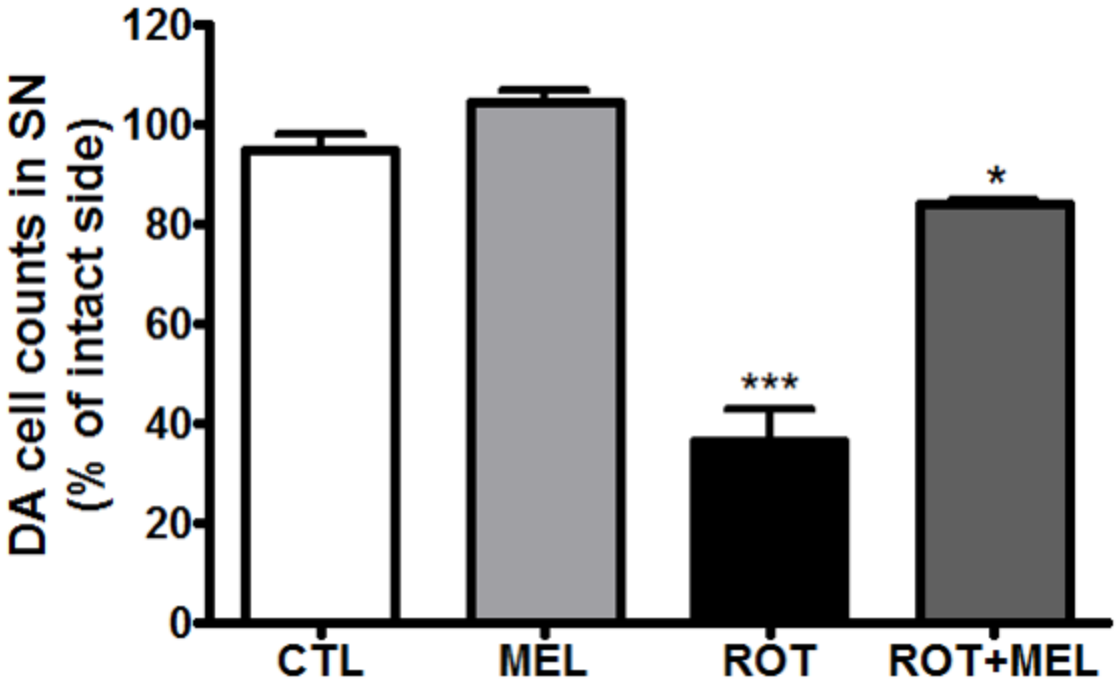

Figure 3.7. Melatonin prevents the loss of tyrosine hydroxylase positive (dopamine) neurons in the substantia nigra of rotenone lesioned rats. One-way ANOVA revealed a significant ($p<0.001$) decrease in the number of tyrosine hydroxylase positive neurons in the substantia nigra (SN) of rotenone lesioned animals. ***$p<0.001$ vs sham-vehicle, sham-melatonin and rotenone plus melatonin treatment groups; *$p<0.05$ vs. sham-melatonin treated animals (Newman-Keuls analysis).

CHAPTER 4: BILATERAL UPREGULATION OF NIGRAL α-SYNUCLEIN IN A NOVEL INTRACEREBRAL ROTENONE MOUSE MODEL OF PARKINSON'S DISEASE

Candace H. Carriere, Na Hyea Kang, and Lennard P. Niles. Bilateral upregulation of nigral α-synuclein in a novel intracerebral rotenone mouse model of Parkinson's disease. *Journal of Neurodegenerative Diseases* 2015 (submitted).

Contributions of Authors:

LPN: Conceived and planned the study and edited the manuscript

CHC: Assisted in planning the study, performed the surgeries, conducted behavioural testing, performed the immunohistochemical studies, and wrote the manuscript

NHK: Assisted with the surgeries and behavioural testing

Abstract

Rotenone, an insecticide, has been shown to cause systemic inhibition of mitochondrial complex I activity, with consequent degeneration of dopaminergic neurons along the nigrostriatal pathway, as observed in Parkinson's disease. A novel intracranial model was used to explore whether rotenone infusion in mice could provide a model of Parkinson's disease. Specifically, this study examined if intracranial infusion of rotenone results in degeneration of dopaminergic neurons as shown by decreased tyrosine hydroxylase expression, which has yet to be documented in mice treated with this neurotoxin. Mice were unilaterally infused with either vehicle or rotenone (2 µg/site) in both the medial forebrain bundle and the substantia nigra. Motor function was assessed using the forelimb asymmetry test, which indicated a significant decrease in use of the contralateral forelimb in lesioned animals as compared to the sham group during the first week of testing. Densitometric analysis revealed a significant depletion of tyrosine hydroxylase immunofluorescence within the ipsilateral striatum and substantia nigra of lesioned animals, and a significant bilateral overexpression of α-synuclein in the substantia nigra, as compared to control animals. These findings validate this rotenone model of Parkinson's disease, which will be useful for studies of neurodegenerative mechanisms or neuroprotective approaches.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, characterized by the loss of tyrosine hydroxylase (TH) positive dopaminergic neurons within the substantia nigra (SN) pars compacta, which in turn contributes to a depletion

of postsynaptic dopamine within the striatum [1]. This loss of dopamine functionality results in motor behavioural deficits including uncontrollable tremor, postural imbalance, rigidity and bradykinesia [2]. The severity of these motor symptoms seems to be due to the loss of tyrosine hydroxylase (TH) positive dopaminergic neurons in the substantia nigra (SN) [3].

Administration of rotenone, a potent rotenoid used in insecticides, inhibits mitochondrial complex I activity, which leads to the degeneration of dopaminergic neurons within the SN and striatum [4]. Intracranial administration of rotenone in rats has been shown to result in motor dysfunction and decreased TH immunoreactivity along the nigrostriatal pathway [5], however its efficacy in a mouse model of PD has not been established. In a recent study, it was demonstrated that infusion of rotenone into the MFB of mice resulted in overexpression of α-synuclein and small ubiquitin-related modifier (SUMO)-1 [6]. It has yet to be explored if intracranial infusion of rotenone results in decreased TH expression in the mouse striatum and SN, which is an essential marker of dopaminergic degeneration. Therefore, we have investigated the behavioural and immunohistochemical effects of unilateral rotenone infusion into both the medial forebrain bundle (MFB) and the SN in mice, on TH immunoreactivity and other characteristics related to PD.

2. Methods

2.1 Experimental design

All experimental procedures were carried out in accordance with the guidelines set by the Canadian Council for Animal Care and approved by the McMaster University Animal Research Ethics Board. Male C3H/He mice weighing 25-29 g at the start of testing,

were used. Mice were group housed at an ambient temperature of 23±2 °C, under a 12-h light/dark cycle (lights on at 0700h), and they were randomly assigned to sham or lesioned groups. Sixteen days after lesioning, mice were sacrificed by transcardial perfusion and brains were harvested for immunohistochemical analysis.

2.2 Surgery

Unilateral lesioning of the MFB and the SN was conducted under isoflurane anesthesia (4% in O2 for induction and 2.5% in O2 for maintenance) in a stereotaxic apparatus with a mouse adapter. Results from the pre-operative forelimb asymmetry testing was used to determine the hemisphere that was to be lesioned (based on the dominant forelimb, the contralateral hemisphere was lesioned). Lesioned mice received rotenone (Sigma-Aldrich Canada Co. Oakville, ON) that was dissolved in a vehicle of dimethyl sulfoxide, Cremophor® (Sigma-Aldrich Canada Co. Oakville, ON) and saline (1:1:18) and sham-lesioned mice received an equal volume of vehicle [7]. The MFB was lesioned by infusion of 2 µL at a rate of 1 µL min^{-1} (4 minutes for diffusion) at the stereotaxic coordinates AP -1.22, ML 1.10 (from bregma) and DV -5.2 to -5.1 below Dura (needle was advanced to -5.2 and then retracted to -5.1) [8]. The SN was lesioned by infusion of 2 µL at 1 µL min^{-1} (4 minutes for diffusion) at the stereotaxic coordinates AP -3.08, ML 1.2 (from bregma) and DV -4.6 to -4.5 below Dura (needle was advanced to -4.6 and then retracted to -4.5). All mice received a total of 4 µL infusion of either vehicle (2 µL/site) or rotenone (2 µg in 2 µL/site).

2.3 Behavioural testing

A forelimb use asymmetry test was used to evaluate motor performance in both treatment groups. Acclimation to the testing environment was conducted one week before surgery and before each set of testing that began one week after lesioning. Animals were placed in a transparent Plexiglas cylinder (22 cm diameter and 30 cm height) for 5 minutes before testing. Subsequently, forelimb use during explorative behaviour was videotaped for 5 minutes. This behaviour was quantified based on the independent use of either the left or right forelimb for contacting the wall during a full rear [9]. All behavioral measurements were blind, with a coding system used to identify animals only after data compilation.

2.4 Immunohistochemistry

Animals were deeply anaesthetized and sacrificed by transcardial perfusion with a pre-wash of saline (0.9% NaCl) followed by 4% paraformaldehyde fixative (PFA; Caledon Canada Ltd., Georgetown, Ont.). Brains were harvested, post-fixed in 4% PFA overnight and then transferred into phosphate buffered saline (PBS) with 0.06M sodium azide and stored at 4°C until slicing. Brains were cryoprotected and tissue was sectioned at 25 μM. The primary antibodies used were a rabbit anti-TH antibody (1:1000; AB152; Millipore, Temecula, CA) and a rabbit anti-α-synuclein antibody (1:1000; SC-7011; Santa Cruz Biotechnology, Inc., Dallas, TX). The secondary antibodies used were a fluorescein (FITC)-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch, Inc., West Grove, PA) and a goat anti-rabbit IgG-Texas Red (1:200; SC-2780; Santa Cruz Biotechnology, Inc., Dallas, TX). Samples from the striatum and the SN were imaged using a confocal

microscope (Leica TCS SP5 and LSM 510, Carl Zeiss MicroImaging, Inc.) with excitation using a 488 nm Argon laser and 543 nm Helium Neon laser. To control for any variability that may occur throughout processing, full sets of tissue samples, one brain from each treatment, were sliced, stained and imaged at the same time.

2.5 Densitometric analysis

Four images were acquired from two sections from separate animals, for a total of 8 slices imaged per group [10]. The striatum and SN were analyzed for any luminosity changes in TH and α-synuclein fluorescence. The parameters used for image acquisition remained identical for both groups. Images from equivalent regions were converted to RGB images using ImageJ, and the mean luminosity value was recorded.

2.6 Statistics

Behavioural data were analyzed by a two-way analysis of variance (ANOVA) and significant group differences (with $p<0.05$ taken as the level of significance) were determined by a Bonferroni test. Ipsilateral and contralateral luminosity values were separately analyzed using one-way ANOVA and post-hoc analysis. Data shown are expressed as means ±S.E.M.

3. Results

Unilateral infusion of rotenone into the MFB and the SN causes contralateral forelimb motor dysfunction in C3H/He mice

Analysis of forelimb use data revealed that during the first week post-surgery, rotenone-lesioned animals showed a significant ($p<0.05$) decrease in the use of their contralateral forelimb, as compared to sham-lesioned (control) animals. A decrease in the use of the

contralateral forelimb continued in lesioned animals during the second week of testing, but the increase in variance precluded significance. There were no significant group differences in use of the ipsilateral forelimb.

Unilateral infusion of rotenone into the MFB and the SN causes nigrostriatal degeneration in C3H/He mice

Unilateral rotenone infusion into the MFB and the SN depleted TH immunoreactivity in the striatum and SN. One-way ANOVA of luminosity values indicated a significant rotenone effect on TH immunofluorescence within the lesioned striatum ($F_{3, 4}$=30.74; p<0.01; Fig. 1b) and the SN ($F_{3, 4}$=10.26; p<0.01; Fig. 2b), as compared to both the intact (contralateral) hemisphere of the same animals and that of control animals. It was also found that intracranial infusion of rotenone induced a significant bilateral increase in α-synuclein aggregation in the SN ($F_{3, 4}$=41.99; p<0.01; Fig. 3) of lesioned animals, as compared to control animals. In contrast, α-synuclein was not detected in the striatum of either lesioned or control animals.

4. Discussion

The present study describes the effects of intracranial rotenone infusion on dopaminergic integrity in the mouse nigrostriatal system. The most important observations made herein are: (1) a significant decrease in contralateral forelimb use by rotenone-lesioned animals, (2) a significant loss of TH fluorescence in the striatum and the SN of lesioned animals (Fig. 1 and Fig. 2), and (3) a significant aggregation of α-synuclein in the SN of lesioned animals (Fig. 3), as compared to control animals.

In assessing possible motor dysfunction, we used an asymmetry (cylinder) test, which revealed a significant decrease in contralateral forelimb use and a trending increase in

ipsilateral forelimb use by rotenone lesioned animals, as compared to control animals. This finding presumably represents a contralateral motor deficit inflicted by rotenone lesioning. This is in accordance with evidence that intrastriatal infusion of rotenone causes contralateral motor dysfunction in rats [7].

Moreover, immunohistochemical examination revealed a loss of TH immunoreactivity in the striatum and the SN of lesioned animals (Fig. 1 and Fig. 2), and α-synuclein aggregation in the SN of lesioned animals (Fig. 3). The reduced levels of TH, the rate-limiting enzyme for dopamine synthesis, in the striatum and SN following rotenone treatment (Fig. 1 and Fig. 2), indicate the degeneration of dopaminergic neurons along the nigrostriatal pathway.

The marked accumulation of α-synuclein found both ipsilateral and contralateral to the lesioned SN (Fig. 3), as compared to control animals, may be due to the severity of the neurotoxin-induced insult, as both the MFB and SN were lesioned. This suggests that lesioning at both of these sites induced symptoms of a later-stage parkinsonian model, with increased α-synuclein aggregation in the SN. These findings are in accordance with earlier studies which showed that injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice cause dopaminergic neurodegeneration along the nigrostriatal pathway, as shown by a decrease in TH immunoreactivity and an increase in α-synuclein-immunoreactive neurons in the SN [11]. The similarity in the effects of rotenone and MPTP, in causing an increased accumulation of α-synuclein in the mouse SN, may be related to their ability to interfere with complex I of the electron transport chain, with consequent mitochondrial dysfunction and associated dopaminergic neurodegeneration [12].

The overexpression of α-synuclein in the SN following rotenone-induced injury, as observed in the present study, is consistent with western blot data which show an increase in the expression of this protein in the mouse SN following unilateral infusion of rotenone into the MFB [6]. The function of α-synuclein, which is a small protein mainly localized in nerve terminals [13], is not fully understood. Studies have shown that it is involved in regulating synaptic vesicle formation, neurotransmitter release and neuronal plasticity [14]. The overexpression of α-synuclein is known to inhibit neurotransmitter release at the presynaptic terminal [13] and to impair mitochondrial function [15]. Moreover, when α-synuclein is present in high concentrations, there is an increased likelihood that aggregates of insoluble fibrils will occur [16]. There is evidence that mutations of α-synuclein lead to a misfolding of the protein and consequently result in aggregation, thus losing the function of α-synuclein and ultimately leading to neuronal cell death [2,13] One of the key pathological hallmarks of Parkinson's disease is the Lewy body, which is mainly composed of α-synuclein aggregates that are found in the cell soma [2].

Therefore, our finding that rotenone induces overexpression of α-synuclein that is accompanied by the depletion of TH, is similar to the pathology of Parkinson's disease, and further supports the usefulness of this model for future studies.

Interestingly, the overexpression of α-synuclein may be due to its physiological neuroprotective function at the synapse, as shown in past knockout studies [17,18]. Following the knockout of the synaptic co-chaperone cysteine-string protein alpha (CSPα), mice underwent progressive neurodegeneration and impaired synaptic functioning. This impairment was reversed following transgenic expression of α-

synuclein, which also reduced the inhibition of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex assembly [17], which is involved in vesicle fusion and exocytosis. Although normal levels of α-synuclein appear to be neuroprotective, overexpression of this protein results in toxicity, degeneration [19] and increased susceptibility to systemic pro-inflammatory challenge [20,21]. Furthermore, synaptic accumulation of α-synuclein is accompanied by an age-dependent redistribution of SNARE proteins as well as a decrease in dopamine release, which is similar to that observed in Parkinson's disease [22]. Thus, the upregulation of α-synuclein we observed may represent a cellular attempt to survive the damage to dopaminergic neurons following rotenone infusion.

5. Conclusion

In summary, we have presented behavioural and neuropathological evidence of the neurodegenerative effects of intracranial infusion of rotenone in a mouse model of PD. Due to this model's effects on TH and α-synuclein immunoreactivity, intracranial rotenone infusion may be useful for future studies aimed at evaluating the effects neuroprotective drug therapies on dopaminergic degeneration, α-synucleinopathy and other genetic or molecular markers of PD.

Acknowledgement

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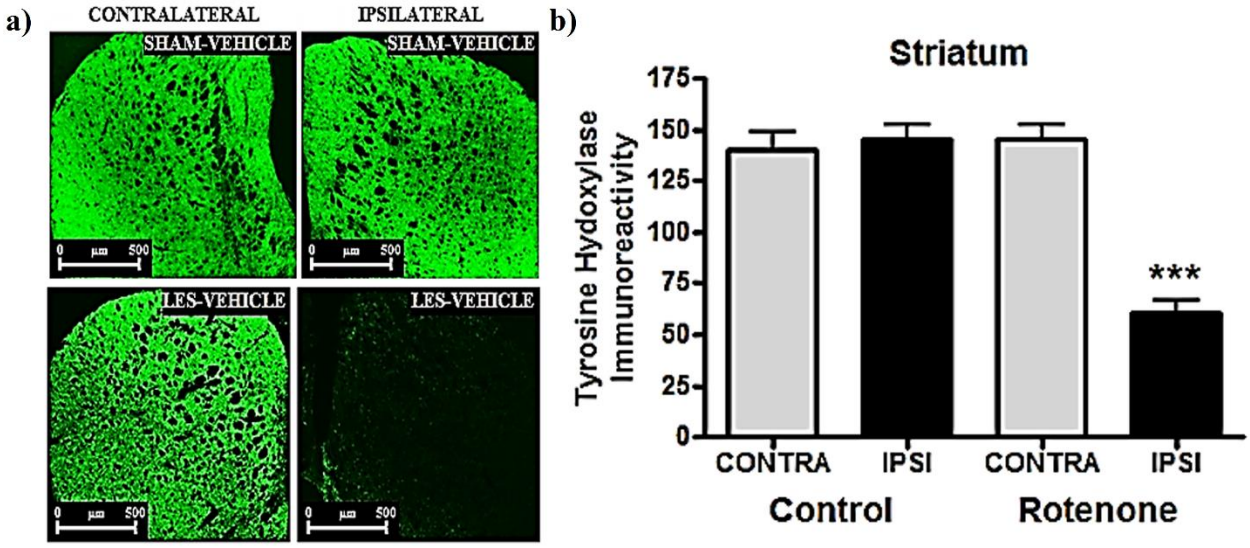

Figure 4.1. Rotenone lesioning of the nigrostriatal pathway depletes striatal TH immunoreactivity in C3H/He mice. Contralateral and ipsilateral images of FITC-stained TH in (a) the striatum of sham-lesioned and rotenone-lesioned animals at 5x magnification, at 2 weeks post-surgery. (b) Densitometric analysis of luminosity values reveal a significant (p<0.01) loss of TH fluorescence in the striatum.

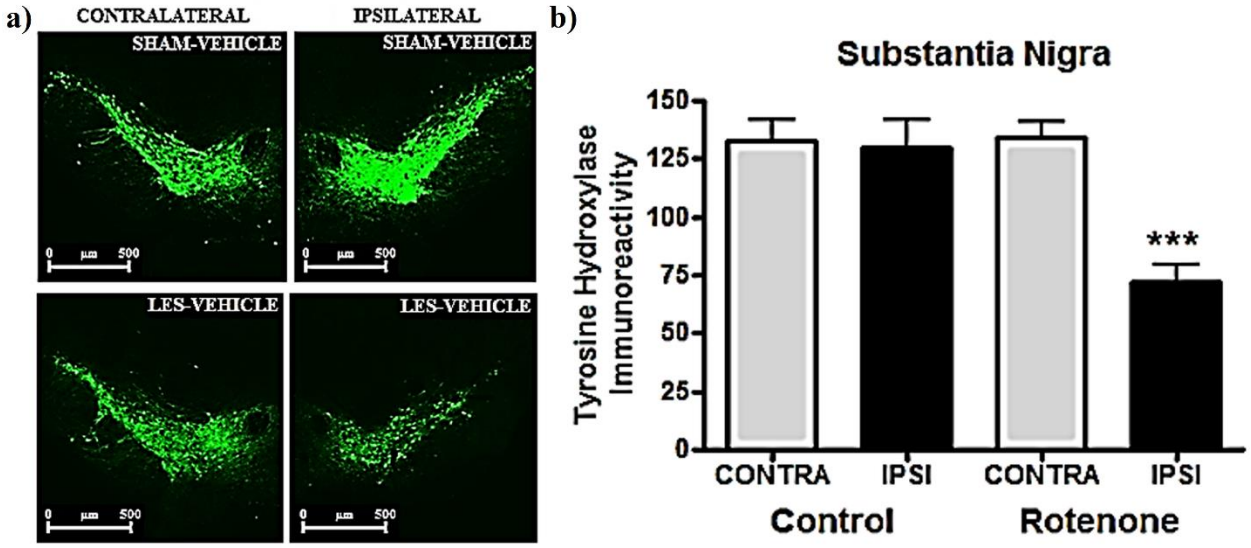

Figure 4.2. Rotenone lesioning of the nigrostriatal pathway depletes nigral TH immunoreactivity in C3H/He mice. Contralateral and ipsilateral images of FITC-stained TH in (a) the SN of sham-lesioned and rotenone-lesioned animals at 5x magnification, at 2 weeks post-surgery. (b) Densitometric analysis of luminosity values reveal a significant (p<0.01) loss of TH fluorescence in the SN.

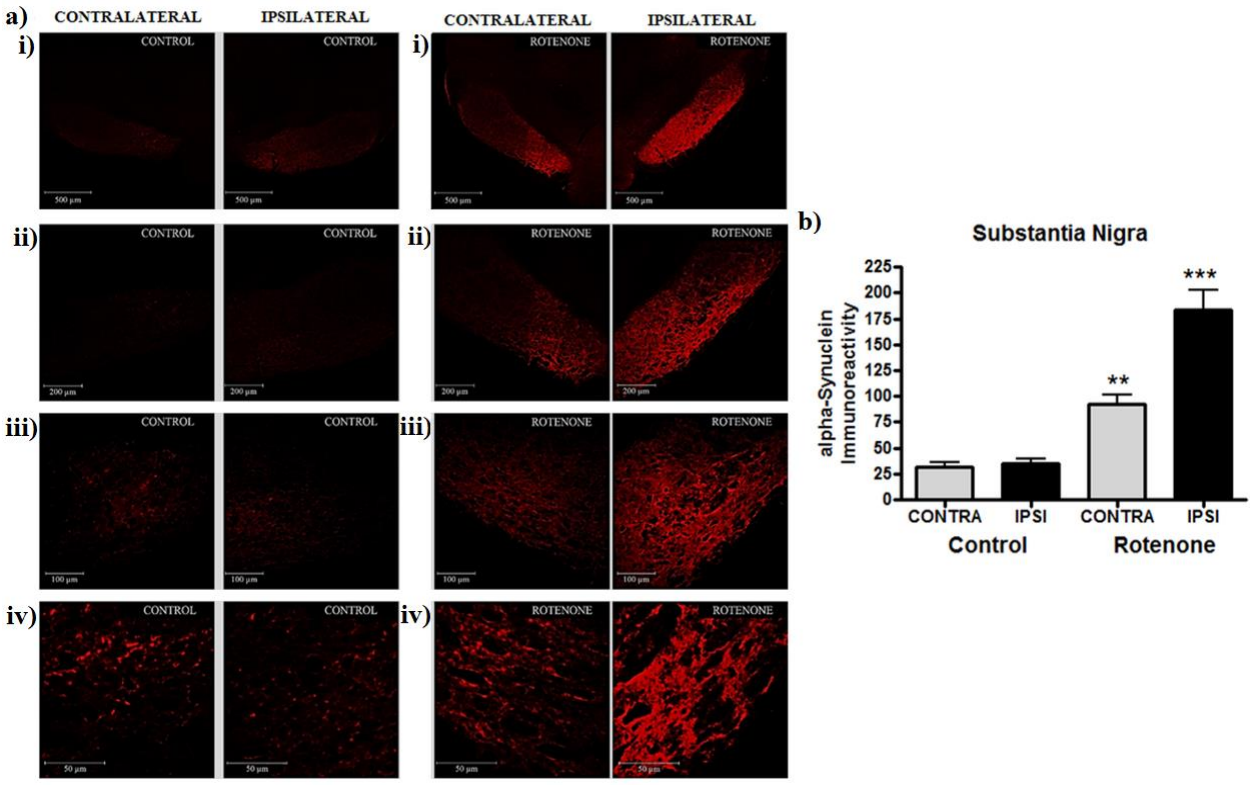

Figure 4.3. Rotenone lesioning of the nigrostriatal pathway induces α-synucleinopathy in C3H/He mice. (a) Contralateral and ipsilateral images of Texas Red-stained α-synuclein in the substantia nigra at (i) 5x, (ii) 10x, (iii) 20x, and (iv) 63x magnification, at 2 weeks post-surgery. (b) Densitometric analysis of luminosity values indicate a significant accumulation of α-synuclein ($p \leq 0.01$), especially in the ipsilateral SN of lesioned mice, as compared to control animals.

CHAPTER 5: DISCUSSION AND CONCLUDING REMARKS

5.1: Summary of Findings

The first study investigated the neuroprotective effects of VPA in an intrastriatal rotenone model of Parkinson's disease. Unilateral infusion of rotenone (6 µg) into the striatum resulted in a significant decrease in contralateral forelimb use, while at the same time caused a significant increase in the use of the ipsilateral forelimb. Chronic treatment with VPA in drinking water, at a dose of 4 mg/mL, significantly abolished contralateral forelimb motor dysfunction, as well as attenuated the increase in ipsilateral forelimb use. In addition to the forelimb asymmetry we observed, infusing rotenone into a single hemisphere of the striatum resulted in decreased tyrosine hydroxylase immunoreactivity within the striatum and the substantia nigra, and a significant loss of dopamine neurons within the substantia nigra. Further, treatment with VPA prevented tyrosine hydroxylase depletion within the striatum and the substantia nigra, and significantly blocked dopaminergic neuronal cell death within the substantia nigra, as confirmed using stereology. These findings strongly support the therapeutic potential of VPA treatment in Parkinson's disease.

The second study examined the protective effects of melatonin in a similar intrastriatal rotenone model of Parkinson's disease. Following the unilateral infusion of a higher dose of rotenone (12 µg) into the striatum, we discovered that apomorphine administration resulted in a significant increase in ipsilateral rotations. Apomorphine also induced changes in locomotor activity in rotenone-treated animals, such that these animals performed significantly fewer full rears/wall climbs and spent significantly less total and average time in a rear/wall climb. Chronic treatment with melatonin that began one week before surgery, in drinking water at a physiological dose of 4 µg/mL,

significantly attenuated most apomorphine-induced behaviours in rotenone-treated animals. Again, we found that infusing rotenone into the striatum resulted in a significant decrease of tyrosine hydroxylase activity within the striatum and the substantia nigra, and a significant loss of dopamine neurons within the substantia nigra. Importantly, chronic treatment with melatonin significantly ameliorated the depletion of tyrosine hydroxylase immunoreactivity within the striatum and the substantia nigra, and significantly prevented the loss of dopamine cells within the substantia nigra. These findings encourage the therapeutic potential of a long-term treatment with low-dose melatonin in Parkinson's disease.

The third study explored a novel intracranial rotenone model of Parkinson's disease, to determine if the unilateral infusion of rotenone into the mouse medial forebrain bundle and the substantia nigra could provide a useful model of Parkinson's disease. We discovered that infusing rotenone (4 µg total; 2 µg/site) into a single hemisphere resulted in a significant decrease in contralateral forelimb use, as compared to animals that were infused with vehicle. Immunohistochemical analysis revealed that rotenone infusion significantly increased bilateral expression of α-synuclein within the substantia nigra, as compared to sham-infused (control) animals. Further, we discovered that this route of rotenone administration caused a significant decrease in tyrosine hydroxylase immunoreactivity within the striatum and the substantia nigra, as compared to the intact (contralateral) hemisphere of the same animals and both hemispheres of control animals. These findings support this intracranial rotenone model of Parkinson's disease, which could prove to be useful for studies of neurodegenerative mechanisms and neuroprotective approaches.

In summary, these studies provide neuropathological evidence of the neurodegenerative effects of rotenone infusion into the striatum or the medial forebrain bundle/substantia nigra as rat or mouse models of Parkinson's disease. We also discovered that treatment with VPA abolished forelimb motor dysfunction and treatment with either VPA or melatonin attenuated retrograde degeneration of the nigrostriatal pathway, with preservation of tyrosine hydroxylase immunoreactivity in the striatum and dopamine neurons in the substantia nigra, supporting their therapeutic potential in Parkinson's disease.

5.2: Neuroprotection by Valproic Acid (Study #1)

Very few *in vitro* and *in vivo* studies have demonstrated that treatment with therapeutic doses of VPA are protective against the toxic effects of drugs that mimic many of the characteristics of Parkinson's disease, such as 6-OHDA, MPTP, and rotenone. In one study that examined the potential advantages of VPA treatment in a 6-OHDA model of Parkinson's disease, the researchers found that when VPA was added to standard chow (2% VPA) for 4 weeks, dopaminergic degeneration was significantly reduced in both the striatum and the substantia nigra (Contestabile et al., 2012). Similar protective dopaminergic effects were found when MPTP (25 mg/kg, twice daily; subcutaneous injection) and VPA (400 mg/kg; intraperitoneal injection) were co-administered for five days with VPA treatment continuing for an additional two weeks (Kidd and Schneider, 2011). In an *in vitro* study, SH-SY5Y cells were pretreated with VPA (3 mM) and then incubated in rotenone (200 nM) for 24 hours. The results from this study revealed that VPA treatment significantly improved cell viability, enhanced mitochondrial membrane potential, reduced reactive oxygen species formation, and decreased rotenone-induced

nuclear fragmentation and cell death (Xiong et al., 2011). In addition to these studies, one group showed that pretreatment with VPA added to standard chow (2% VPA) for 4 weeks prior to systemic rotenone treatment (3 mg/kg/day, for 7 days; osmotic subcutaneous minipumps) significantly prevented tyrosine hydroxylase and dopamine depletion in the striatum and the substantia nigra, as well as attenuated α-synuclein alterations (Monti et al., 2010). Unlike these previous animal studies, we administered VPA in a novel intrastriatal rotenone model for six weeks in drinking water. Due to the chronic and progressive nature of Parkinson's disease, it appears beneficial to examine the effects of a drug that could potentially be prescribed for long-term use. In order to assess the therapeutic efficacy of VPA, we used a novel Parkinsonian animal model, where rotenone (6 µg) was infused at three sites within the striatum. A major advantage of this stereotaxic model is its suitability for long term studies of potential neuroprotective agents. It is important to note that throughout this study, normal body weights and a 100% survival rate were maintained in not only those animals treated with VPA, but those infused with rotenone as well. These results promote the use of stereotaxic rotenone infusion, as opposed to systemic administration, which results in high morbidity and mortality rates. In addition, these findings suggest that the extended use of VPA, which is widely used clinically for neurological disorders including epilepsy (Belcastro et al., 2013), would be safe for the treatment of Parkinson's disease.

In the first study, we used a forelimb asymmetry (cylinder) test to assess possible motor dysfunction. This test revealed a significant decrease in left (contralateral) forelimb use but an increase in right (ipsilateral) forelimb use in animals that were infused with rotenone, as compared to all other groups for the first week. This finding suggests that

the infusion of rotenone into the striatum results in a contralateral motor deficit, as all animals were infused in the right striatal hemisphere. Previous reports have postulated that there is variability in the sensitivity of experimental animals to rotenone, which may be due to many biological factors, including age and weight (Fleming et al., 2004; Lapointe et al., 2004; Zhu et al., 2004; Phinney et al., 2006). The issue of inconsistent susceptibility to this neurotoxin could account for the lack of contralateral motor dysfunction in rotenone-infused animals, as contralateral forelimb use for the remaining three weeks of testing was not significant. In one study, researchers noted that lower doses of intrastriatal rotenone did not elicit consistent contralateral motor differences, however, more reliable deficits in motor activity were observed at the highest rotenone dose (10.8 µg) (Mulcahy et al., 2011). Further, they also found that behavioural dysfunction corresponded with a decrease in dopamine concentration and neuronal loss within the striatum (80-90%) and substantia nigra (60-80%), which corroborates other reports that suggest that clinical symptoms of Parkinson's disease do not appear until 50% of striatal dopamine concentration is lost and the cell death within the substantia nigra reaches 80% (Iancu et al., 2005; Toulouse and Sullivan, 2008; Mulcahy et al., 2011). Thus, the lower dose of rotenone (6 µg) may not have been enough to incite such a marked loss of dopamine concentration and neuronal death to produce a consistent behavioural deficit. Our observation of a significant increase in ipsilateral forelimb use in this model may be due to a compensatory response in rotenone-infused animals, as a result of contralateral forelimb impairment. Interestingly, these abnormalities in forelimb use were not seen in rotenone-infused animals treated chronically with VPA, indicating its neuroprotective ability in this Parkinsonian model.

In this study we found that the infusion of rotenone into a striatal hemisphere results in a marked loss of tyrosine hydroxylase activity within the striatum and a significant loss of dopaminergic neurons within the substantia nigra. Immunohistochemical examination revealed a partial or complete preservation of tyrosine hydroxylase immunoreactivity in the striatum and the substantia nigra, respectively, of rotenone-infused animals that were treated with VPA. This finding was confirmed using unbiased stereological cell counting, where we showed that intrastriatal injections of rotenone cause a significant decrease in tyrosine hydroxylase positive neurons in the substantia nigra on the infusion side, as compared to the contralateral (left) hemisphere of the same animal or either side of animals infused with vehicle. This discovery signifies retrograde degeneration along the nigrostriatal pathway in this intrastriatal model. Importantly, treatment with VPA prevented the rotenone-induced loss of dopamine neurons in the substantia nigra. Therefore, six weeks of VPA treatment is neuroprotective in this rotenone model of Parkinson's disease.

There are several biological mechanisms that could account for the neuroprotective effects of VPA in this rotenone model of Parkinson's disease. As mentioned earlier, there are no known receptors for VPA, however its lipophilic nature allows easy entry into target cells where it is known to promote cell survival through multiple signal transduction cascades, including PI3K/Akt, MAPK/ERK 1/2, and the Wnt/β-catenin pathways (Monti et al., 2009). VPA has also been reported to bind directly to the catalytic core of class I and also act on class lIb of HDACs to inhibit their activity (Göttlicher et al., 2002; Abraham et al., 2014), with consequent hyperacetylation of histone proteins and activation of gene expression (Phiel et al., 2001; Harrison and

Dexter, 2013). Researchers suggest that this inhibition of HDAC activity by VPA plays a crucial role in its neuroprotective effects in models of neurodegeneration (Leng et al., 2008; Monti et al., 2009; Chiu et al., 2013).

Evidence also supports that the inhibitory effects of VPA on HDAC function may be involved in its induction of diverse neurotrophic or protective proteins (Fig. 5.1) including BDNF, GDNF, CDNF, MANF and heat shock protein 70 (Hsp70) (Chen et al., 2006; Marinova et al., 2009; Monti et al., 2009; Niles et al., 2012; Almutawaa et al., 2014). Studies have revealed that treatment with VPA increased exon-IV-containing BDNF mRNA levels, while at the same time increased activation of the BDNF promoter IV in cortical neurons, which is believed to be due to HDAC inhibition (Yasuda et al., 2009; Chiu et al., 2013), and has been implicated in neuronal survival in models of neurodegeneration including Parkinson's disease (Lindholm et al., 2007; Voutilainen et al. 2009; Voutilainen et al., 2011; Duarte et al., 2012; Ren et al., 2013). Further, VPA has been demonstrated to increase GDNF mRNA levels and promoter activity in midbrain neuron-glia cultures (Wu et al., 2008b), as well as increased GDNF levels within the rat hippocampus (Varela et al., 2015). In addition, VPA has proven to be protective in 6-OHDA (Contestabile et al., 2012), MPTP (Kidd and Schneider, 2010, 2011), lactacystin (Harrison et al., 2015), and a systemic rotenone models of Parkinson’s disease (Monti et al., 2010), as well as when administered to SH-SY5Y cells treated with rotenone (Xiong et al., 2011).

In summary, we have presented behavioural and neuropathological evidence of the neurodegenerative effects of unilateral infusion of rotenone into the striatum as a rat model of Parkinson's disease. Most importantly, treatment with VPA prevented deficits

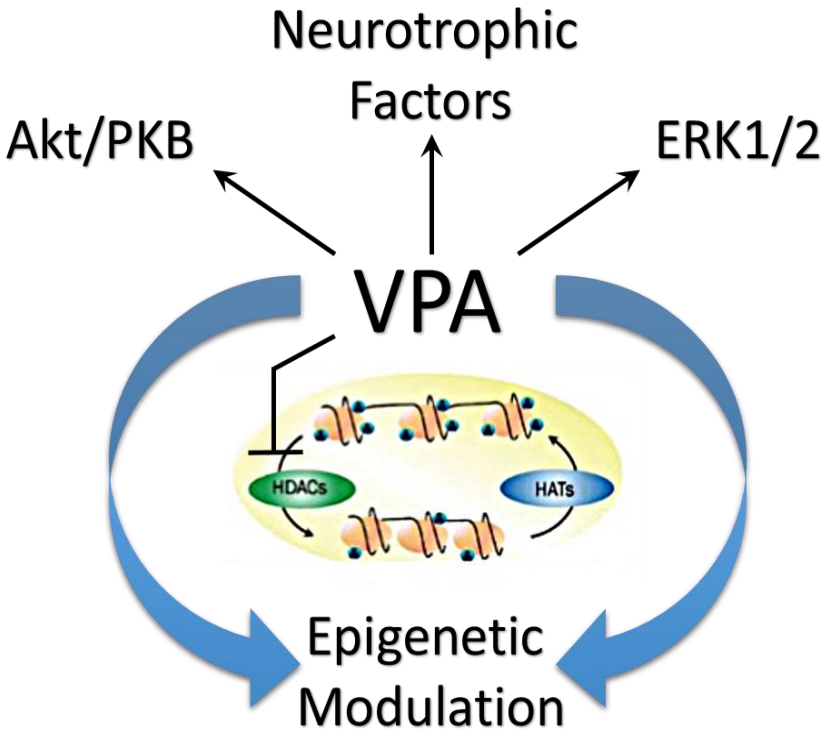

Figure 5.1: Proposed neuroprotective and antioxidant effects of VPA on mitochondrial damage. Through the inhibition of HDAC activity, modulation of PI3K/Akt signalling, activation of the MAPK/ERK 1/2 pathway, and inhibition of GSK-3β activity, VPA is able to activate gene expression, whereby increasing pro-survival mechanisms including protein kinases and neurotrophic factors.

in contralateral forelimb use and retrograde degeneration of the nigrostriatal system, with preservation of tyrosine hydroxylase immunoreactivity in the striatum and dopaminergic neurons in the substantia nigra, indicating its potential efficacy as a treatment for Parkinson's disease.

5.3: Neuroprotection by Melatonin (Study #2)

Following the results found in the first study, this second study examined the neuroprotective effects of a chronic low-dose treatment with the indoleamine hormone, melatonin, against neurotoxicity resulting from rotenone infusion into the striatum. As mentioned earlier, melatonin is able to act through anti-inflammatory, anti-apoptotic and antioxidant mechanisms (Fig. 5.2), resulting in its ability to be protective against damage resulting from such toxins as 6-OHDA (Dabbeni-Sala et al., 2001; Sharma et al., 2006; Borah and Mohanakumar, 2009), MPTP (Thomas and Mohanakumar, 2004; Chen et al., 2005; Capitelli et al., 2008) and rotenone (Sousa and Castilho, 2005; Zhou et al., 2012). In one study that had co-administered rotenone (subcutaneous injection) and melatonin (intraperitoneal injection) for 14 days, researchers showed that melatonin treatment was able to prevent rotenone-induced degeneration along the nigrostriatal system and α-synuclein overexpression (Lin et al., 2008). Another study also demonstrated that following 10 days of rotenone exposure (intraperitoneal injection), chronic exposure to melatonin (intraperitoneal injection), administered 24 hours after rotenone treatment, was able to prevent dopamine concentration loss within the striatum and tyrosine hydroxylase immunoreactivity depletion within the substantia nigra (Bassani et al., 2014). In addition to these systemic examples, melatonin was found to recover the activity of antioxidant enzymes, superoxide dismutase and superoxide

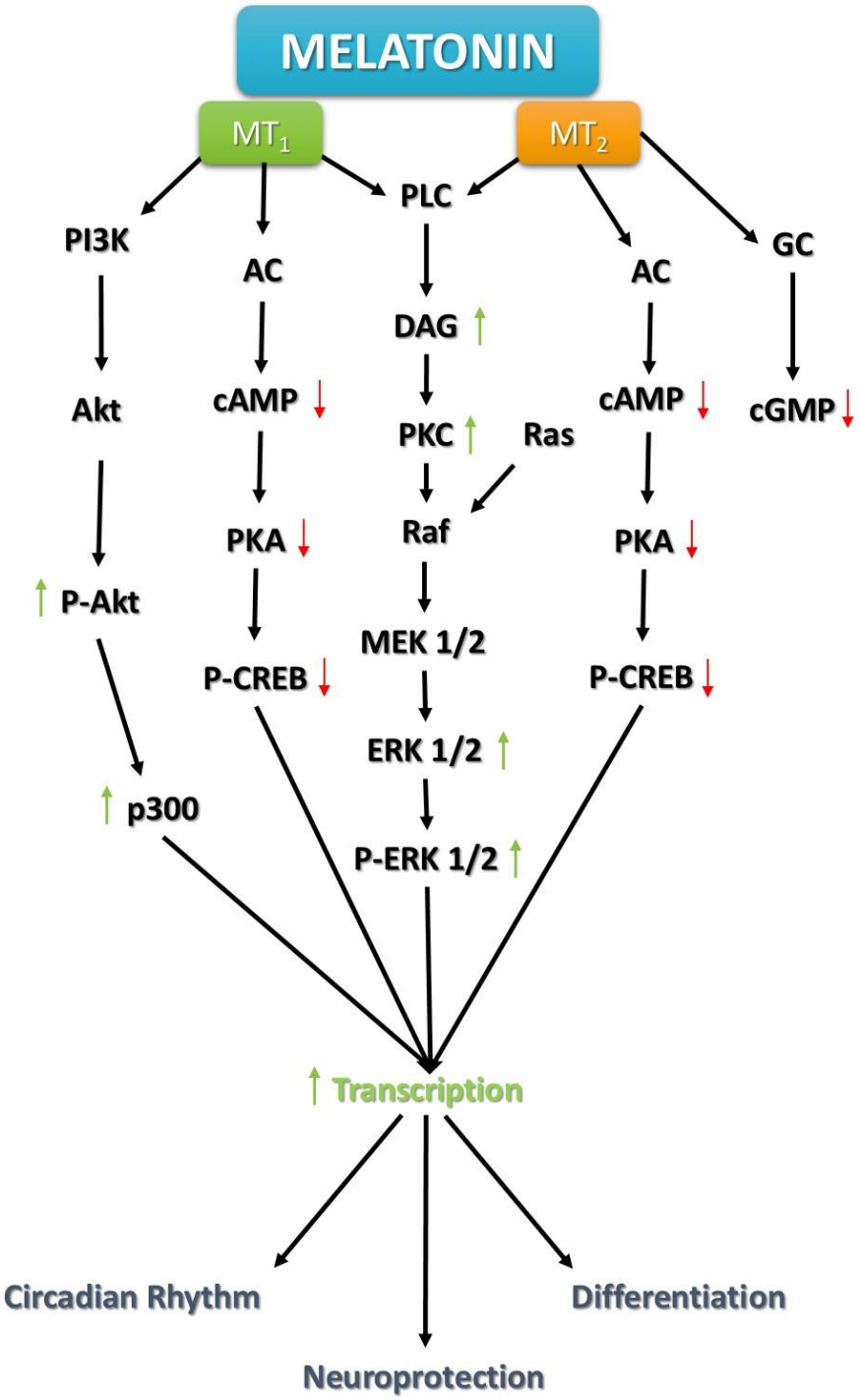

Figure 5.2: Proposed neuroprotective effects of melatonin via MT_1 and MT_2 coupling. All pathways are as described in the text. Through these mechanisms melatonin can increase pro-survival protein kinases and neurotrophic factors, as well as act through epigenetic modulation by stimulating the histone acetyltransferase enzyme, p300, resulting in gene expression.

catalase, as well as attenuate glutathione levels in the substantia nigra, following unilateral intranigral infusion of rotenone (Saravanan et al., 2005). Although studies have demonstrated that melatonin is neuroprotective following systemic rotenone injection, these studies, which used high pharmacological doses of melatonin, were limited to about 14 days or less, due to the high mortality associated with this rotenone model. Additionally, these previous studies have examined the effects of pharmacological doses (e.g.10 mg/kg) of melatonin in systemic (Lin et al., 2008; Bassani et al., 2014; Mattam and Jagota, 2014) and intranigral rotenone models (Saravanan et al., 2007), whereas our study examined the potential protective effects of melatonin on the nigrostriatal system in the same intrastriatal model used for the first study, but with a higher dose (12 µg) of rotenone. Furthermore, our study administered melatonin at a physiological dose (4 µg/mL) in drinking water, which began one week prior to surgery, and continued for the nine weeks that followed. Again, it is important to note that throughout this study, even at an elevated dose of rotenone, normal body weights and survival rates were maintained in animals that were infused with this toxin. Although apomorphine-induced behavioural changes are generally used to evaluate dopaminergic integrity in Parkinsonian models, very few studies are available that have reported the effects of apomorphine in animals that have received rotenone infused into a single striatal hemisphere. Previously, one study found that apomorphine injection caused a significant increase in ipsilateral rotation behaviour in animals that had received an intrastriatal infusion of rotenone (Sindhu et al., 2006). Similarly, in this study we found a significant increase in the number of apomorphine-induced ipsilateral rotations in animals infused with rotenone into the striatum. These results tend to

oppose those observed following infusion of 6-OHDA, where apomorphine administration results in contraversive rotations, but similar apomorphine-induced ipsiversive rotations have been reported in animals following unilateral injections of rotenone or MPP^+ into the striatum or the substantia nigra (Sun et al., 1988; Shimohama et al., 2003; Sindhu et al., 2006; Da Cunha et al., 2008; Dombrowski et al., 2010; Karuppagounder et al., 2013; Madathil et al., 2013a, 2013b). Moreover, contralateral rotations were observed, following injection of MPP^+ or rotenone into the medial forebrain bundle, at 16 and 30 days post-surgery, respectively (Sindhu et al., 2006). Thus, rotational behaviour is influenced not only by the neurotoxin and dose used, but also the site of injection (Sindhu et al., 2006; Da Cunha et al., 2008; Dombrowski et al., 2010). It is possible that the ipsilateral rotations that we observed may be due to the degree of midbrain dopaminergic neuron loss, as infusion of 6-OHDA causes a loss of almost all midbrain dopaminergic neurons, and animals show contraversive rotations, whereas MPTP or rotenone infusion only cause a partial loss of dopamine cells, resulting in ipsiversive rotations (Da Cunha et al., 2008). Also, it has been proposed that rotenone selectively targets dopamine neurons by interfering with sites of dopamine metabolism, such that rotenone would diffuse both pre- and post-synaptically, resulting in the loss of postsynaptic dopamine receptors on the lesioned side (Sai et al., 2008; Lawal et al., 2010; Qi et al., 2014). This loss of postsynaptic receptors would account for ipsilateral rotations following apomorphine administration. In addition to these results, we found that rotenone-infused animals treated with vehicle spent significantly less time, both overall and on average, in a rear/wall climb, and also completed significantly fewer full rears/wall climbs as compared to control groups.

Animals that had been treated with melatonin (rotenone-infused and sham-infused) had significantly fewer full rears/wall climbs, as compared to the control group treated with vehicle. It is possible that the well-known sedative effects of melatonin (Golombek et al., 1996; Pandi-Perumal et al., 2008), were involved. Melatonin has been reported to attenuate neuronal excitation through enhancing GABAergic activity and modulating glutamatergic receptors, which could result in sedative/hypnotic effects with reduced nocturnal activity (Hardeland and Poeggeler, 2012).

The behavioural abnormalities observed in rotenone-infused animals presumably indicate motor deficits due to decreased dopaminergic function in the striatum and the substantia nigra. It is important to note that the total and average times spent in a rear/wall climb and apomorphine-induced rotations were not observed in animals infused with rotenone and treated with melatonin, indicating its neuroprotective potential in this Parkinsonian model.

In corroboration with our first study, we found a significant reduction in tyrosine hydroxylase activity within the striatum and the substantia nigra following a unilateral infusion of rotenone into the striatum, which was confirmed using optical densitometry and stereology. This depletion in tyrosine hydroxylase levels is typical of degeneration of dopaminergic neurons, and from the stereological cell counts, we confirmed retrograde degeneration along the nigrostriatal system in this intrastriatal model. Importantly, chronic treatment with a physiological dose of melatonin prevented the decrease of tyrosine hydroxylase immunofluorescence in the striatum, as well as the loss of dopamine neurons in the substantia nigra, resulting from rotenone infusion.

As noted earlier, several studies have examined the effects of pharmacological doses (e.g.10-30 mg/kg) of melatonin in systemic (Lin et al., 2008; Bassani et al., 2014) and intranigral rotenone models (Saravanan et al., 2007). In contrast, the present study has provided novel evidence that chronic therapy with low-dose melatonin is neuroprotective in an intrastriatal rotenone rat model of Parkinson's disease, as reflected by its ability to significantly prevent degeneration of dopamine neurons along the nigrostriatal pathway. In summary, we have presented neuropathological evidence of the neurodegenerative effects of intrastriatal infusion of rotenone in a rat model of Parkinson's disease. The novel finding that we have demonstrated is that chronic administration of melatonin at a physiological dose is capable of ameliorating apomorphine-induced behavioural disturbances, preserving tyrosine hydroxylase immunoreactivity within the striatum, and protecting dopamine cells from the apoptotic and necrotic effects of rotenone, indicating its therapeutic potential in Parkinson's disease.

5.4: Intracranial Rotenone Infusion and α-Synuclein Overexpression (Study #3)

Until now, the effects of stereotaxic rotenone infusion on dopaminergic integrity within the mouse nigrostriatal system had not been clarified. This third study examined the behavioural and immunohistochemical changes caused by unilaterally infusing rotenone into the medial forebrain bundle and the substantia nigra, in order to determine the potential of this Parkinsonian mouse model for future neuroprotective studies.

To assess possible deficits in motor function, we used a forelimb asymmetry test, which revealed a significant decrease in contralateral forelimb use and a trending increase in ipsilateral forelimb use by those animals infused with rotenone, as compared to sham-infused (control) animals. This finding presumably represents an irregularity in

contralateral motor function inflicted by rotenone insult. This is in accordance with evidence that infusing rotenone into the striatum causes contralateral motor dysfunction in rats (Mulcahy et al., 2011).

Immunohistochemical examination revealed a loss of tyrosine hydroxylase immunoreactivity within the striatum and the substantia nigra of infused animals, and bilateral overexpression of α-synuclein within the substantia nigra of these same animals. The reduced levels of tyrosine hydroxylase in the striatum and the substantia nigra following rotenone treatment indicates degeneration of dopamine neurons along the nigrostriatal pathway. Also, the significant accumulation of α-synuclein found in both the ipsilateral and contralateral hemispheres of the substantia nigra, as compared to control animals, may be due to the severity of the neurotoxin-induced insult, as both the medial forebrain bundle and substantia nigra were lesioned. This suggests that injections at both of these sites produce a similar symptomology as that found in advanced stage Parkinson's disease. Although it has been previously reported that rotenone infusion into the medial forebrain bundle results in α-synuclein aggregation (Weetman et al., 2013), we were able to demonstrate that unilateral infusions of rotenone into the medial forebrain bundle and the substantia nigra not only induced α-synucleinopathy, but also causes behavioural abnormalities and a significant depletion of tyrosine hydroxylase activity within the striatum and the substantia nigra. These effects of rotenone are very similar to the pathology of Parkinson's disease and further supports the usefulness of this model for future studies.

The findings of this third study are consistent with earlier MPTP mouse studies, such that when MPTP was injected into these animals, it resulted in dopamine-specific

neurodegeneration along the nigrostriatal pathway, as shown by a decrease in tyrosine hydroxylase immunoreactivity and an increase in the number of α-synuclein-immunoreactive neurons in the substantia nigra (Vila et al., 2000b). The similarity in the effects of rotenone and MPTP, causing an increased accumulation of α-synuclein in the mouse substantia nigra, may be related to their ability to interfere with complex I activity of the electron transport chain, with consequent mitochondrial dysfunction and associated dopaminergic neurodegeneration (Richardson et al., 2005; Winklhofer and Haass, 2010).

The overexpression of α-synuclein in the substantia nigra following rotenone-induced injury, as observed in this third study, is consistent with western blot data which showed an increase in the expression of this protein in the mouse substantia nigra following unilateral infusion of rotenone into the medial forebrain bundle (Weetman et al., 2013). The function of α-synuclein, which is a small protein mainly localized in nerve terminals (Bendor et al., 2013), is not fully understood. Studies have shown that it is involved in regulating synaptic vesicle formation, neurotransmitter release, and neuronal plasticity (Recchia et al., 2004b). The overexpression of α-synuclein is known to inhibit neurotransmitter release at the presynaptic terminal (Bendor et al., 2013) and to impair mitochondrial function (Martin et al., 2006). Moreover, when α-synuclein is present in high concentrations, there is an increased likelihood that aggregates of insoluble fibrils will occur (Wood et al., 1999). There is evidence that mutations of α-synuclein lead to a misfolding of this protein and consequently result in aggregation, thus losing the function of α-synuclein and ultimately leading to neuronal cell death (Lotharius and Brundin, 2002; Bendor et al., 2013) One of the key pathological hallmarks of

Parkinson's disease is the Lewy body, which is mainly composed of α-synuclein aggregates that are found in the cell soma (Lotharius and Brundin, 2002). Interestingly, the overexpression of α-synuclein may be due to its physiological neuroprotective function at the synapse, as shown in past knockout studies (Chandra et al., 2005; Marques and Outeiro, 2012). Following the knockout of the synaptic co-chaperone cysteine-string protein alpha (CSPα), mice underwent progressive neurodegeneration and impaired synaptic functioning. This impairment was reversed following transgenic expression of α-synuclein, and also reduced the inhibition of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex assembly (Chandra et al., 2005), which is involved in vesicle fusion and exocytosis. Although normal levels of α-synuclein appear to be neuroprotective, overexpression of this protein results in toxicity, degeneration (Scott et al., 2010), and increased susceptibility to systemic pro-inflammatory challenge (Alvarez-Erviti et al., 2011; Couch et al., 2011). Furthermore, synaptic accumulation of α-synuclein is accompanied by an age-dependent redistribution of SNARE proteins, as well as a decrease in dopamine release, which is similar to that observed in Parkinson's disease (Garcia-Reitböck et al., 2010).

To conclude, in this study we have presented behavioural and neuropathological evidence implicating the neurodegenerative effects of intracranial infusion of rotenone in a mouse model of Parkinson's disease. This model will be useful for future studies that are aimed at evaluating the effects of neuroprotective drug therapies on motor dysfunction, dopaminergic degeneration, α-synucleinopathy and other genetic or molecular markers of Parkinson's disease.

5.5: Future Directions & Concluding Remarks

Previously, evidence has shown that independently, VPA and melatonin are able to increase levels of neurotrophic factors and protective genes (Sharma et al., 2008; Wu et al., 2008a; Chiu et al., 2013), and the results from our studies have demonstrated that VPA or melatonin can ameliorate behavioural abnormalities, preserve tyrosine hydroxylase activity along the nigrostriatal pathway, and prevent dopamine neuronal cell death in the substantia nigra. In addition to these findings, recent reports suggest that VPA upregulates the G protein-coupled melatonin receptors, MT_1 and MT_2, both *in vitro* and *in vivo* (Castro et al., 2005b; Jawed et al., 2007; Niles et al., 2012; Almutawaa et al., 2014; Bahna et al., 2014). In light of this, and the involvement of these receptors in mediating physiological neuroprotection by melatonin, future studies would assess the effectiveness of a combinatorial therapeutic treatment with VPA and melatonin in a rotenone model of Parkinson's disease, as compared to either treatment alone. The benefit of a combination therapy could result in the use of lower doses of both drugs, potentially reducing possible side effects. In addition, this approach could result in an enhanced preservation of tyrosine hydroxylase activity and expression of neurotrophic factors, with related dopaminergic neuroprotection and restoration of behavioural function (Fig. 5.3).

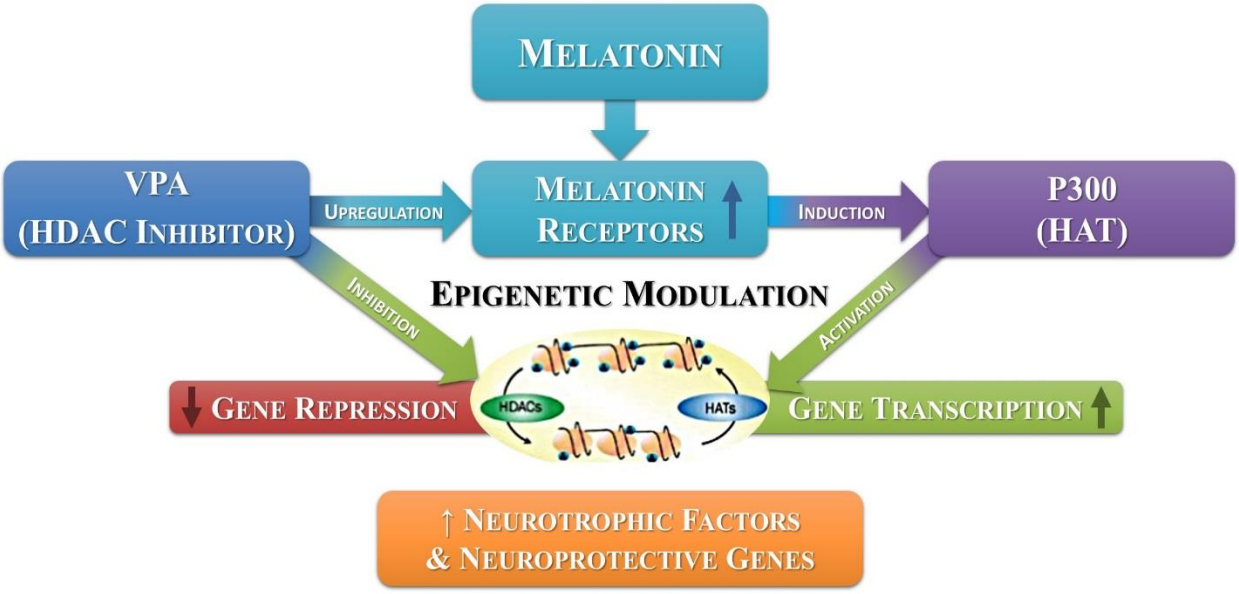

Figure 5.3: Possible mechanisms involved in the neuroprotection by VPA and melatonin

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APPENDIX I: IMMUNOHISTOCHEMISTRY

Transcardial Perfusion

1. Administer a lethal dose of sodium pentobarbital via intraperitoneal injection (0.67cc-1.64cc/rat)
2. Once the animal is under and no longer responding to painful stimuli (tail or limb pinch), place in a supine position on surgical tray in the wet hood
3. Locate the xyphoid process and cut the tissue just below the ribcage using scissors to expose the abdominal organs
4. Lift up the rib cage and carefully cut away the diaphragm to expose the heart
5. Grasp the heart and insert a blunted 18 gauge needle that is attached to an IV bag containing 0.9% saline (NaCl) solution into the left ventricle
6. Quickly make an incision in the right atrium and open the IV
7. Once the liver has lightened in colour and the fluid leaving the right atrium is clear, switch to the IV containing 4% PFA solution
8. Following PFA infusion, you should notice twitching/tremors in the extremities
9. Close the outlet valve once the fixative is nearly finished (200-250 mL) and remove the needle from the heart
10. Remove the animal from the tray and decapitate using scissors
11. Make an incision from the neck to the nose, exposing the skull
12. Trim off any excess muscle and skin to reveal the base of the skull
13. Using rongeurs, carefully clear away the skull around the brain
14. Using a spatula, sever the olfactory bulbs and nervous connections and gently tease the brain away from the skull
15. Remove the brain and place in 4% PFA solution overnight at 4°C
16. Remove the fixative and replace with 1x PBS and 0.06M sodium azide
17. Store at 4°C until needed

Immunohistochemisty

1. Remove PBS and replace with 30% sucrose solution for 72-96 hours at 4°C
2. Using dry ice, add a layer of OTC reagent to the cryostat chuck and freeze
3. Slice the end of the cerebellum so that the edge is flat and stand the brain on the frozen OTC, adding more OTC around the base of the brain to secure it in place
4. Freeze the OTC around the brain using dry ice and transport to the cryostat
5. Allow the brain to freeze in the cryostat (-12 to -16°C)
6. Secure the brain in the cryostat and begin to slice away tissue until you reach the striatum (100 µm thickness/slice)
7. Using an atlas (Paxinos and Watson, 1982; Franklin and Paxinos, 2008), determine where the striatum begins
8. Once the striatum is visible, start collecting tissue at 35 µm (rat) or 25 µm (mouse) in 24 well plates (one slice/well) containing 1x PBS
9. After the striatum has been collected, advance to the substantia nigra following directions from the atlas

10. Once the substantia nigra is visible, start collecting tissue at 35 µm (rat) or 25 µm (mouse) in 24 well plates (one slice/well) containing 1x PBS
11. Store these dishes at 4°C until needed
12. For immunostaining, take a plate and rinse slices 3 times with 1x PBS + 0.05% Triton-X for 10 minutes each
13. Incubate tissue in 1.5% NDS (rat) or 10% NGS in PBS for 1 hour
14. Incubate tissue in the primary antibody for 72 hours at 4°C
15. Rinse slices 3 times with 1x PBS + 0.05% Triton-X for 10 minutes each
16. Incubate tissue in the secondary antibody for 2 hours at room temperature
17. Rinse slices 3 times with 1x PBS + 0.05% Triton-X for 10 minutes each
18. Using a small paintbrush, pick up a slice and place it on a slide
19. Add 1x PBS + 0.5% Triton-X to open the sections up on the slide
20. Use a Kimwipe to gently collect excess liquid around the tissue and allow tissues to air dry in darkness
21. Once the slices have dried, add fluorescent mounting media and coverslip
22. Leave overnight at room temperature in a slide box to dry and examine as needed

APPENDIX II: STEREOLOGY

Samples from the substantia nigra were imaged using a confocal microscope (LSM 510, Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) equipped with a Plan-Apochromatic 63× 1.4 Oil DIC, with excitation using a 488-nm Argon laser for high resolution imaging. Every sixth section throughout the substantia nigra was collected with the first section being randomly selected, allowing for an unbiased series of sections. Estimation of volume was performed using the Cavalieri principle (Mayhew and Gundersen, 1996; Nyengaard, 1999; Tang et al., 2003), where volume is expressed as the function of an intersection between an object and a plane orthogonal to a fixed sampling point x, and bound to the domain [a,b], allowing an orthogonal linear projection onto the sampling axis of which a scale has been previously defined:

$$\mathbf{V(obj): = t \times (a/p) \times \Sigma P}$$

Where,	t	is the thickness of the stack sampled.
	(a/p)	is the calculated area of the stack.
	ΣP	is the sum of all of the points sampled across all sections.

The relationship between the area function and the volume is true regardless of orientation of planes or shape of objects giving an accurate method to estimate the volume of the substantia nigra. Following this principle, volume was then estimated under 5x objective with each section superimposed with a matrix at a randomly selected position with distances 254.43 µm x 254.43 µm in both X and Y axes. The distance for the X and Y axes were calculated by dividing the sum of the areas of each substantia nigra image that appeared on the sections that were to be used by 150, and then taking the square root of this value (West, 2012) (Fig. 1.). Using the vertices of the matrix within the area of interest, a point count occurred on each section, creating a volume probe. Summing up the corresponding points from all sections of an individual animal and multiplying that value with the uniform distance between each section (175 µm) and by the area of each matrix (254.43 µm x 254.43 µm), gives an estimated total volume of the substantia nigra in the rat brain (Mayhew and Gundersen, 1996).

Each section was then inspected at a higher magnification to perform point counts to create a three dimensional probe. Going to a high power magnification (63x water immersion), z-stack images were collected using a Leiss LSM 510 confocal microscope at 2 µm intervals, which resulted in a total thickness of ~32 µm per stack. A guard zone of 5 µm at each superficial portion of the tissue was used to prevent bias due to super-saturation of cells or artefacts resulting from the cutting process (Gundersen, 1977). These images were saved at 512 pixels by 512 pixels for analysis. Each cell was identified and counted using unbiased design-based stereological procedures. LSM and Zen software were used for data analysis.

On each stack of images an unbiased counting frame was superimposed, consisting of two exclusion lines and two inclusion lines, creating an unbiased virtual counting space.

Cells were counted provided they were found entirely within this unbiased counting frame or hit at least one of the inclusion lines but not any of the exclusion lines. Cells were counted when they first came into focus to avoid double counting cell profiles. The unbiased counting frame served as the basis for an Optical Disector, with an area of 120 µm x 120 µm (Fig. 2). The Optical Fractionator method (Gundersen, 1986; Bonthius et al., 2004; Hosseini-Sharifabad and Nyengaard, 2007) of calculating estimated total cell numbers was performed by multiplying the number of cells counted within all counting spaces (Q) with the reciprocal value of the sampling fraction (sampling probability). The sampling probability was calculated by three levels of fractions:

1) The number of investigated sections compared to the total number of sections (section sampling fraction, ssf)
2) The area of the counting frame compared to the area of the matrix used (area sampling fraction, asf)
3) The height of the counting frame compared to the average section thickness (thickness sampling fraction, hsf)

$$\mathbf{N = \frac{1}{asf} \times \frac{1}{hsf} \times \frac{1}{ssf} \times \Sigma Q-}$$

Where,
- ΣQ- is the total number of cells counted within the unbiased counting space for all sections.
- asf is the area of the counting frame compared to the area of the matrix used.
- hsf is the height of the counting frame compared to the average section thickness.
- ssf is the number of investigated sections compared to the total number of sections.

From this, estimated total cell numbers were calculated. Densities were calculated by taking the total estimated number of cells and dividing by the substantia nigra estimated volume, and converting each number to cells per mm3. Statistical analyses were run using total cell counts rather than densities to avoid potential bias (West, 1999).

$$\mathbf{N/V = \frac{\Sigma Q-}{V(obj)}}$$

Where,
- ΣQ- is the total number of cells counted within the unbiased counting space for all sections.
- V(obj) is the reference volume estimate calculated using Cavalieri's method.

The Coefficient of Error variance for the Cavalieri method was calculated by taking the squared root of variance related to point count (expressed as noise) plus the variance of the estimation using systematic random sampling and dividing by the total number of points hitting the area of interest (Gundersen and Jensen, 1987; Nyengaard, 1999; Tang et al., 2003; Hosseini-Sharifabad and Nyengaard, 2007).

$$\mathbf{CE(V)} = \frac{\sqrt{\mathbf{VarNoise + VarSRS}}}{\mathbf{\Sigma P}}$$

$$\mathbf{VarNoise} = \mathbf{0.0724x}\left(\frac{\mathbf{b}}{\sqrt{\mathbf{a}}}\right)\mathbf{x}\left(\mathbf{n\ x\ \Sigma P}\right)$$

$$\mathbf{VarSRS} = \frac{\sqrt{\left[\left(\mathbf{3A\ -\ VarNoise}\right) - \mathbf{4B + C}\right]}}{\mathbf{n}}$$

Where,

P	is the number of points counted within a section.
A	= ΣPi x ΣPi
B	= ΣPi x ΣPi+1
C	= ΣPi x ΣPi+2
n	is the number of tissue sections.
$\left(\frac{b}{\sqrt{a}}\right)$	is the shape factor for a given region, where b is the boundary length and a is the calculated area for this region of interest (Gundersen and Jensen, 1987).

The Coefficient of Error variance for the estimated cell numbers were calculated for the fractionator method by taking the inverse of the square-rooted sum of cells counted for each section (Nyengaard, 1999; Bonthius et al., 2004).

$$\mathbf{CE(\Sigma Q^-)} = \frac{1}{\sqrt{\mathbf{\Sigma Q^-}}}$$

Where,

ΣQ^-	is the total number of cells counted within the unbiased counting space for all sections.

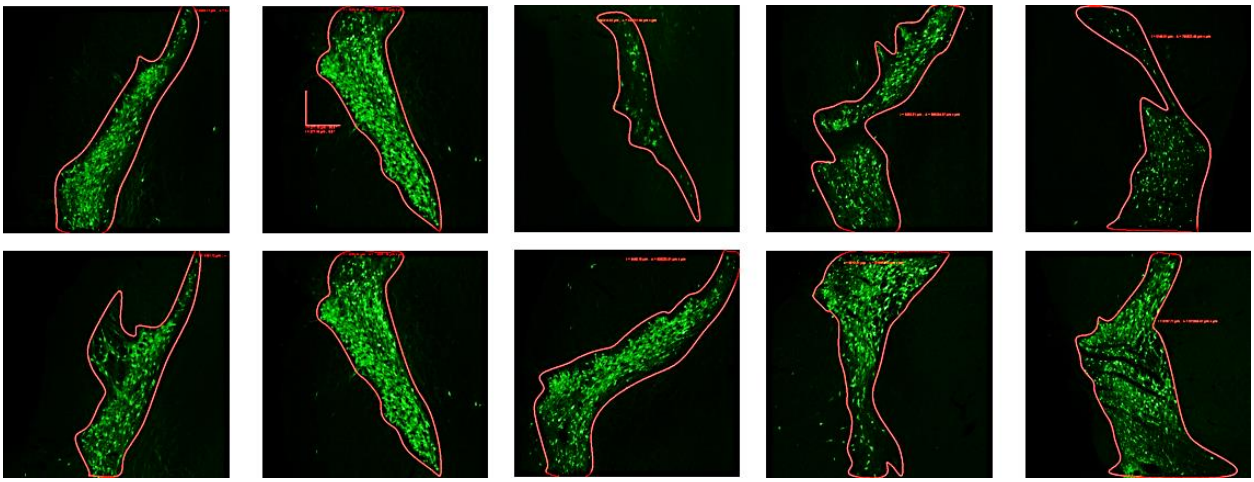

Figure AII.1: Calculation of the optical dissector length and width by taking the square root of the areas of the substantia nigra from all of the slices collected divided by 150 (West, 2012).

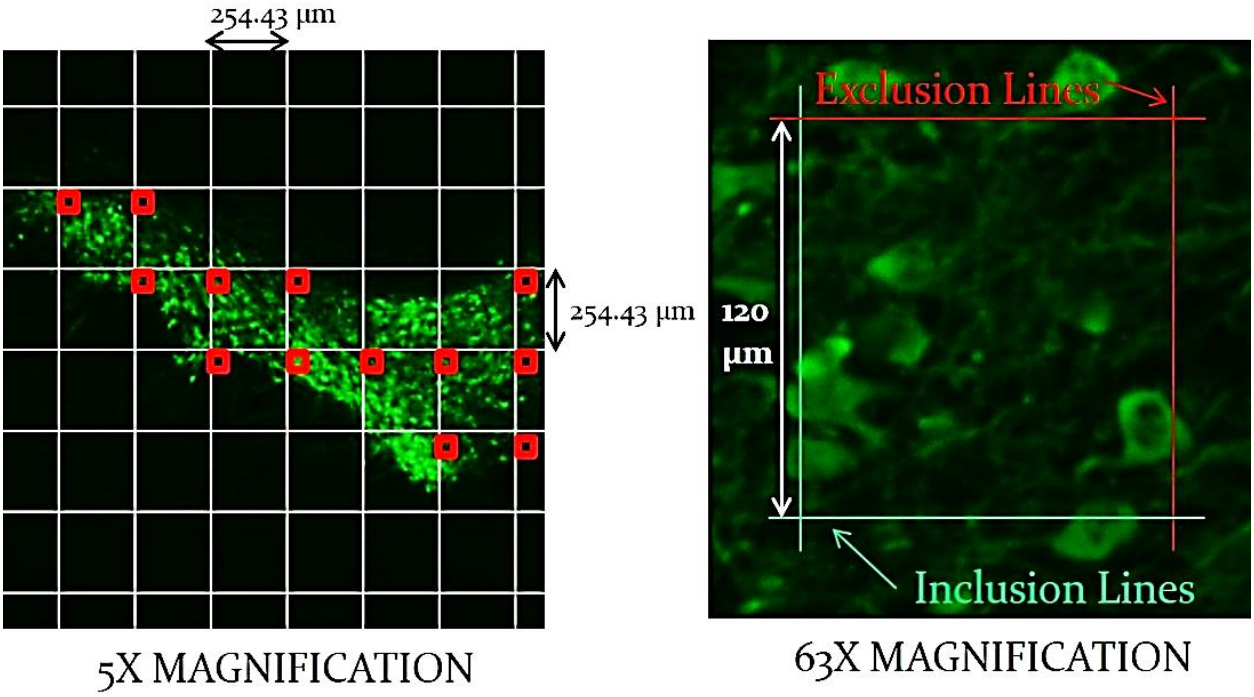

Figure AII.2: Stereology protocol. Every sixth section throughout the substantia nigra was collected with the first section being randomly selected, allowing for an unbiased series of sections. Optical stacks of images were acquired from the substantia nigra at 63x magnification for stereological procedures. Estimation of volume was performed using the Cavalieri principle. Each cell was identified and counted using unbiased design-based stereological procedures. Using the Optical Fractionator method, estimated total cell numbers were calculated (Mayhew and Gundersen, 1996).