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A Model of Sporadic Parkinson's Disease: Herbicide Induced Parkinson's Disease in Rat and Neuroprotection by Water Soluble CoQ10

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A Model of Sporadic Parkinson's Disease: Herbicide Induced Parkinson's Disease in Rat and Neuroprotection by Water Soluble CoQ$_{10}$

by
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Declaration of Co-Authorship / Previous Publication

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I hereby declare that this thesis incorporates material that is result of joint research, as follows:

This thesis also incorporates the outcome of a joint research undertaken in collaboration with Dr. Jafar Naderi under the supervision of Professor Pandey. The collaboration is covered in Chapter 3 of the thesis. In this case, the key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of co-authors was primarily through the provision of carrying out some experiments as well as writing and proof reading the Manuscript.

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This thesis includes two original papers that have been previously published/submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status*
Chapter 3 (Section 3.1)	*Paraquat induces oxidative stress, neuronal loss in substantia nigra region and parkinsonism in adult rats: neuroprotection and amelioration of symptoms by water-soluble formulation of coenzyme Q_{10}*	*published in BMC Neuroscience, 2009*
Chapter 3 (Section 3.3)	*Water-soluble formulation of Coenzyme Q10 inhibits Bax-induced destabilization of mitochondria in mammalian cells*	*published in Apoptosis, 2006*
Chapter 1	*Role of Environmental and Inflammatory Toxicity in Neuronal Cell Death*	*published in the Open Toxicology Journal, 2008*

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Abstract

Parkinson's disease is caused due to the progressive loss of dopaminergic neurons in the brain. Less than 10% of cases have a strict familial etiology while more than 90% are sporadic. The mechanisms underlying neuronal cell death in Parkinson's disease have not been fully elucidated. Furthermore, there is currently no therapy available to prevent the progressive loss of neurons in Parkinson's disease. The objective of my research was to establish a sporadic model of Parkinson's disease and evaluate the neuroprotective effects of water soluble Coenzyme Q_{10}. In this study, a model of paraquat-induced neurodegeneration in rats was used to evaluate the efficacy of water soluble Coenzyme Q_{10} as a neuroprotectant. The results revealed a loss of dopaminergic neurons in the substantia nigra *pars* compacta region of the brain in paraquat-injected rats. Increased levels of oxidative stress and activated astrocytes were also observed in rats treated with paraquat. Furthermore, the behavioural tests uncovered deficiencies in fine motor skills in these rats. In parallel, rats that received water soluble Coenzyme Q_{10} in their drinking water showed significant neuroprotection against paraquat toxicity including behavioral improvements, reduced loss of dopaminergic neurons and lowered levels of oxidative stress. Interestingly, this neuroprotection was accompanied by an increased numbers of activated astroglia in the midbrain. The ability of this formulation of Coenzyme Q_{10} as a therapeutic agent was also studied; preliminary results indicate that it offers neuroprotection. Pilot studies revealed that paraquat-induced dopaminergic loss increases with age. Altogether, this study confirmed the neurotoxicity of paraquat, especially towards dopaminergic neurons, and provided a rat model of Parkinson's disease suitable for mechanistic and neuroprotective studies. This is the first preclinical evaluation of

water soluble Coenzyme Q_{10} as a neuroprotectant for Parkinson's disease at clinically

relevant doses.

Dedication

This work is dedicated to my family for all their love, encouragement and support as well as my supervisor Dr. Siyaram Pandey for his guidance.

Acknowledgements

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Abbreviations

4-HNE	4-hydroxy nonenal
6-OHDA	6-hydroxydopamine
ADP	adenosine diphosphate
AIF	apoptosis inducing factor
ANOVA	analysis of variance
ATP	adenosine triphosphate
BDNF	brain derived neurotrophic factor
BG	basal ganglia
BSA	bovine serum albumin
CNS	central nervous system
CNTF	cytokine ciliary neurotrophic factor
COMT	cytoplasmic catechol-O-methyltransferase
COX-2	cyclooxygenase-2
DA	dopaminergic
DAB	Diamino benzamidine
DAPI	4',6-diamidino-2-phenylindole
DAT	dopamine transporter
dH$_2$O	distilled water
DNA	deoxyribonucleic acid
DOPA	dihydroxyphenylalanine

DOPAC	dihydroxyphenylalanine carboxylase
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GDNF	glial cell derived neurotrophic factor
GFAP	glial fibrillary acid protein
GPe	globus pallidus *pars* externa
GPi	globus pallidus *pars* interna
GSH	reduced glutathione
GSR	glutathione reductase
GSSG	oxidized glutathione
H & E	hematoxylin and eosin
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HVA	homovanillic acid
IgG	immunoglobulin G
IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthase
JNK	c-Jun-N-terminal kinase

L-Dopa	levodopa
MAO	mitochondrial monoamine oxidase
MB	maneb
MDA	malondialdehyde
MPP^+	1-methyl-4-phenyl pyridinium ion
MPTP	1-methyl- 4-phenyl-1,2,5,6-tetrahydropyridine
NAD^+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NeuN	Neuronal nucleii
NGF	nerve growth factor
NMDA	N-methyl-d-aspartate
NO	nitric oxide
$ONOO^-$	peroxynitrite anion
PBS	phosphate buffer saline
PD	Parkinson's disease
PEG	polyethylene glycol
PHPA	p-hydroxy phenyl acetic acid
PMSF	phenylmethanesulphonylfluoride
PQ	paraquat (chemical name: 1, 1'-dimethyl-4, 4'-bipyridinium)
PTP	permeability transition pore
PTS	polyoxyethanyl α-tocopheryl sebacate
RNS	reactive nitrogen species
ROS	reactive oxygen species

SDS-PAGE	sodium dodecyl sulphate polyacrilamide gel electrophoresis
SN	substantia nigra
SNc	substantia nigra *pars* compacta
SNr	substantia nigra *pars* reticulata
SOD	superoxide dismutase
ST	striatum
STN	subthalamic nucleus
TBS	tris-buffered saline
TBST	tris-buffered saline with 0.2% tween
TEMED	tetramethylethylenediamine
TH	tyrosine hydroxylase
TNF-α	tumor necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
VDAC	voltage dependent anion channel
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area
WS-CoQ$_{10}$	water soluble coenzyme Q$_{10}$

Chapter 1

Introduction

Parkinson's disease (PD) is characterized by the loss of dopamine producing neurons present in the substantia nigra *pars* compacta region in the brain. Motor function is affected in this disease. This chapter introduces the anatomy of the brain region affected by PD, as well as the different dopaminergic pathways and the pathway associated with PD. The causes of PD, the various models used for studying PD and the mechanisms of cell death in PD are also addressed in this chapter. Lastly, the different mechanisms underlying neuronal cell death in PD, and the neuroprotective strategies that are used to treat people with PD are discussed.

1.1 The Basal Ganglia Circuitry

The basal ganglia (BG) consist of a large and functionally diverse set of nuclei, which lie deep within the cerebral hemispheres. The subset of nuclei involved in motor control include the caudate nucleus, putamen (caudate and putamen together form the striatum), the globus pallidus: *pars* externa (GPe), and *pars* interna (GPi), subthalamic nucleus (STN) and the substantia nigra (SN) (Bjarkam and Sorensen 2004) (Figure 1.1). According to the classical model, the basal ganglia form a complex network that integrates areas of the cerebral cortex, basal ganglia nucleus and the thalamus (Alexander and Strick 1986). In this complex circuit, the neurons from the cortical areas project into the striatum, especially the putamen. The striatum also receives projections from the amygdala and hippocampus (Groenewegen 2003). These inputs into the striatum are all excitatory. The transfer of cortical and thalamic information in the

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striatum is modulated by dopaminergic inputs from the substantia nigra *pars* compacta (SNc). The globus pallidus and the substantia nigra pars reticulata are the main sources of output from the basal ganglia.

The signals for intentional movements are initiated in the cerebral motor cortex and eventually reach the brain stem. From here they reach the muscles, but before they do so, centers such as the cerebellum and the basal ganglia pose their influence on these signals. Both these centers exert their influence on the final motor signals via the thalamus (Groenewegen 2003).
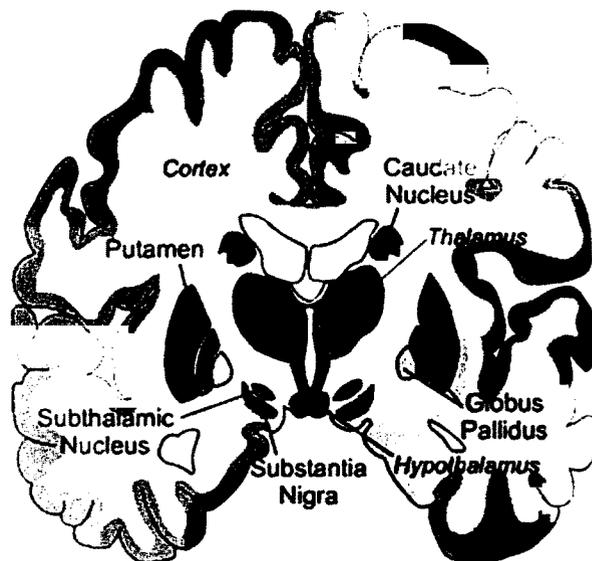
Figure 1.1 A coronal section showing the components of the basal ganglia in the human brain. The figure shows the arrangement of the caudate nucleus, putamen, and globus pallidus relative to the substantia nigra, in the midbrain.

The efferent projections from the striatum to the basal ganglia are segregated into two pathways (for a representation of the basal ganglia circuitry, refer to Figure 1.2). In the direct pathway, the neurons from the putamen directly project into the globus pallidus *pars* interna and the substantia nigra *pars* reticulata (SNr). Neurons in this pathway bear the D1 dopamine receptor subtype and provide a direct inhibitory effect on GPi/SNr (Figure 1.2A). In the indirect pathway, the neurons from the putamen connect with the GPi/SNr via the globus pallidus *pars* externa (GPe) and the subthalamic nucleus. The neurons in this pathway have D2 dopamine receptor subtype, and cause the excitation of GPi/SNr (Bjarkam and Sorensen 2004) (Figure 1.2B). Under normal circumstances, the dopaminergic neurons present in the substantia nigra *pars* compacta will supply dopamine to the striatum via the nigrostriatal pathway, stimulating neurons expressing D1 receptors and inhibiting neurons expressing D2 receptors (Bjarkam and Sorensen 2004). Thus the output from the basal ganglia is influenced by the opposing effects of the direct and indirect pathways. An abnormal decrease or increase in the activity of the basal ganglia output, is associated with Parkinsonism and dyskinesias (Obeso *et al.* 2006).

A
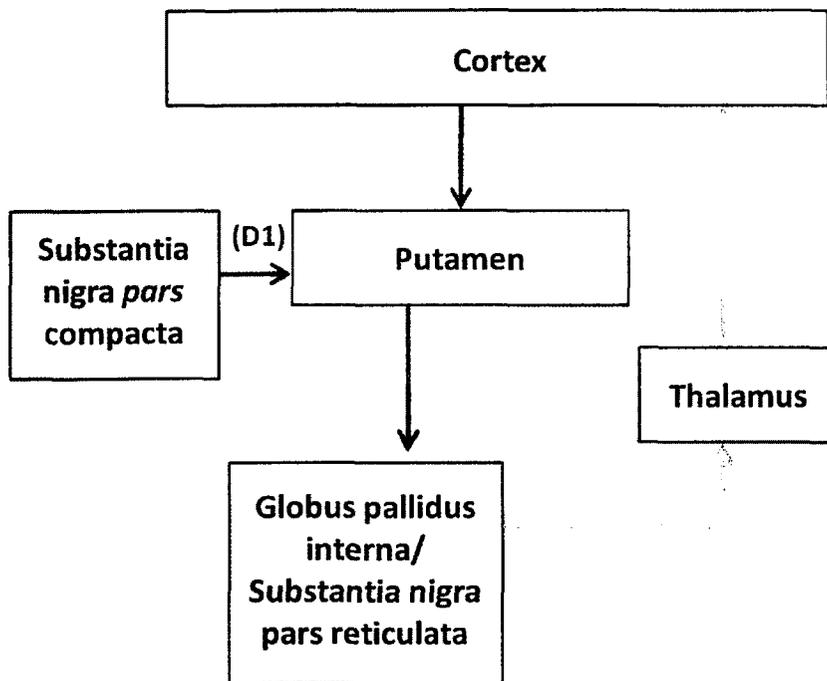
B
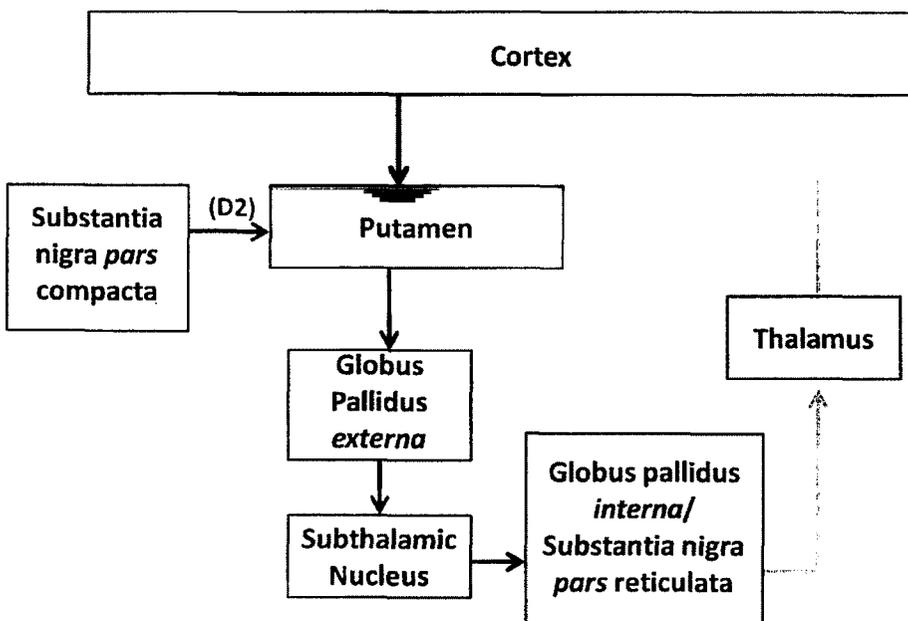
Figure 1.2 The basal ganglia circuitry. The efferent projections from the striatum to the basal ganglia are segregated into two pathways namely the direct pathway (A) and indirect pathway (B).

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1.2 Dopamine in the Nigrostriatal Tract

Dopamine is a member of the class of catecholamines, along with epinephrine and norepinephrine. The first, and rate-limiting, step in catecholamine synthesis is the enzymatic conversion of tyrosine to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (Figure 1.3). Dopamine is synthesized in the cytoplasm of the presynaptic terminals and stored in vesicles by a vesicular monoamine transporter (VMAT). Upon release in the synaptic cleft, its action is terminated by re-uptake into the terminal or surrounding glial cells by the dopamine transporter (DAT) (Dziedzicka-Wasylewska 2004).
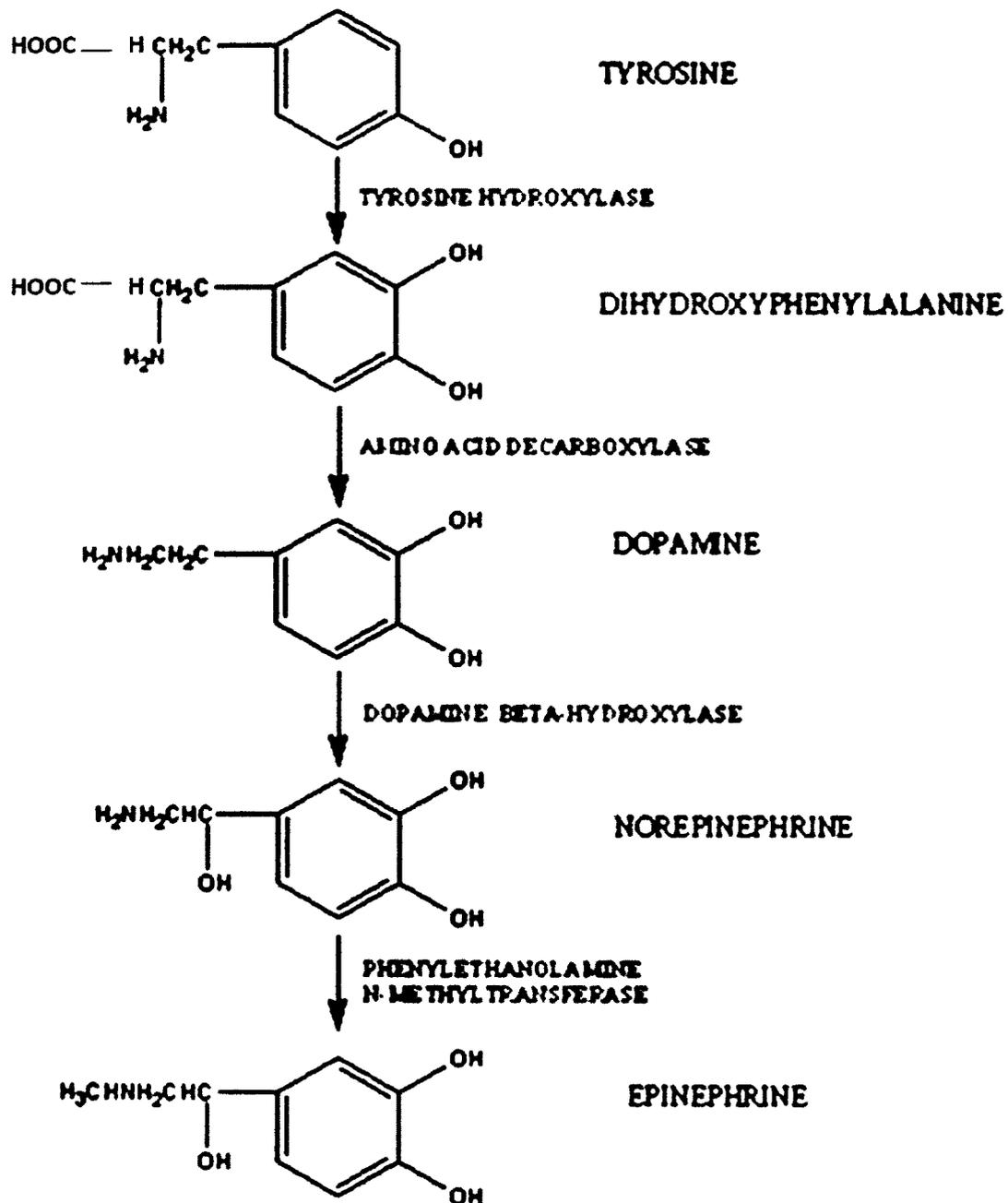
Figure 1.3 Catecholamine synthesis. Dopamine functions as both a neurotransmitter and a precursor for other catecholamines. Tyrosine hydroxylase is the rate-limiting enzyme.

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Two enzymes are involved in the catabolism of dopamine: mitochondrial monoamine oxidase (MAO) and cytoplasmic catechol-O-methyltransferase (COMT). The final product of the breakdown of dopamine yields homovanilic acid (Figure 1.4) (Dziedzicka-Wasylewska 2004).
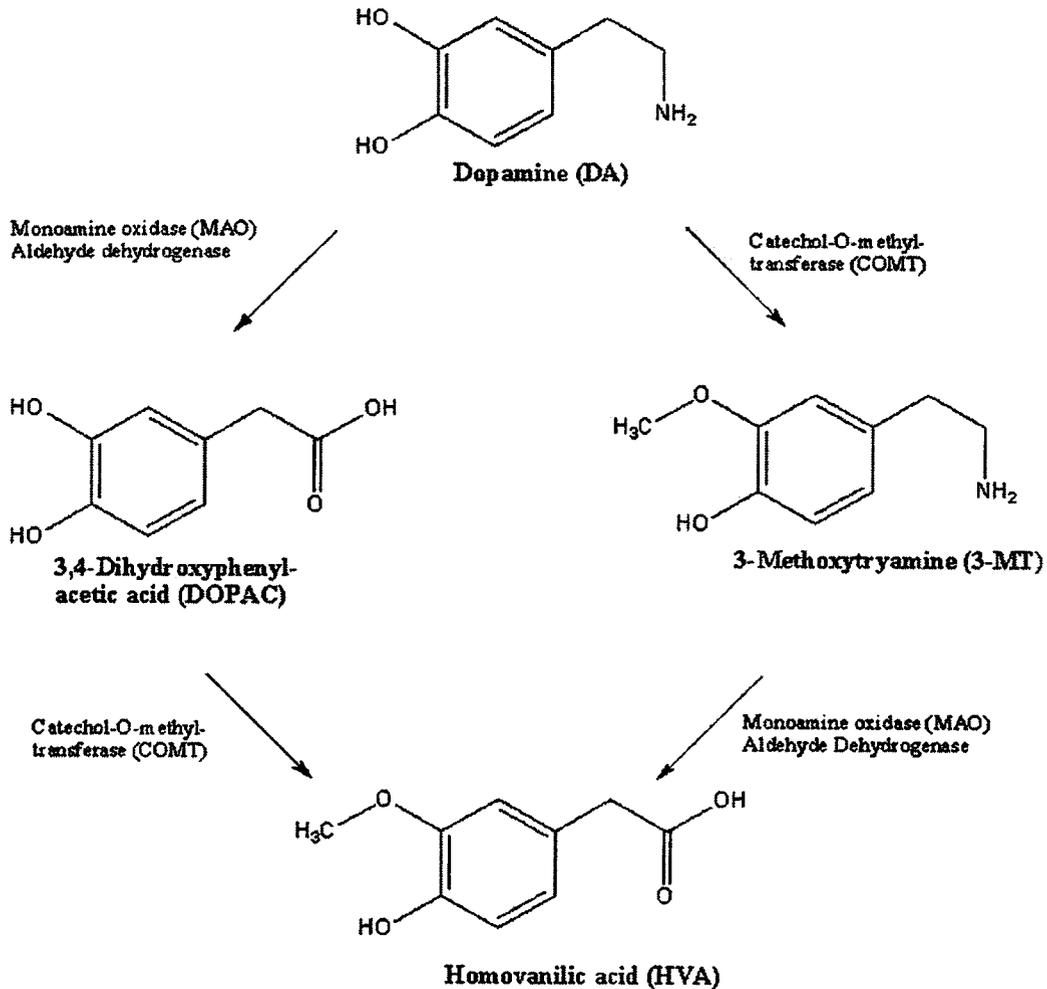
Figure 1.4 Dopamine catabolism. Dopamine is broken down into homovanilic acid by two different enzymes.

1.3 Dopaminergic Pathways

Many of the dopaminergic neurons in the central nervous system are positioned in the midbrain, and are divided into four functional and anatomical groups: mesolimbic, mesocortical, nigrostriatal and tuberoinfundibular dopaminergic neurons. These circuits are involved in the regulation of a wide range of processes, from circadian rhythms to depression and reward-based behavior (Dziedzicka-Wasylewska 2004).

The mesolimbic pathway arises in the ventral tegmental area (midbrain) and innervates the nucleus *accumbens* and olfactory tubercle in the ventral striatum and parts of the limbic system including the septum, amygdyla and pyriform cortex. This pathway is thought to be essential for motivated behaviours such as activity related to reward and the positive reinforcement from frequently abused drugs, for instance alcohol, cocaine, amphetamines and opiates. The mesocortical dopaminergic pathway also originates in the ventral tegmental area and innervates the frontal, cingulate and entorhinal cortices. These areas are involved in emotional, motivational and cognitive functions, such as certain aspects of learning and memory. It is broadly acknowledged that the mesocorticolimbic dopamine system plays a pivotal role in mediating acute rewarding effect of drugs of abuse. Finally, the nigrostriatal pathway projects from the substantia nigra *pars* compacta and innervates the dorsal striatum and is involved in motor control (Dziedzicka-Wasylewska 2004). Imbalance in this pathway is associated with Parkinson′s disease and is associated with bradykinesia and hypokinesia (Groenewegen 2003).

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1.4 Parkinson 's disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder, and was first described by James Parkinson in 1817 (Schober 2004). The prevalence of PD is 1-2 per 1000 (Bartels and Leenders 2009). The cardinal feature of this disease is dopamine deficiency arising due to a progressive degeneration of dopaminergic (DA) neurons in the nigrostriatal pathway. The dopamine neurons play an important role in coordinating normal motor function. Post mortem studies have revealed a decrease in the levels of dopamine and its metabolites in the different areas including the globus pallidus, putamen, caudate, nucleus *accumbens* and the substantia nigra of PD patients. Clinical symptoms such as resting tremors, bradykinesia, rigidity and postural instability arise when about 60-70% of the DA neurons are lost, leading to a decline in dopamine levels in the nigrostriatal pathway (Lang and Lozano1998).

Besides the dopaminergic defects, PD is assumed to be a multi-centric disease. It is also believed that there is an association between the nigrost riatal degeneration and degenerative process elsewhere in the central and peripheral nervous system. Three possible scenarios are hypothesized: damage to the SNc and other regions occur simultaneously, the disease primarily begins in the SNc and this influences the involvement of other areas; or, the involvement of the SNc occurs later in the disease (Lang and Lozano 1998). Lewy bodies are found in the degenerating DA neurons of PD patients and are comprised of aggregated α- synuclein and ubiquitin (Bartels and Leenders 2009).

1.5 Etiology

The prevalence of Parkinson's disease (PD) in individuals over 50 years of age is 1 to 2% (Bartels and Leenders, 2009 2009). Most cases (95%) are of unknown etiology (Corti *et al.* 2005). Epidemiological studies have shown PD concordance rates to be similar between monozygotic and dizygotic twins (50 years of age), indicating that heredity is not a major etiologic component. Tanner screened 19,000 monozygotic and dizyogotic twins for PD and found that there were very similar concordance rates between the two types of twins (1989a). This result led Tanner to conclude that "genetic factors do not play a major role in causing PD." Studies designed to understand the role of environmental toxins explored geographic differences in PD prevalence. Evidence pointed to a higher incidence of PD in industrialized nations. For example, PD occurrence was correlated to exposure to industrial chemicals, quarries and printing plants in China (Tanner *et al.* 1989b). Similarly, extensive pesticide use was associated with increased incidence of PD in California. In fact, the incidence rate of PD between 1984 and 1994 was reportedly higher in California, a state which uses almost a quarter of all pesticides in the US (Ritz and Yu 2000).

1.6 Models to Study Sporadic/Idiopathic Parkinson's disease

Much of the information regarding the etiology and pathogenesis of PD is obtained from clinical studies, post-mortem studies, and epidemiological studies. Accessibility to a human brain is limited because of ethical approval, availability of postmortem brain tissue, and difficulty in drawing definitive conclusions due to individual variability and differences in tissue quality (Emborg 2004). Animal models are therefore an important

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tool to study the pathogenesis and strategies for therapeutic intervention of PD. Parkinson's disease does not spontaneously develop in animals; therefore neurotoxic agents have to be used to induce the characteristic functional changes associated with PD. Ideally, animal models of PD should show all the clinical characteristics and pathological features observed in people. Some of the prominent toxin-induced PD models are discussed below.

1.6.1 MPTP Model

A major breakthrough in PD research came in the 1980's when a model to study sporadic/idiopathic PD was accidentally discovered. In 1983 Langston and his colleagues reported parkinsonian syndrome in young addicts that was clinically indistinguishable from PD after injecting themselves with a synthetic narcotic. The toxic compound was identified as 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) by chemical analysis. Post mortem analysis revealed selective loss of neurons in the substantia nigra and Lewy-body-like inclusions. Intravenous use of MPTP caused all the key signs of Parkinsonism. The striking similarities between sporadic PD and MPTP-induced Parkinsonism suggested that compounds similar to MPTP in structure or biological activity might be the primary cause of sporadic PD (Smeyne and Jackson-Lewis 2005).

MPTP by itself is not toxic. It is a lipophilic compound that easily crosses the blood brain barrier upon administration. MPTP is converted to its toxic metabolite 1-methyl-4-phenyl pyridinium ion (MPP^+) by monoamine oxidase B especially in non–

dopaminergic cells such as astrocytes (Di Monte 2003, Przedborski and Vila 2003, Schober 2004). Figure 1.5 illustrates the metabolism of MPTP.
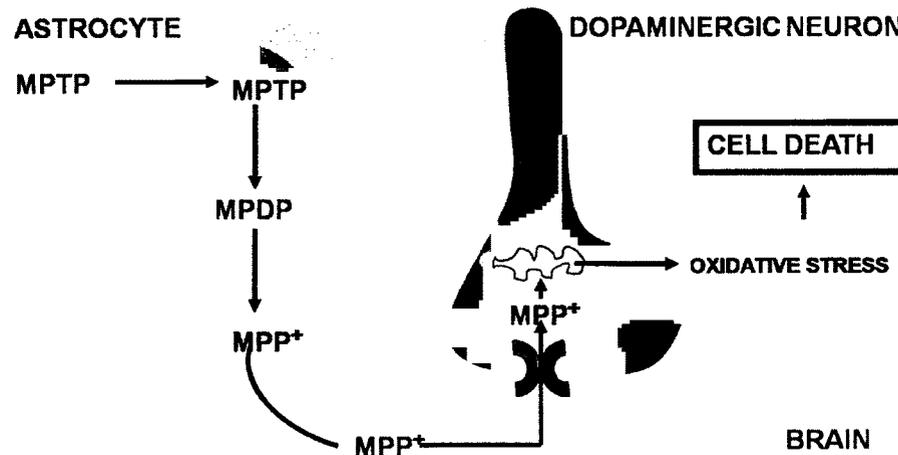
Figure 1.5 Metabolism of MPTP in glial cells. In the presence of monoamine oxidase, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) is converted to 1-methyl-4-phenyl pyridinium ion (MPP$^+$) which is the toxic to the dopaminergic neurons.

MPP$^+$ is a substrate for the vesicular monoamine transporter (VMAT), which translocates MPP$^+$ into synaptosomal vesicles, and also for the dopamine transporter (DAT) present on dopaminergic neurons and for norepinephrine-serotonin transporters. (Javitch *et al.* 1985, Daniels and Reinhard 1988, Przedborski and Vila 2003). Upon entering a dopaminergic neuron, MPP$^+$ can be concentrated by an active process within the mitochondria where it impairs electron flow through the electron transport chain by inhibiting complex-I.

MPTP induces the symptoms of PD in rodents, cats, non-human primates and mini-pigs (Terzioglu and Galter 2008). Neuropathological studies in humans and monkeys indicate that MPTP induces damage to the dopaminergic neurons in the nigrostriatal pathway identical to that seen in PD (Bove *et al.* 2005). MPTP is usually administered systemically (subcutaneous, intraperitonial, intravenous or intramuscular). In non-human primates, unilateral intracarotid infusion of MPTP is often used to administer the drug. Susceptibility to this compound varies in different species. A multitude of regimens and doses of MPTP (acute, chronic) are used to study PD (Betarbet *et al.* 2002). Acute MPTP administration induces specific degeneration of dopaminergic neurons in the substantia nigra (between 50 and 93% loss) while chronic administration of MPTP induces about 50-60% loss of dopaminergic neurons (Betarbet *et al.* 2002). Although numerous studies with MPTP have been conducted in monkeys, molecular mechanisms underlying MPTP-induced toxicity are largely studied in rodents such as mice (systemic injections) and, albeit to a lesser extent, in rats (intracerebral injections) (Bove *et al.* 2005).

Neurochemical changes that accompany MPTP exposure include decreased levels of dopamine and its metabolites in the striatum as well as and increased oxidative damage (Betarbet *et al.* 2002). Systemic MPTP administration in mice induces PD-like symptoms, including bradykinesia, rigidity and posture anomalies. These symptoms parallel the low dopamine neuron counts that also result due to MPTP exposure (Sedelis *et al.* 2001). Studies have also investigated the role of MPTP on motor behaviour including locomotion, circling, rearing and/or stereotyped behaviour (Sedelis *et al.* 2001).

1.6.2 Pesticide/ Herbicide Induced PD Models

Several epidemiological studies have revealed a link between environmental factors such as farming and exposure to chemicals used in agriculture, and increased incidence of PD (Tanner *et al.* 1989b). A number of environmental stimuli are associated with the disease including herbicides, pesticides, cyanide, carbon monoxide and heavy metals (Uversky 2004). Over the years there has been an increased focus on the association between pesticide exposure and incidence of PD. Studies have shown a strong correlation between long term pesticide exposure and increased risk of PD (Brown *et al.* 2006). It has been hypothesized that many chemicals used in agriculture are capable of selectively targeting dopaminergic neurons, thereby accelerating the development of PD. In order to better understand the role of environmental toxins in PD many toxin-based models have been developed.

1.6.2.1 Paraquat Model of PD

Paraquat (PQ), or 1, 1'-Dimethyl-4, 4'-bipyridinium, is a quaternary nitrogen herbicide commonly used to kill broad leaf weeds. Although banned in the United States and countries of the European Union, it is still used in many developing countries. For many years, experimental studies using PQ focused on its deleterious effects on the lungs, liver and kidneys because acute exposure can induce toxicity and even death (Bove *et al.* 2005). Epidemiological studies in Taiwan, where PQ is commonly sprayed on rice fields, showed that people exposed to PQ for at least 20 years had almost a 600% higher chance of developing PD (Liou *et al.* 1997). These studies raised the possibility that PQ could be

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an environmental Parkinsonian toxin. Also interesting to note is that the structure of PQ is strikingly similar to that of MPP^+ (Figure 1.6).

PARAQUAT
1,1-DIMETHYL-4,4'-BIPYRIDINIUM

MPP+
1-METHYL-4-PHENYLPYRIDINIUM

MANEB
MANGANESE ETHYLENEBIS(DITHIOCARBAMATE)
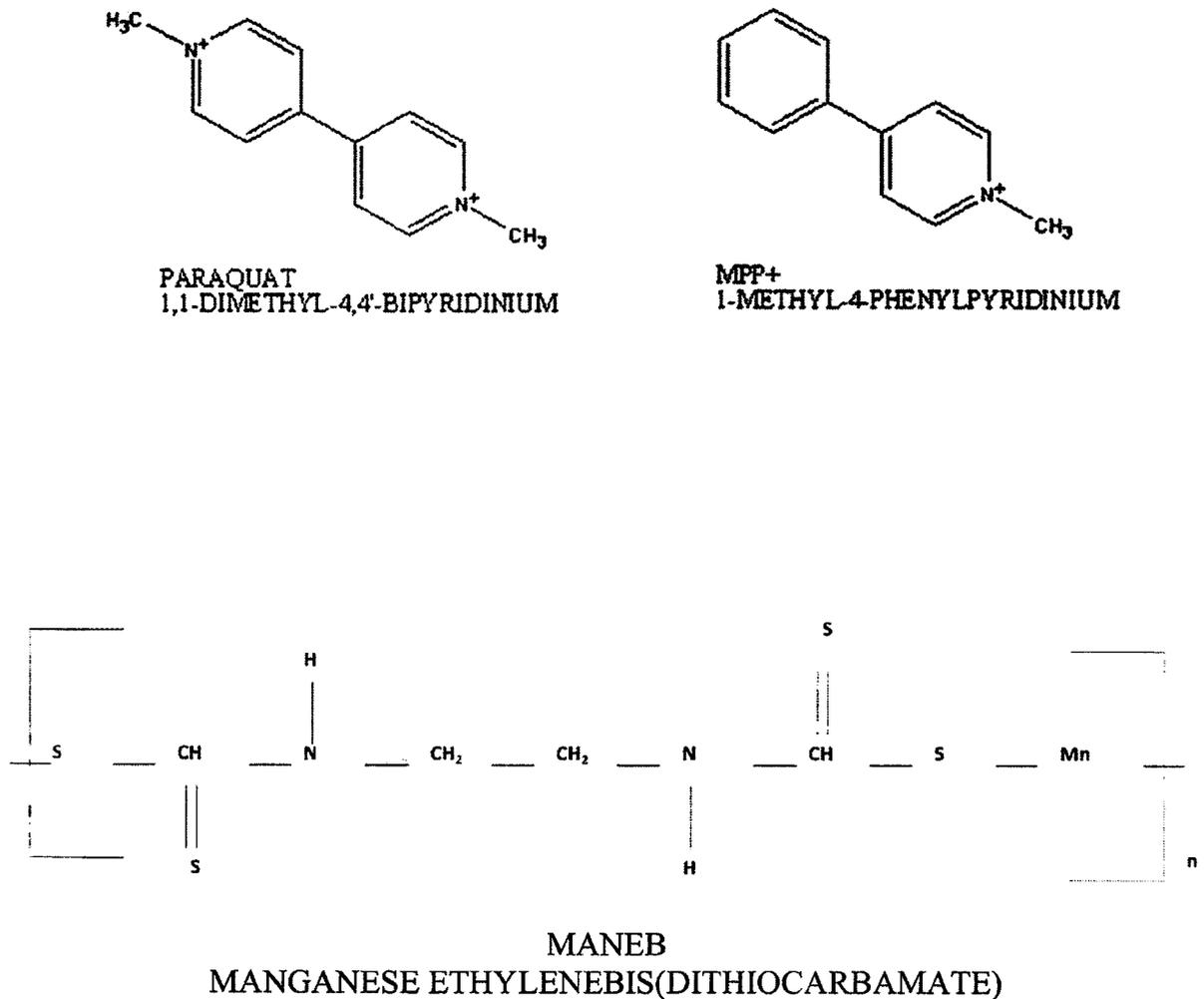
Figure 1.6 The chemical structures of PQ, MPP^+ and MB.

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Paraquat is a hydrophilic, charged molecule and therefore does not diffuse across cell membranes, including the blood brain barrier (Dinis-Oliveira *et al.* 2006). Studies have shown that PQ enters the central nervous system through neutral amino acid transporters located in the blood brain barrier (McCormack and DiMonte 2003). Co-administration studies using PQ and competitive inhibitors of neutral amino acid transporters (e.g., L-valine, L-phenylalanine, L-dopa), revealed a prevention in PQ induced neurotoxicity (McCormack and DiMonte 2003).

The cellular toxicity of PQ is mainly due to its' ability to participate in reactions that produce reactive oxygen species (ROS). In the cell, PQ is reduced to form a monocation free radical (PQ^{*+}) by either NADPH-cytochrome P450 reductase (Clejan 1989), cytochrome *c* reductase (Fernandez *et al.* 1995), or complex I of the mitochondrial electron transport chain (Fukushima *et al.* 1993) (Figure 6). The monocation free radical form of PQ then reacts with oxygen to form a super-oxide radical (O_2^{*-}). Once the super-oxide radical is formed, it can react through well known mechanisms to create other reactive oxygen species, all of which are harmful to cells (Dinis-Oliveira *et al.* 2006). Moreover, it is believed that the redox cycling process of PQ depletes intracellular stores of NAD(P)H due to its increased oxidation, thus contributing to the toxicity of this herbicide (Dinis-Oliveira *et al.* 2006). PQ^{*+} can also reduce iron (III) and iron (III) chelates, in turn catalyzing the formation of hydroxyl radicals via the Fenton reaction (Hastings 1995). PQ, like MPTP has been used to induce PD in rodents (mice and rats) as well as in monkeys (Bove *et al.* 2005). Systemic exposure to PQ in mice and rats results in loss of dopaminergic neurons especially in the SN region, and reduction in the density of striatal TH–fibres (von Bohlen und Halbach *et al.* 2004). Intraperitonial injections of

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10 mg/kg PQ in mice have shown to create a selective loss of dopaminergic neurons in the SN region (McCormack *et al.* 2002).
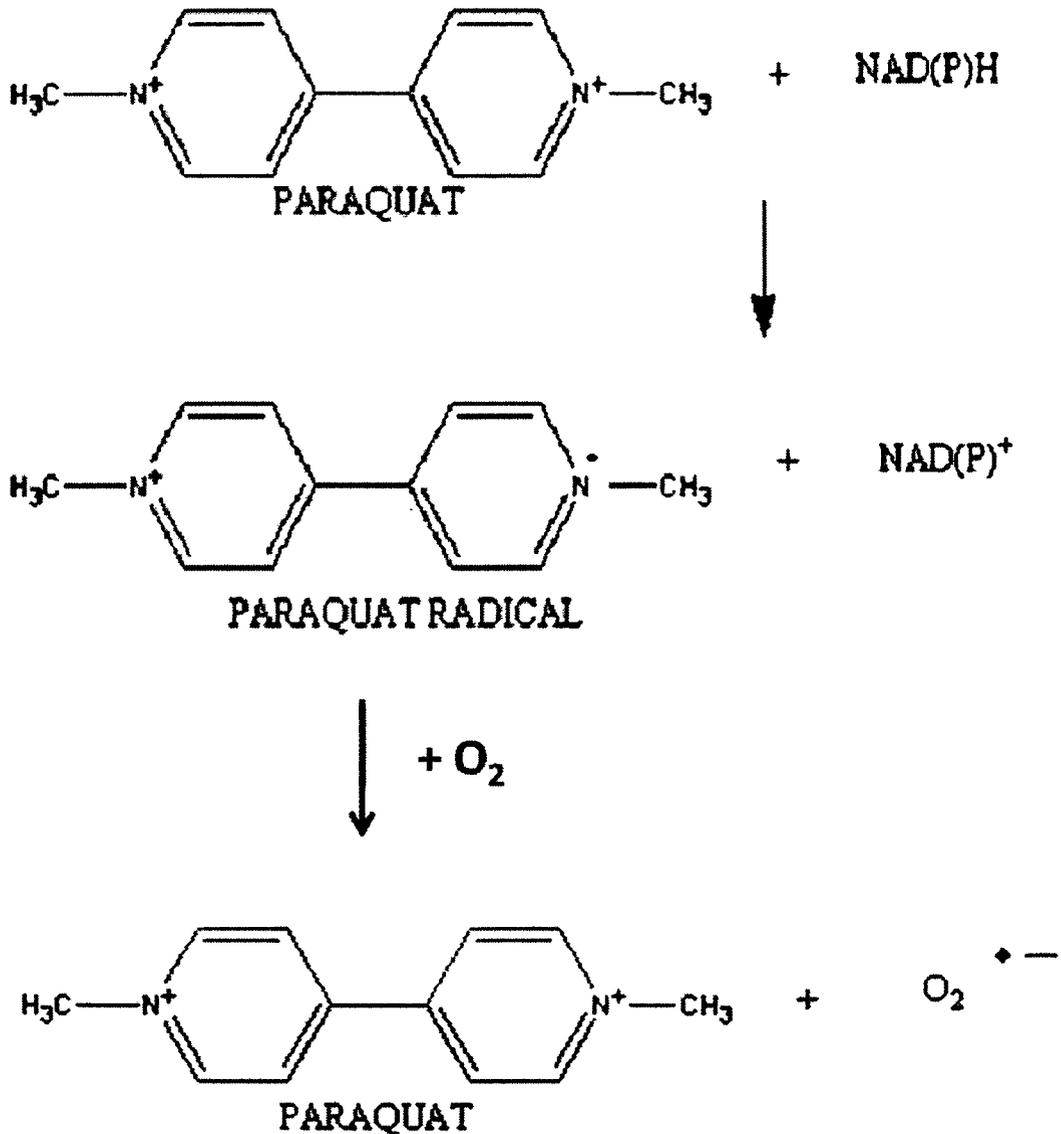
Figure 1.7 Reduction-oxidation cycling of PQ. PQ is reduced to form a monocation free radical (PQ[*+]) by either NADPH-cytochrome P450 reductase cytochrome c reductase. cytochrome c reductase, or complex I of the mitochondrial electron transport chain. The monocation free radical form of PQ then reacts with oxygen to form a super-oxide radical (O$_2$[*-]).

Early studies of PQ-induced PD in animal models were unsuccessful in observing any changes in the nigrostriatal DA pathway following systemic exposure to PQ (Bove *et al.* 2005). However, with the introduction of stereological cell counting following sublethal dosing of PQ over a 3-week period shows a decrease in TH-positive cells in the SNc. In addition, DA neurons in the SN and striatum appear particularly sensitive to PQ, as other subpopulations of neurons were unaffected (Bove *et al.* 2005). Reduced motor activity and dose dependent losses of striatal DA nerve fibres were also reported in mice receiving multiple PQ injections (Brooks *et al.* 1999). While no significant depletion of striatal DA has been observed following PQ injections *in vivo*, evidence for enhanced DA turnover is suggested by increases in TH activity and altered DA metabolite levels (McCormack and DiMonte 2003).

Additional evidence to support the status of PQ as a parkinsonian toxin comes from data indicating upregulation and aggregation of α-synuclein within SNc neurons in treated mice (Manning-Bog *et al.* 2002). Significant amplification in 4-hydroxynonenol positive neurons and nitrotyrosine immunoreactivity in nigral cells of PQ-treated mice provide further evidence for oxidative injury in the SNc (McCormack *et al.* 2005). Although PQ exerts its toxicity via oxidative stress, it also triggers the activation of c-Jun-N-terminal kinase (JNK) and caspase-3 in both *in vitro* and *in vivo* conditions, suggesting that JNK pathways could mediate paraquat-induced neurodegeneration (Peng *et al.* 2004).

1.6.2.2 Paraquat-Maneb Model: The Multi-hit Hypothesis

Most studies identifying environmental toxins that target DA neurons have focused on the use of a single chemical to induce PD. Nonetheless, humans may be exposed to multiple toxins in their environment. The multi-hit hypothesis proposes that the brain is able to resist the effects of an individual chemical that targets DA neurons; however, the defence machinery may be compromised when several toxins target different sites in the DA system, ultimately leading to neuronal damage and cell death (Brown *et al.* 2006). Of the different models using combinations of toxins to study PD, the PQ-Maneb combination has been shown to act synergistically to induce PD-like pathology. Maneb is a commonly used fungicide (for structure, refer to Figure 1.6). Studies using a combination of Maneb (MB) (30 mg/kg) and PQ (10 mg/kg) have shown greater neuronal damage than what is induced by either chemical alone (Cory-Slechta *et al.* 2005). The PQ-MB model has been used to study the effect of exposure to multiple environmental neurotoxins on the risk for PD development (Thiruchelvam *et al.* 2000a).

1.7 Molecular Mechanisms Contributing to Toxin-induced Neurodegeneration in PD

The mechanisms involved in the progressive loss of dopaminergic neurons in PD are of foremost interest in the field of PD. Though the exact mechanism of neurotoxicity is not yet fully known, oxidative stress, mitochondrial dysfunction, excitotoxicity and neuroinflammation are all believed to play an important role in the death of SN neurons. These mechanisms are discussed below.

1.7.1 Oxidative Stress

Mounting evidence supports the theory that oxidative stress may contribute to PD pathogenesis (von Bohlen und Halbach *et al.* 2004). The brain depends mainly on energy produced from the mitochondria, and approximately 95% of the molecular oxygen that is inhaled is metabolized by the mitochondrial electron transport chain. This causes the mitochondria to be exposed to high levels of reactive oxygen species (ROS) and oxidative stress that can damage distinct neuronal populations (Tritschler *et al.* 1994).

High levels of ROS cause oxidative stress, which refers to the undue oxidation of biomolecules leading to cellular damage. ROS include a number of reactive molecules derived from oxygen (Valko *et al.* 2007). Stepwise reduction of molecular oxygen leading to the formation of ROS like superoxide and hydrogen peroxide is shown in figure 1.8.

$$O_2 \xrightarrow{e^-} O_2^{\bullet-} \xrightarrow[2H^+]{e^-} H_2O_2 \xrightarrow{e^-} {}^{\bullet}OH + OH^- \xrightarrow[2H^+]{e^-} 2H_2O$$
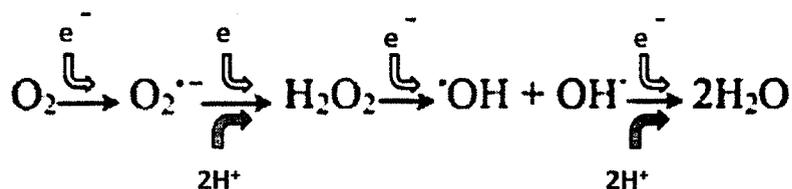
Figure 1.8 Stepwise reduction of molecular oxygen.

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Production of the superoxide anion radical occurs mostly in the mitochondria and it is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 is not a free radical but can penetrate cell membranes making it very toxic to the cell. In the presence of iron, the Fenton reaction converts H_2O_2 to the OH* radical. This radical is likely capable of causing more damage than any other ROS (Betteridge 2000). These oxygen radicals can induce mitochondrial dysfunction, accelerating the production of more reactive oxygen species, thereby escalating and perpetuating the vicious cycle (von Bohlen und Halbach *et al.* 2004). Additionally, superoxide radical can react with nitric oxide (NO) to form peroxynitrite anion (ONOO⁻), which is highly reactive and can be neurotoxic (von Bohlen und Halbach *et al.* 2004).

ROS can react with proteins, DNA and lipids. *In vitro* studies revealed that ROS can directly react with several amino acid residues leading to less active enzymes and malfunctioning proteins. ROS can cause modifications in DNA leading to mutagenesis. ROS have also been shown to target mitochondrial DNA. In many cases apoptosis, a form of programmed cell death is mediated by ROS (Valko *et al.* 2007).

1.7.1.1 Oxidative Stress in PD

The contribution of oxidative stress in PD nigral cell death has been demonstrated by post-mortem studies in PD patients as well as by toxin-induced PD models (Jenner 2003; Dickson 2007). Even under normal conditions, the substantia nigra (SN) region is subjected to oxidative stress due to production of ROS during dopamine metabolism, high levels of essential cofactors iron and copper for catecholamine metabolism, and/or high levels of oxidized GSH (Spina and Cohen 1988, Dickson 2007). Dopamine

autooxidation leads to production of H_2O_2, which is converted to the extremely toxic hydroxyl radical (Youdim et al. 1989) (Figure 1.9).
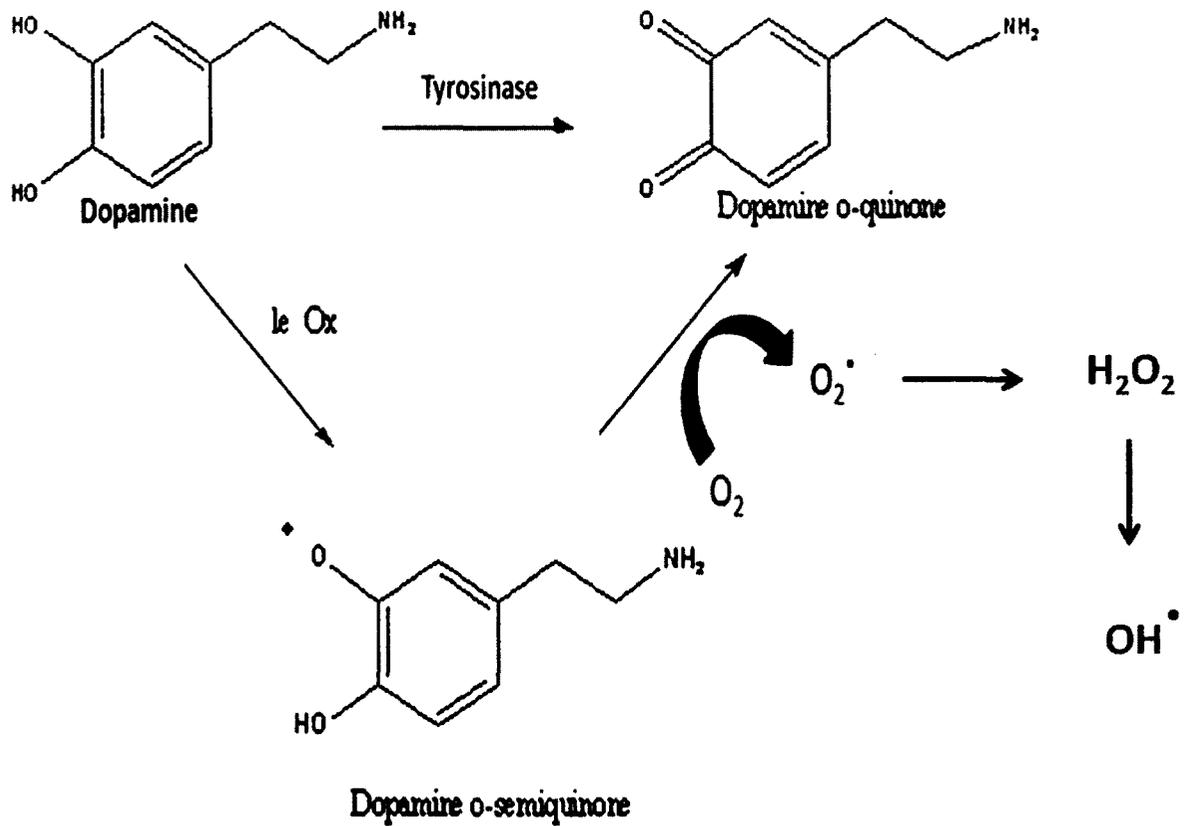
Figure 1.9. Dopamine autooxidation. Autooxidation of dopamine leads to the production of superoxide anion radicals, which can then be converted to hydroxyl radical.

Post mortem studies of PD patients have shown higher than normal iron levels in the SN region (Andersen 2004).

Although ROS levels cannot be measured directly, the assessment of their reaction products and the concomitant damage in post mortem tissues serve as indirect evidence of increased or decreased levels. The evidence for increased oxidative damage include increased levels of lipid peroxidation, DNA damage and protein oxidation observed in the SN region in PD patients (Sanchez-Ramos 1994, Yoritaka *et al.* 1996, Alam *et al.* 1997a, Alam *et al.* 1997b, Floor 1998, Zhang *et al.* 1999). In the brain, GSH is known to play a vital role in detoxification of ROS (Dringen 2000). A decrease in the levels of reduced GSH by about 50% in PD substantia nigra is also evidence of increased oxidative stress (Perry 1982, Perry 1986, Riederer 1989, Beal 2003a). Lower glutathione content appears to be the earliest marker for oxidative stress during the progression of PD (Nakamura 1997).

Interestingly, nitric oxide (NO) can cause nitrosative damage by displacing iron from ferritin, leading to degeneration of the cells in PD (Beckman *et al.* 1990, Reif and Simmons 1990, Shergill *et al.* 1996). NO inhibits glutathione reductase and also causes DNA single–strand breaks and increases DNA oxidation (Sanchez-Ramos 1994, Alam *et al.* 1997a, Barker *et al.* 1996). It is known that parkin has ubiquitin-3 ligase like activity and S-nitrosylation of parkin may impair its ability to ubiquitinate proteins (Schapira 2008). NO also inhibits the mitochondrial respiratory chain and enhances damage caused to the mitochondria by toxins such as MPP^+ (von Bohlen und Halbach *et al.* 2004).

It is yet to be determined which of the two species, ROS or reactive nitrogen species (RNS), is the main culprit in the development of PD. Furthermore, oxidative stress is linked to other cellular processes such as cell death, inflammation, excitotoxicity

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and mitochondrial dysfunction, so it is not easy to establish whether oxidative stress is the primary initiating agent or a product of these events (Jenner 2003, Andersen 2004).

1.7.2 Mitochondrial Dysfunction in PD

Mitochondria are the primary site for energy production, which is regulated by five respiratory complexes. There is mounting evidence suggesting the involvement of mitochondrial dysfunction and impairment of complex I in the onset of PD (Koller and Cersosimo2004). Many environmental toxins including rotenone, MPP^+ and PQ are known to inhibit complex I of the mitochondrial electron transport chain (Fukae *et al.* 2007). Studies involving toxin-induced PD in animal models have revealed a loss in the activity of complex I by MTPT and rotenone. Post mortem studies of PD patients indicate a 30-40% loss of activity of complex I in the SN region (Bove *et al.* 2005).

Reactive oxygen species (ROS) such as the superoxide anion radical can reversibly and irreversibly inhibit complexes I and IV, and although the mechanism of action of reactive nitrogen species (RNS) remains vague it is hypothesized that NO may be involved in the S-nitrosylation of thiol groups in these complexes (Barker *et al.* 1996)

Mitochondria are responsible for generating ATP through oxidative phosphorylation and mitochondrial dysfunction caused by oxidative stress can lead to a decrease in ATP production, which may contribute to cell degeneration in PD (Andersen 2004). Indeed, *in vitro* studies in our lab indicate that oxidative stress causes a decline in mitochondrial ATP production in neuroblastoma cells (McCarthy *et al.* 2004). Reduced ATP levels causes inhibition of PA700, an activator of the 26S proteasome, resulting in decreased ubiquitination (Jenner 2003). Additionally, mitochondrial dysfunction leads to

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the failure of ATP-dependent magnesium inhibition of N-methyl-d-aspartate (NMDA) receptors thereby escalating vulnerability to excitotoxicity (Koller and Cersosimo2004).

1.7.3 Excitotoxicity in PD

There is no direct evidence to implicate excitotoxicity in PD; however, it has been suggested that glutamate may have a toxic effect on DA neurons in the SN region since this region receives glutaminergic projections from the subthalamic nucleus (STN) (Groenewegen 2003, Henchcliffe and Beal 2007). Under physiological conditions in striatum there exists a neurotransmitter balance between the activation of striatal neurons through N-methyl-D-aspartate (NMDA) receptors and inhibition by the D2 dopamine receptors. In PD patients, depleted nigrostriatal dopamine results in disinhibition of the neurons in the striatum and therefore leads to glutamatergic overactivity (Lang and Obeso2004a). Due to increased glutamate binding onto the NMDA receptors, there are changes in cell permeability to calcium. The pathological activation of NMDA receptors causes massive Ca^{2+} entry into neurons, where Ca^{2+} accumulates in the mitochondria. Matrix Ca^{2+} accumulation is proposed to produce high levels of superoxide and other reactive oxygen species that damage the mitochondria and thus the cell, ultimately causing neuronal cell death. The two mechanisms involved in calcium dependent NMDA neurotoxicity are: excessive nitric oxide (NO) formation and mitochondrial dysfunction (von Bohlen und Halbach *et al.* 2004).

Increased activity of glutamate in the STN has been reported in animal models and post-mortem studies (Symeyne and Jackson-Lewis 2005). MPTP induced animal

models of PD have shown altered glutamate activity, while co-injection of MPTP with glutamate antagonists offered protection to DA neurons from the deleterious effects of glutamate (Symeyne and Jackson-Lewis 2005).

1.7.4 Neuroinflammation in PD

The role of inflammation in neurodegenerative diseases such as PD is not well understood and the events triggering an inflammatory response in the brain are unclear. Glial cells are the most abundant cells in the brain and make up 90 percent of the cells in the brain. These cells do not carry nerve impulses. However, they perform many important functions, including providing physical and nutritional support for neurons. Different types of glial cells exist, which include Microglia, Oligodendroglia, Astroglia, Schwann's Cells, and Satellite Cells (Mena and De Yebenes 2008).

Astrocytes are the most abundant glial cells, and are considered to be critical for neuronal support, nutrition, eliminating toxic compounds, and production of neurotrophic substances. Astrocytes are of interest to PD researchers because PD patients have a lower number of astrocytes in the substantia nigra (SN) than in other brain areas (Mena and De Yebenes 2008). It is hypothesized that DA neurons of the SN region have increased susceptibility to toxic insults because they are less protected than neurons of other regions. DA neurons spontaneously produce free radicals during the metabolism of dopamine, and the astrocyte guardian cells that offer protection from oxidative stress have high free radical scavenging properties (Mena and De Yebenes 2008). Indeed, there is evidence to suggest that astrocytes support the differentiation, survival, pharmacological properties, and resistance to injury of DA neurons. Dysfunctional

astrocytes enhance neuronal degeneration by a diminished secretion of trophic factors. Glial cells can also instigate damage of nigrostriatal DA neurons via the release of cytokines and amplified production of reactive oxygen molecules (Mena and De Yebenes 2008).

Over the last decade the concept of "neuroinflammation" has become more accepted in the field of neurodegenerative diseases, and particularly in PD (Mena and De Yebenes 2008). There is an abundance of reactive astrocytes and reactive microglia in the SN of PD patients, indicating a robust inflammatory state (Mena and De Yebenes 2008). Glial cells are normally neuroprotective but given adverse stimulation, they may possibly contribute to chronic inflammation. Microglia, the phagocytes of brain, might be the main contributors of inflammation as they can produce large amounts of superoxide anion radicals and other neurotoxins (Mena and De Yebenes, 2008). Oligodendrocytes do not seem to play a part in promoting inflammation but may be sensitive to damage by inflammatory processes (Mena and De Yebenes 2008).

In PD, degeneration of dopaminergic neurons is coupled with a drastic increase in microglial activity, which could be a consequence of neuronal cell death or due to participation of microglia in the cell death process (McGeer and McGeer 1999). Altered glial function contributes to the demise of DA neurons as observed upon co-incubation of these neurons with toxins such as MPTP and 6-hydroxydopamine (6-OHDA) (McNaught and Jenner 1999). The detection of elevated levels of pro-inflammatory cytokines and increased oxidative stress induced damage in post mortem PD patient samples suggests that microglial activation plays a significant role in the degenerative process leading to PD (Teismann and Schulz 2004).

McGeer *et al.* reported that the response of glial cells is more robust in the SN than the striatum, albeit damage to DA neurons is more severe in the striatum (1999). Microglia become activated in the brain, proliferate and start expressing noxious molecules such as ROS, RNS, cytokines and pro-inflammatory prostaglandins in response to various insults (Kreutzberg 1996). Amplified levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) have been observed in the cerebrospinal fluid and striatum of PD patients (Mogi 1994). These cytokines are potent activators of inducible nitric oxide synthase (iNOS) in rodents (Teismann and Schulz 2004). Cytokines released from glial cells bind to their specific receptors found on DA neurons. Upon binding to their corresponding receptors, these cytokines can trigger apoptosis by activating caspase-3 via the extrinsic pathway (Hartmann *et al.* 2000)

All of these cellular events play an important role in the progression of PD. There is a complex inter-relationship that exists, making it is very difficult to elucidate the initiating event.

1.8 Advances in Therapeutic Approaches for PD

There are several options available for the potential treatment of PD. Current therapies are symptomatic and focus on treating the symptoms of PD. These include dopamine replacement strategies, non-dopaminergic pharmacological therapies, surgical approaches (directed at improving motor function, or at replacing striatal dopamine/dopaminergic neurons), and neuroprotective therapies. Undoubtedly, these therapies have significantly improved the quality of life and survival of PD patients;

however, with disease progression clinical features emerge that cannot be controlled with available medical or surgical therapies (Olanow 2008). The development of a neuroprotective therapy that slows, impedes, or ideally reverses neurodegeneration occurring in Parkinson's disease is a most important and fruitful area of research but also the area most abounding in failures (Lang and Obeso2004b).

Surgery is an option held in reserve for complex cases of PD in which motor complications or medical intolerance has led to a drastic decline in quality of life (Savitt 2006). Surgery for Parkinson's disease may involve destruction of certain parts of the brain (thalamus, the globus pallidus and the subthalamic nucleus) or insertion of electrodes into these areas for electrical stimulation. Cell transplantation to replace striatal DA neurons is under investigation even though there are many technical and methodological issues (Lang and Obeso2004b). Many studies in rodents indicate that embryonic mesencephalic cell grafts can survive, reverse dopamine deficit and even compensate for the behavioural disturbances arising from depletion of dopamine. Clinical trials using embryonic mesencephalic cell grafts on PD patients have increased survival and can secrete dopamine in response to pharmacological stimuli (Lang and Obeso2004b).

Pharmacological replacement of dopamine with dopamine agonists, such as Levodopa (L-dopa), is the most effective symptomatic treatment for PD (Olanow 2008). Although L-dopa is extremely effective; there are two main concerns with its long-term use. The first relates to the toxicity of L-dopa to dopamine neurons; L-Dopa can generate harmful reactive oxygen species (ROS) by oxidative metabolism of dopamine or via autooxidation, which may hasten the rate of degeneration in PD. The second concern is

the high frequency of motor complications associated with chronic L-dopa treatment (Olanow 2008).

There is ample evidence for the role of inflammation in PD. Numerous case-control studies suggest that people who use non-steroidal anti-inflammatory agents have a lower risk of developing PD. Many neuroprotective agents such as monoamine oxidase (MAO-B), inhibitors such as rasagiline, anti-excitatory drugs such as glutamate antagonists, and trophic factors such as GDNF have been studied for their potential as possible therapeutics (Olanow 2008). Bioenergetic compounds such as creatine, riboflavin, nicotinamide and Coenzyme Q_{10} (CoQ_{10}) are potential candidates as neuroprotective agents for PD (Beal, 2003b).

1.8.1 CoQ_{10} as a Neuroprotective Agent

Coenzyme Q_{10} (CoQ_{10}) is a hydrophobic, naturally occurring compound that transfers electrons from complex-I and complex-II to complex-III of the mitochondrial electron transport chain. It is a very important antioxidant in the mitochondria as it readily scavenges free radicals (Beal 2003b). The redox cycling of CoQ_{10} is shown in figure 1.10. CoQ_{10} also called ubiquinone can accept one electron and form a semiubiquinone radical, which can then accept one more electron to get reduced to ubiquinol.

Neuroprotective effects of CoQ_{10} in the central nervous system have been demonstrated *in vivo* by the prevention of reduction in the GSH and ATP levels, as well as protection during experimental ischemia (Beal 2003b). An open-label phase trial of CoQ_{10} in PD patients found good CoQ_{10} absorption and tolerability. Although

encouraging results were obtained from this study, Beal and his colleagues have cautioned that the data is preliminary and would only recommend that PD patients take a high dosage of CoQ_{10} once a larger trial has been completed (Beal 2003b).
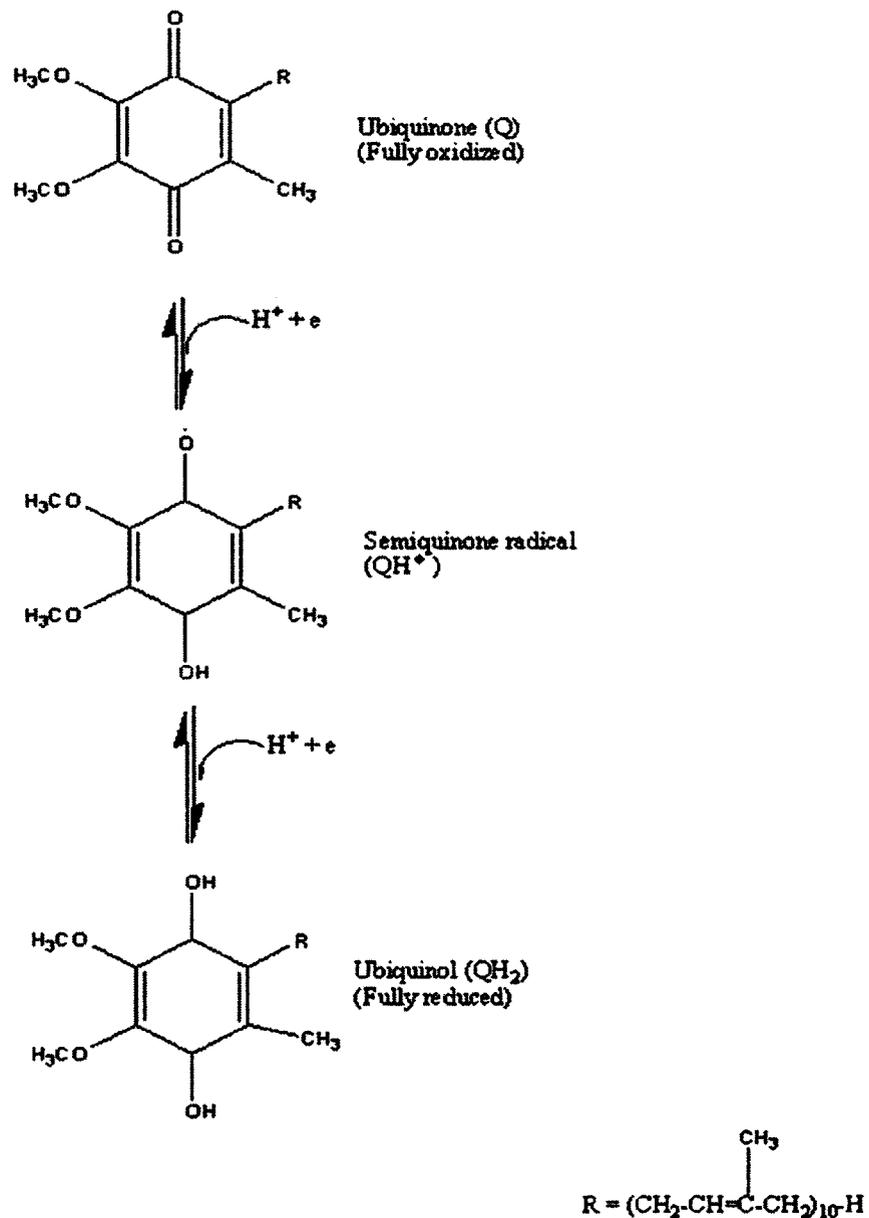
Figure 1.10 The oxidized and reduced forms of CoQ_{10}. The oxidized form of CoQ_{10} (ubiquinone) can accept one electron to form the semiubiquinone radical, which can accept one more electron to form the reduced form of CoQ_{10} (ubiquinol).

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A major drawback in working with CoQ_{10} is that it is very lipophilic and so studying its protective properties in cell culture is difficult due to its hydrophobic nature; however, through a patented protocol (US patent #6 045 826) a water-soluble formulation of CoQ_{10} (WS-CoQ_{10}) was developed by Sikorska and Borowy-Borowsky of the National Research Council of Canada. Vitamin-E and polyethylene glycol have been used as carriers to solubilize CoQ_{10} and help in the uptake of this compound by the cells. Studies aimed at evaluating the bioavailability of this formulation established that cells were capable of internalizing WS-CoQ_{10} when it was added to the media as an aqueous solution (Borowy-Borowski et al. 2004). A 3-day exposure to 10 μg/ml of WS-CoQ_{10}, caused an increase in cellular mitochondrial membranes and cell membranes. The treatment also caused an increase in total cellular levels of ATP (Sandhu et al. 2003).This formulation has effectively been used to prevent oxidative stress induced cell death (McCarthy et al. 2004, Somayajulu et al. 2005). Protection by WS-CoQ_{10} of differentiated neuronal cells against cell death induced by glutamate excitotoxicity was also reported (Sandhu et al. 2003). Recently, WS-CoQ_{10} was shown to inhibit Bax activity and thus prevent Bax-induced destabilization of mitochondria in mammalian cells (Naderi et al. 2006). I have shown WS-CoQ_{10} to be protective against PQ-induced toxicity in vitro; therefore evaluatation of the efficacy of WS-CoQ_{10} in vivo was the next step. This project is focussed on evaluating the efficacy of this unique formulation of CoQ_{10} in preventing dopaminergic (DA) cell death occurring in PD.

Studies in my lab have shown that PQ induces neuronal cell death (McCarthy et al. 2004) and pre-treatment of these cells with WS-CoQ_{10} can prevent PQ mediated cell death in vitro. I have tested the efficacy of this compound in two different cell lines and

using different insults. The results from the *in vitro* work were very encouraging and therefore roused interest to test the efficacy of this compound *in vivo*.

As mentioned earlier, occurance of sporadic PD is correlated with exposure to toxins. Toxin induced PD model could be a good model to study the efficacy of the water soluble formulation of CoQ_{10}. As described before, many mice models of PQ induced PD are used, but there is a lack of behavioural outcomes in these models. I wanted to establish a model to assess the deficits in motoer and balancing behaviour. This project is a collaborative work between the department of Biochemistry and Pschology at the University of Windsor, Ontario. Dr. Cohen, our collaborator has a well established pschology lab where he uses rats to study different behaviours. Since there exists a functional lab to assess behavioural outcomes in rats, they were the ideal choice for a sporadic PD model for this study.

Previous work has shown that PQ causes oxidative stress induced cell death of DA neurons and WS-CoQ_{10} helps prevent this loss of neurons. PQ induces selective loss of dopaminergic iof the neurons in the SNc. Toxin-induced sporadic models of PD have been developed, where exposure to toxins such as herbicides can lead to the loss of dopaminergic neurons in the SNc. PQ-induced PD models have been well characterised in mice (McCormack *et al.* 2002) in which the neurodegenerative changes induced by PQ are dose-dependent and differentially affect specific cell populations (McCormack *et al.* 2002, Manning-Bog *et al.* 2003). Our working hypothesis was based on the fact that PQ induces death of DA via oxidative stress and mitochondrial dysfunction. WS-CoQ_{10} can prevent mitochondrial dysfunction as observed in *in vitro* studies previously. If animlas are fed with this formulation, it can provide resistance aginst PQ mediatd toxicity. Since a behavioural component is used to assess motor damage, PQ mediated DA degeneration

would lead to motor and balance deficits. If WS-CoQ$_{10}$ is neuroprotective, it would result in redox balance favouring low oxidative stress, decrease the number of DA neurons undergoing cell death, and ultimately reduce the motor and balance deficits induced by PQ.

1.9   Objectives

The objectives of this research are to:

1. Develop a paraquat-induced sporadic Parkinson's disease model in Long Evan's hooded rats.

2. Assess the neuroprotective effects of water soluble Coenzyme Q$_{10}$ (WS-CoQ$_{10}$) by evaluating behavioural, biochemical and histochemical parameters and to evaluate the mechanism of neuroprotection offered WS-CoQ$_{10}$.

Chapter 2

Materials and Methods

2.1 Animals and General Supplies

Long-Evans hooded rats were purchased from Charles River Breeding Farms in St. Constant, Quebec. Superfrost slides were purchased initially from VWR (catalogue number 48311-703) and recently from Fisher (catalogue number 12-550-18, 12-550-16A); tissue embedding rings (catalogue number 25602-766) and paraffin (catalogue number 72050-030) were purchased from VWR. Square glass Coplin jars with lids that hold 10 slides were a gift from NRC Ottawa. Brain matrix for coronal sectioning for an adult rat weighing between 300-600 g (catalogue number 15054) was purchased from Ted Pella Inc. Disposable microtome blades (catalogue number DMB-LP) were purchased from Triangle Biomedical Sciences. Antibody diluent (catalogue number S0809) and blocking solution (catalogue number X0909) were purchased from DAKO. Ultracruz mounting medium for fluorescence (catalogue number B2808) was purchased from SantaCruz Biotechnology. Cytoseal™ 60 (catalogue number 8310-16) was obtained from Richard-Allan scientific.

2.2 Stains, Kits and Antibodies

Hematoxylin mercury-free acidified solution (catalogue number 245-677) was purchased from Fisher and Eosin Y alcoholic saturated solution (catalogue number 1502-130) was purchased from VWR. Cresyl Violet acetate (catalogue number C1791) and Diamino benzamidine (DAB) (catalogue number D8001-5G) were purchased from Sigma. Luxol fast-blue and FD-Neurosilver kit (PK 301) were purchased from FD

Neurotechnologies. Fluoro-jade B (catalogue number AG310) was bought from Chemicon. Peroxidase substrate kit (catalogue number SK- 4100) was purchased from Vector laboratories.

Primary antibodies: Rabbit anti-Tyrosine Hydroxylase polyclonal antibody (catalogue number P40101-0) was purchased from Pelfreeze, and mouse anti-tyrosine hydroxylase monoclonal antibody (catalogue number T2928) was bought from Sigma. Rabbit anti-dopamine transporter polyclonal antibody (catalogue number AB1591P), rabbit anti-vesicular monoamine transporter 2 c-terminus polyclonal antibody (catalogue number AB1767), rabbit anti-glial fibrillary acidic protein polyclonal antibody (catalogue number AB5804), rabbit anti-synuclein polyclonal antibody (catalogue number AB5038), rabbit anti-parkin polyclonal antibody (catalogue number AB9244) and mouse anti-NeuN clone 60 Alexa Fluor 488-tagged monoclonal antibody (catalogue number MAB377X) were purchased from Chemicon. Rabbit anti-4-hydroxy nonenal polyclonal antibody (catalogue number 393207) was purchased from VWR. Rabbit anti-nitrotyrosine polyclonal antibody (catalogue number A-21285) and Apo-brdU TUNEL assay kit (catalogue number SKU#A23210) were purchased from Molecular Probes.

Secondary antibodies: Polyclonal goat anti-rabbit HRP conjugate (catalogue number P0448) was purchased from DAKO; Anti-Rabbit IgG (whole molecule) FITC antibody produced in sheep (catalogue number F7512) and anti-mouse IgG (whole molecule) peroxidase conjugate (catalogue number A9044) were purchased from Sigma.

2.3 Reagents for Biochemical Assays

For Adenosine 5'-triphosphate (ATP) assay: lyophilized luciferin luciferase mix (catalogue number FLAAM-1VL) was purchased from Sigma and ATP (catalogue

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number 93168322) was purchased from Roche. For reduced glutathione (GSH) assay: GSH (catalogue number G-6529), Glutathione reductase (GSR) (catalogue number G3664-500U) and DTNB (catalogue number D8130) were purchased from Sigma. The proteome profiler rat cytokine array kit (catalogue number ARY008) was purchased from R&D systems. For protein assays: Bovine serum albumin (BSA) (catalogue number A7906) was purchased from Sigma and Bio-Rad protein dye assay reagent concentrate (catalogue number 500-0006) was purchased from BIORAD. For lipid peroxidation assay: tricholoro acetic acid (catalogue number T9159) and 2-thiobarbituric acid (catalogue number T5500) and the MDA standard (1,1,3,3-tetramethoxypropane) (catalogue number 108383) were purchased from Sigma. For SDS-PAGE and western blotting: TEMED and bis-acrylamide were purchased from BIORAD; Tris-HCl, Glycine, Tris-base and SDS were purchased from BD. Nitrocellulose membranes (catalogue number 66485) were purchased from PALL Gelman Labs; low fat milk powder, Nestle Carnation brand, was purchased from Canadian Tire. Chemiluminescent peroxidase substrate (catalogue number CPS-160KT) was purchased from Sigma.

2.4 Water-soluble CoQ$_{10}$ and Placebo

2.4.1 Water-soluble Formulation of Coenzyme Q$_{10}$ (WS-CoQ$_{10}$)

The WS-CoQ$_{10}$ and placebo solution used in this experiment were supplied by NRC Ottawa. The WS-CoQ$_{10}$ was produced through a patented protocol (US patent #6 045 826) and contains both oxidized and reduced forms of CoQ$_{10}$ in a 2: 1 (w/w) ratio. Coenzyme Q$_{10}$ (CoQ$_{10}$) is contained within a water soluble "cage" made of polyethylene

glycol and α-tocopherol (vitamin E). This water-soluble formulation was prepared from CoQ$_{10}$ (Kyowa Hakko, New York, NY) and polyoxyethanyl α-tocopheryl sebacate (PTS) by directly combining both components in a molar ratio of 1: 2 mol/mol (1: 3 w/w) and heating them to a temperature higher than their respective melting points to form a clear melt, which is water-soluble and can be diluted with aqueous solutions (e.g., phosphate buffered saline (PBS), water, saline) to a desired concentration as previously described (Borowy-Borowski *et al.* 2004) , (www.Zymes.com).

2.4.2 PTS or Placebo

PTS/placebo was synthesized by conjugating polyethylene glycol 600 to α-tocopherol via bi-functional sebacic acid (Sigma-Aldrich, St. Louis, MO) as previously described (Borowy-Borowski *et al.* 2004), (www.Zymes.com). Typically, stock solutions of WS-CoQ$_{10}$ (50 mg of CoQ$_{10}$ and 150 mg of PTS per ml in PBS) or a placebo (150 mg of PTS per ml of PBS) were made and stored at 4°C. These samples were then shipped to Dr. Pandey at the University of Windsor. Stock solutions were diluted with regular drinking water to a final concentration of 50 μg WS-CoQ$_{10}$ and 50 μg PTS per ml (placebo) for the experiments.

2.5 Instruments

A Genesys 10UV Spectrophotometer was used for measuring absorbance for Bradford assays and lipid peroxidation assays. Centrifugation was performed using CR3i Jouan centrifuge (fixed angle rotor: 13,000 r.p.m. (12520 xg) and swing bucket rotor:

4,000 r.p.m. (3023 xg)), DESAGA Starstedt-Gruppe centrifuge and Biofuge 15 centrifuge from Baxter Scientific Inc. A SpectraMax GEMINI XPS from Molecular Devices was used for luminescence (ATP assay) and fluorescent assays. The chemidoc from ALPHA INNOTECH Corporation was used for gel imaging. Microscopes from MOTIC AE20 (attached to a Moticam 2300 camera, 3.0 MP Live resolution), Leica DM IRB, Nikon ECLIPSE E800 (Dr. Zielinski's lab), ZIESS Axioskop 2 mot plus and (Dr. Crawford's lab) were used for taking phase and fluorescent pictures. Microtomes from American Optical Company (Dr. Crawford's lab) and MICROM HM 320 were used for sectioning. Sorvall tissue grinder was used for homogenizing tissues. EL_X808 IU Ultra microplate reader from BIO-TEK INSTRUMENTS, Inc. was used for measuring absorbance in 96 well plates. Labconco freeze dry system/freezone 4.5 lypholizer was used for concentrating samples. Other general laboratory equipments used included the following:

A pH meter (Beckman, $\Phi310$) with buffer solutions from ACP, an Adventurer™ balance and Scout ™ Pro balance (OHAUS), Vortex GVLab from Gilson[R] Industries Inc., a 1296-002 DELFIA^R plate shaker from Wallac, a Gibco BRL heatblock from VWR, a Rocking platform model 200 from VWR, a Corning stirrer from Fisher Scientific, a Sorvall OMNI-MIXER, a BIORAD Power Pac 200 and Eppendorf pipettes from Fisher Scientific, VWR, and Gilson.

2.6 Animal Care

Twenty five male Long-Evans hooded rats (60-90 days old) were purchased from Charles River, Quebec. Upon arrival, the rats were randomly split into two groups: those that would receive injections of PQ and those that would receive injections of saline.

These two groups were further divided into groups based on their water regimen: whether they received WS-CoQ$_{10}$ (50 µg/mL), or placebo (50 µg/mL) in their drinking water or plain drinking water. Throughout the experiment, developmental changes were made to the model involving group division depending on water supplementation. The average rate of water consumption for a rat is 10 ml per 100 g of body weight each day. At this rate, an average sized rat (500 g) consumed 5 mg of either WS-CoQ$_{10}$ or placebo per day.

Each one of the groups was housed in separate cages, which can hold between four to five rats. During the weekdays, rats were placed in individual cages, fed 20 – 25 g of rat chow, and given two hours to consume their meal. During the weekends, rats remained in group cages and received group feedings. Plain water or water supplemented with either WS-CoQ$_{10}$ or placebo was available to the rats every day. On this feeding schedule, the rats were kept at 90% of their normal, free-feeding weights verified through weekly weighing. Given that rats are nocturnal, to ensure that they were awake during the day for behavioural testing, the rats were kept on a reversed 12 h: 12 h light: dark cycle with the temperature maintained at 20°C. All animal care, treatments, and procedures were approved by the University of Windsor's Animal Care Committee (AVPP060010706) in accordance with the Canadian Council for Animal Care guidelines. Please refer to Appendix A for further details on injecting animals.

2.7 Injection Regimes

2.7.1 Three Injections of PQ

Two different experimental designs were used for the three injection study. Figure 2.1 describes the time-line and group allocation for both experiments. Previous

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studies have used three intraperitoneal injections of PQ (10 mg/kg) to induce selective damage to dopaminergic neurons in the SN in mice; we used a similar protocol in rats (McCormack *et al.* 2002).

Experiment #1: Sixteen naïve adult male Long-Evans hooded rats were used in the first experiment, designed to establish the regimen of PQ-injections, the degree of neurodegeneration and potential neuroprotection by WS-CoQ$_{10}$. Figure 2.1A outlines the procedures applied in this experiment: three weeks prior to the first saline or PQ injection (and for the duration of the experiment) the rats received either plain water or water supplemented with WS-CoQ$_{10}$ or placebo. The rats were given three weekly intraperitoneal injections of PQ (10 mg/kg body weight dissolved in saline) or saline. The rats were further divided according to the treatments they received: one group received saline injections and plain water; a second group received PQ injections and plain water; a third group received PQ injections and drinking water supplemented with placebo and the fourth group received PQ injections and WSCoQ$_{10}$ supplemented drinking water (Figure 2.1A). Since placebo (PTS) is a pro-drug form of vitamin E (in which tocopherol was chemically derivatised by sebacic acid and PEG) that is used as a component (carrier) of the WS-CoQ$_{10}$ formulation (Sikorska *et al.* 2003), the effect of WS-CoQ$_{10}$ was compared to placebo and water treated groups. Rats were sacrificed one week after the last injection and the brains from these rats were extracted, fixed in formaldehyde. This experiment was repeated with sixteen rats. For this set of rats, biochemical parameters were performed for 2 rats from each group.

Experiment #2: Twenty-five naïve adult male Long-Evans hooded rats were used for this experiment and were exposed to a similar injection schedule as in Experiment 1. The PQ-induced neurodegenerative and WS-CoQ$_{10}$ neuroprotective effects that resulted

from Experiment 1 prompted us to conduct a second experiment (Figure 2.1B) which is modified not only to assay histological/biochemical parameters of PQ-induced neurodegeneration, but also to determine PD-like behavioural changes from such exposure and to determine whether WS-CoQ$_{10}$ treatment could prevent PQ-induced behavioural impairment. Due to the fact that no significant differences in nigral neuronal counts were observed between the PQ-Placebo and PQ-Water treatment groups in Experiment 1, we eliminated plain water treatment from Experiment 2, and gave the rats either WS-CoQ$_{10}$ or its carrier solution (Placebo) in the drinking water. Thirteen rats that received drinking water supplemented with WS-CoQ$_{10}$ and were divided into two injection groups: Saline-CoQ$_{10}$ (n=6) and PQ-CoQ$_{10}$ (n=7). Twelve rats that received placebo supplemented drinking water were divided into two groups based on the injection regime: Saline-Placebo group (contained six rats) and the PQ-Placebo group (contained six rats). Rats were sacrificed two weeks after their last injection as opposed to one week in experiment 1. This additional week allowed us to carry out post-injection behavioural assessment of gait performance on the rotorod apparatus. Three rats from each group were used for staining and three were used for biochemistry experiments.

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A

◄ Regular drinking water or water supplemented with placebo or WS-CoQ₁₀ ►

Paraquat or saline injections

0wk   3wk   4wk   5wk   6wk

Experimental Groups
saline-injected/water (n=4)
PQ-injected/water (n=4)
PQ-injected/placebo (n=4)
PQ-injected/WS-CoQ. (n=4)

Perfusion, sacrifice and
collection of brain tissue

B

◄ Drinking water supplemented with placebo or WS-CoQ₁₀ ►

Paraquat or saline injections

0wk   2wk   6wk   7wk   8wk   9wk

Acclimatization   **Pre-injection phase**   **Injection phase**   **Post-injection phase**

Experimental Groups
saline-injected/placebo (n=6)
saline-injected/WS-CoQ..(n=6)
PQ-injected/placebo (n=6)
PQ-injected/WS-CoQ..(n=7)

Perfusion, sacrifice and
collection of brain tissue

Figure 2.1 Schematic outlines for the three injection PQ regime. (A) Experiment 1 scheme. (B) Experiment 2 scheme.

2.7.2 Five Injections of PQ

Since eight injections proved to be very toxic to rats, we decided to see if five injections of PQ would achieve a greater neuronal loss than the three injection regime. After arrival, rats were randomly grouped and subjected to three weeks of behavioural testing to establish a baseline of their motor abilities (Figure 2.2). Following this, the rats were injected once a week for five weeks giving a total of five injections. After conducting the post injection behavioural studies the rats were dissected and tissues were collected for various experiments. This regime was followed for two sets of experiments: One to study the prophylactic versus therapeutic effects of WS-CoQ$_{10}$ and two, to compare the effects of PQ between old and young rats.
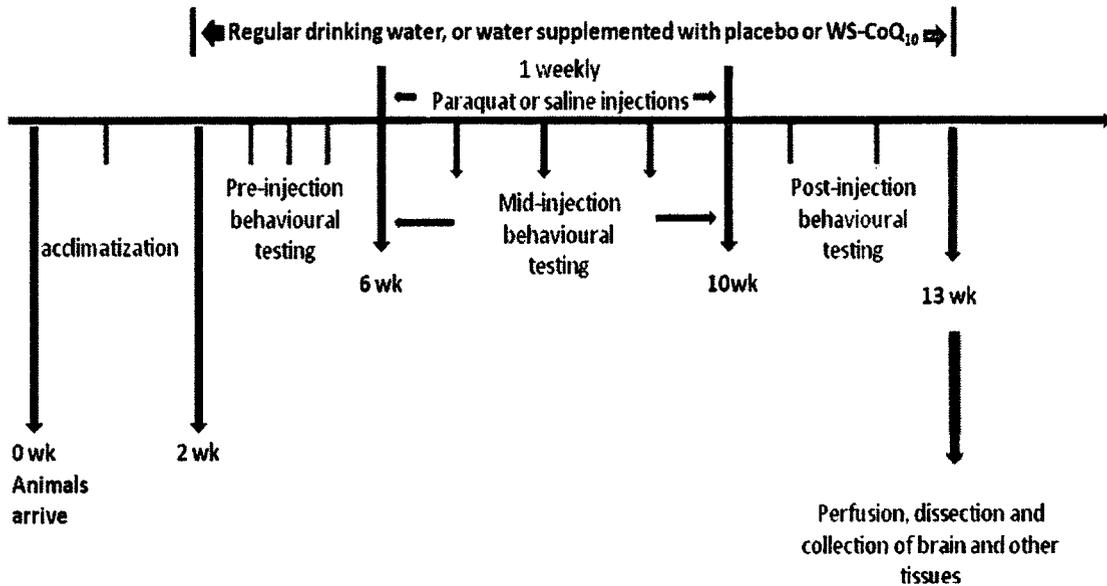
Figure 2.2 Timeline for five injection regime.

2.7.2.1 Prophylactic vs. Therapeutic Effects of WS-CoQ$_{10}$

In order to study the effects of various neuroprotective agents, they are either administered before inducing the disease condition (prophylactic use) or after inducing the disease condition (therapeutic). To design an experiment to study the prophylactic and therapeutic effects of WS-CoQ$_{10}$, 24 naïve rats were divided into six groups. One group received intraperitoneal injections of saline. The other five groups received intraperitoneal injections of PQ (10 mg/kg). After the fifth injection, the drinking water for two of the five PQ-injected groups was changed in order to investigate the effects of WS-CoQ10 administered after PQ-induced neurodegeneration. One of the two groups received WS-CoQ10 supplemented water, starting immediately after the last injection (the next day), while the other received placebo (Table 1). The drinking solutions were prepared the same for all experiments. The behavioural assessments continued throughout the experiment to obtain post-injection measurements. Three weeks after the last injection, two rats from each group were sacrificed for biochemical analyses and two from each group were processed for histochemistry. This experiment was repeated with the same number of rats, groupings and water supplementation.

Table 1. WS-CoQ$_{10}$ feeding regime prior to and after injections of PQ.

Group Number	Number of Animals	Injection	Water Regimen before and during injections	Water Regimen after injection
1	4	Saline	Water	Unchanged
2	4	PQ	Water	
3	4	PQ	Placebo	
4	4	PQ	WS-CoQ$_{10}$	
5	4	PQ	Water	Placebo
6	5	PQ	Water	CoQ$_{10}$

2.7.2.2 Old vs. Young Rats

This experiment was designed to compare the effects of PQ-induced neurodegeneration in young versus old rats as well as to evaluate the neuroprotective effects of WS-CoQ$_{10}$. Sixteen naïve young rats (purchased between 60-90 days old) and 16 old rats (purchased between 300-350 days old). The young and old rats were divided into four groups on the basis of injection and water regime (Table 2). The behavioural assessments continued in the pre-, mid- and post-injection phases. Two rats from each group were processed for histochemistry while the other two were used for performing biochemical analyses. This study is yet to be repeated.

Table 2. Grouping young and old rats on the basis of injections and water regimen.

Group Number	Number of Animals	Injection	Water Regimen	Age
1	4	Saline	Water	Young
2	4	PQ	Water	Young
3	4	PQ	WS- CoQ$_{10}$	Young
4	4	PQ	Placebo	Young
5	4	PQ	Water	Old
6	4	PQ	WS- CoQ$_{10}$	Old
7	4	Saline	Water	Old
8	4	PQ	Placebo	Old

2.8 Tissue Harvesting for Brain Histochemistry

The rats were placed into a plexi-glass chamber and anaesthetized with isoflurane until the rat was unresponsive to a noxious stimulus (i.e., pinched leg). Following this, an anaesthetic muzzle was placed on the rat and the rat was then transferred onto a dissection tray. An incision was made from the pelvis to the neck and both the pleural and thoracic cavities were opened. To perfuse the rat, a 12 gauge needle was inserted into the left ventricle of the heart. Tyrodes solution (8.76 g NaCl, 0.298 g KCl, 0.222 g CaCl$_2$, 2.603 g HEPES, 1 g dextrose, 1 g NaHCO$_3$, 0.06 g Na$_2$HPO$_4$•4H$_2$O per litre) containing 1 U/ml heparin to prevent blood clotting, was gravity fed through the twelve gauge needle (Cassella *et al.* 1997). Once a noticeable discoloration of the liver was observed, the Tyrodes solution was exchanged for a PBS (8 g NaCl, 0.2 g KCl, 2.68 g Na$_2$HPO$_4$-7H$_2$O and 0.24 g KH$_2$PO$_4$ in 800 ml water, pH=7.4) containing 10% formaldehyde. This perfusion which used 10% buffered formaldehyde was continued until a noticeable rigidity developed in the muscles of the rat. Following this, the lungs, heart, kidney, liver, spleen and brain of the rat were harvested and placed in 10% buffered formaldehyde for 24 hours at 4°C to fix the tissues. Following fixation the tissues were transferred to 70% ethanol and stored at 4°C. Please refer to Appendix B for further details.

2.9 Tissue and Slide Preparation

2.9.1 Brain

After harvesting and fixing the tissues, a 4 mm thick coronal section was cut out of the midbrain using a brain matrix for coronal sectioning for an adult rat. To prepare the brain tissue for slicing on the microtome, it was dehydrated and embedded in paraffin wax as described below.

After removal from 70% ethanol, the brain tissue was moved through dehydrating solutions: 95% ethanol for 60 minutes; 100% ethanol for 60 minutes; Xylene for 30-45 minutes; 50/50 (v/v) mixture of Xylene and paraffin wax for 45 minutes at 60°C; and finally 100% paraffin wax overnight at 60°C in a water bath. Please refer to Appendix D for more details.

Once the tissues were embedded in paraffin, they were placed into metal moulds and left to cool for approximately three hours at room temperature. Coronal sections, 8 µm thick, were cut across the entire SN region and processed for immunohistochemistry using a stereotaxic rat brain atlas. The sections were placed on slides and dried overnight in an incubator at 40°C.

2.9.2 Peripheral Organs

Dehydration and paraffin embedding of the peripheral organs was performed using the protocol as mentioned in Section 2.8.1. Using a microtome, 8 µm sections were taken for immunohistochemical analysis.

2.10 Preparation and Staining for Immunohistochemistry of the Brain Tissues

2.10.1 Using 3,3'-diaminobenzidine (DAB)

Our DAB staining procedure was slightly modified from a previously used protocol. To begin immunohistochemistry, the tissues on slides were subjected to deparaffinization and rehydration. To deparaffinize, these slides were incubated in xylene for 20 minutes (2 x 10 minutes). To rehydrate, the slides were then incubated in 100% ethanol for 10 minutes; 95% ethanol for 10 minutes; 70% ethanol for 5 minutes; immersed in 50% ethanol; immersed in 30% ethanol; immersed in dH_2O; and incubated in 50 mM TBS (50 mM Tris-HCl, 150 mM NaCl, pH=7.6) for 5 minutes. The slides were then incubated for 30 minutes in methanol containing 3% H_2O_2 to block endogenous peroxidase activity of the tissue. The slides were then processed for antigen retrieval by incubation in 1 mM EDTA pH=8.0 heated to 95°C in a water bath for 20 minutes and then cooled for 20 minutes at room temperature. Finally, the slides were washed for 5 minutes in 50 mM TBS, washed for 5 minutes in distilled water and incubated for 15 minutes at room temperature with a universal blocking solution. Excess solution was removed and the slides were subjected to the primary antibody of interest in a humid chamber overnight at 2-8°C. The slides were then washed with TBS for 15 minutes (3 x 5 minutes) and were subjected to the appropriate secondary antibody for 1 hour at room temperature and again washed in TBS for 15 minutes (3 x 5 minutes). Negative controls included the omission of primary or secondary antibodies.

Immunolabelling was accomplished by using 0.02% diaminobenzidine tetrachloride and 0.006% H_2O_2 for 10 minutes at room temperature. Sections were washed in running water and counterstained with Mayer's hematoxylin for 90 seconds.

The slides were washed in TBS for 5 minutes to remove excess stain, and then immersed in a 0.2% HCl in ethanol destaining solution followed by 10 dips in 10% NH_4OH. The tissue was then dehydrated in 95% ethanol for 10 minutes; 100% ethanol for 10 minutes; and xylene for 10 minutes (2 x 5 minutes). The slide was then sealed with cytoseal and a cover slip.

2.10.1.1 Tyrosine Hydroxylase (TH) Staining using DAB

For the three injection regime, neurons were counted using DAB staining. Staining for TH using DAB began with the same steps as outlined in section 2.10.1. After blocking the slide for 15 minutes with blocking solution, the brain sections were incubated with rabbit polyclonal anti-tyrosine hydroxylase antibody (1:500, using DAKO antibody diluent) overnight at 4°C. These slides were washed in TBS for 15 minutes (3 x 5 minutes), and then incubated for 1 hour at room temperature with goat anti-rabbit IgG peroxidase conjugated secondary antibody. The rest of the protocol was followed as outlined in 2.10.1.

Loss of dopaminergic neurons (three injection regime) was determined by counting TH-immunopositive cells (cell body) under bright-field illumination. Sections were taken across the SN (bregma -5.04 mm to bregma -6.24mm) and stained for TH-immunopositive neurons. The region for counting was delineated using a stereotaxic rat brain atlas. After delineation of the SNc using 10X objective, TH-immunopositive neurons were counted manually at different levels. The mean numbers of TH-immunopositive neurons per section were calculated for each level. Analysis of the three injection regime was performed as a blind study where neurons were counted manually by two people.

2.10.1.2 Glial Fibrillary Acidic Protein (GFAP) Staining using DAB

Staining for GFAP was performed by using a DAB-based detection protocol. Brain sections were incubated with rabbit polyclonal anti-GFAP (1:500, DAKO Cytomation Inc.) overnight at 2-8°C. Slides were washed (refer to 2.10.1) and the sections were subjected to secondary antibody conjugated to goat anti-rabbit IgG (1:500) for 60 minutes at room temperature. Immunolabelling was detected using DAB according to the protocol described in 2.10.1. This procedure is used by our collaborators at NRC Ottawa.

2.10.2 Using Immunofluorescence

A modified version of a previously published protocol was used (Thiruchelvam *et al.* 2000b). Briefly, tissue sections were deparaffinized in xylene for 20 minutes; 100% ethanol for 10 minutes; and incubated in TBS as described above. Non-specific IgGs were blocked with DAKO universal blocking solution for 15 minutes at room temperature. Excess liquid was removed and sections were incubated with primary antibody overnight in a humid chamber at 2-8°C and then washed with TBS as described above. Tissues were incubated with the appropriate secondary antibody tagged to fluorescein isothiocyanate (FITC) for 1 hour at room temperature and then washed for 15 minutes in TBST and dehydrated in 95% ethanol for 10 minutes, followed by 100% ethanol for 10 minutes and then xylene incubation for 10 minutes (2 x 5 minutes). The slides were mounted with mounting media containing DAPI and the cover slips were sealed with clear fingernail polish. The slides were stored at -20°C.

2.10.2.1 Tyrosine Hydroxylase (TH) Staining with Fluorescence

For the five injection regime, neurons were counted using immunofluorescence staining. Staining for TH began with the same steps as outlined in 2.10.2. After blocking the slides for 15 minutes with blocking solution, the brain sections were incubated with rabbit polyclonal anti-tyrosine hydroxylase antibody (1:500, using DAKO antibody diluent) overnight at 2-8°C. These slides were then washed in 50 mM TBS for 15 minutes (3 x 5 minutes) and then incubated for 1 hour at room temperature with anti-rabbit FITC conjugated secondary antibody (1:200 using DAKO antibody diluent). Next, the slides were washed for 15 minutes (3 x 5 minutes) in 50 mM TBS, mounted with mounting media containing DAPI and cover slips were sealed with clear fingernail polish. All slides were then stored at -20°C. Images were captured Nikon ECLIPSE E800, ZEISS Axioskop 2 Mot Plus or Leica DM IRB microscopes.

For the analysis of the five injection regime, the SNc neurons were counted manually by two people: one person was familiar to the groups while the other was not. In this pilot study, evaluating the Prophylactic vs. Therapeutic effects of WS-CoQ$_{10}$, neurons were counted from two rats per group while in the five injection regime studying the effects of aging (Old vs. Young), neurons were counted from one rat per group. Every sixth section across the entire SNc was counted (both the left and the right sides) to obtain TH-immunopositive neuronal numbers.

2.10.2.2 Glial Fibrillary Acidic Protein (GFAP) Staining with Fluorescence

Staining for GFAP began with the same steps as outlined in 2.10.2. After blocking the slide for 15 minutes with blocking solution, the brain sections were incubated with

rabbit, monoclonal anti-GFAP antibody (1:500, using DAKO antibody diluent) overnight at 2-8°C. These slides were then washed in 50 mM TBS for 15 minutes (3 X 5 minutes) and then incubated for 1 hour at room temperature with anti-rabbit FITC conjugated secondary antibody (1:200, using DAKO antibody diluent). The slides were then washed for 15 minutes (3 x 5 minutes) in 50 mM TBS and then mounted with mounting media containing DAPI; the sides of the cover slip were sealed with clear fingernail polish. All slides were then stored at -20°C until before and after photography. For counting activated astrocytes, no particular parameters were set for this batch of experiments. However, it is recommended to set parameters that define activated conditions of Astrocytes.

2.10.2.3 NeuN (neuronal nuclei) staining

Staining for NeuN began with the same steps as outlined in 2.10.2. The manufacturer's protocol (Chemicon) was followed for antigen retrieval and antibody dilution. After incubation in 50 mM TBS for 15 minutes the sections were subjected to antigen retrieval using fresh 10mM sodium citrate (pH=6.0) for 20 minutes at 90°C in a water bath. Following this sections were allowed to cool down to room temperature for 30 minutes. Next, the sections were washed with 50 mM TBS for 5 minutes and incubated with DAKO universal blocking solution for 15 minutes in a humid chamber at room temperature. Excess blocking solution was removed and slides were incubated with NeuN antibody (1:100, using antibody diluent) overnight at 2-8°C. The slides were then washed with 50 mM TBS and dehydrated, incubated with xylene and cover slipped using

mounting media containing DAPI and sealed with clear fingernail polish. All slides were then stored in -20°C.

2.10.3 Hematoxylin and Eosin (H&E) Staining for Brain and Peripheral Histopathology

The protocol was modified slightly from the original version obtained from NRC, Ottawa. The same protocol was used for staining the brain and peripheral tissues (lung, liver and kidney). Slides were subjected deparaffinization in xylene for 20 minutes (10 x 2 minutes), rehydration in 100% ethanol for 10 minutes, 95% ethanol for 5 minutes, 70% ethanol for 5 minutes and dH_2O for 5 minutes. The slides were stained with hematoxylin for 3 minutes, and washed in dH_2O for 5 minutes. They were then immersed twice in acid alcohol (0.2 mL HCl in 100 mL 70% ethanol) and rinsed with running water and dH_2O. Slides were then incubated for about 1 minute in eosin, rinsed and washed in 95% ethanol, followed by 100% ethanol for 10 minutes each and finally immersed in xylene for 20 minutes (2 x 10 minutes). The slides were cover slipped using cytoseal mounting media.

2.11 Tissue Fractionation of the Brain

The rats designated for biochemical analysis (after completion of behavioural studies) were sacrificed by CO_2 inhalation in accordance with a protocol approved by the Canadian Council for Animal Care. Briefly, isolated brains were washed with ice-cold PBS and placed on ice until further dissection. For the three injection regimes, the brain was further dissected into the frontal cortex, the midbrain and the striatum. Rats were

sacrificed and experiments were done on the same day. Therefore, fresh midbrain and striatum homogenates were used for the three injection assays. The tissues were weighed and homogenized in 10 volumes (10 ml buffer for 1g tissue) of ice-cold homogenization buffer (50 mM Tris-HCl, 0.15 mM NaCl, 10 mM EDTA, 1% TritonX-100, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, pH=7.5) (Akita *et al.* 2001). The homogenates were centrifuged for 10 minutes at 3,000 r.p.m using a Jouan CR3i centrifuge and a T40 swing bucket rotor at 4°C. The nuclear pellet (and debris) was discarded and the cytoplasmic/cytosolic fraction (supernatant) was used for GSH analysis, lipid peroxidation, cytokine array and Westernblots. Half of this supernatant (obtained after centrifugation at 3000xg) was subjected to centrifugation again at 12,520 xg to separate the mitochondrial fractions that were used for ATP assay.

Unless utilised immediately, the cytoplasmic fractions were frozen at -80°C in 250 μl aliquots. If the assay was performed on frozen lysate, then the lysate was subjected to centrifugation at 3000 r.p.m for 7-10 minutes at 4°C and the pellet was discarded, prior to being assayed.

For the five injection regime, there was a slight modification after the dissection. Instead of utilizing the midbrain and the striatum, only the midbrain was utilized for all assays in this regime. Furthermore, instead of utilizing fresh homogenates, the midbrain tissues were snap frozen in liquid nitrogen and stored at -80°C in this regime. (refer to Appendix C). Later these tissue homogenates were prepared and cytoplasmic fractions were obtained as mentioned above. All the assays: GSH, lipid peroxidation, ATP, Western blots, Cytokine analysis were performed on cytoplasmic fractions. Please refer to the summary Table 4 at the end of this chapter.

2.12 Protein Estimation Using Bradford Assay

The concentration of proteins present in the tissue homogenates was estimated using the protocol from BioRad Laboratories. The protein estimation was performed in duplicates by using 2 μl of each sample, 798 μl of dH_2O and 200 μl of BioRad protein assay reagent to a total volume of 1 ml in plastic cuvettes. The mixtures were then vortexed and allowed to stand for 5 minutes at room temperature. The absorbance was then measured by using a Genesys UV-Visible Spectrophotometer at 595 nm. The standard curve was prepared by using 1 to 14 μl of 1mg/ml Bovine serum albumin (BSA) standard, 799 to 786 μl of dH_2O and 200 μl of BioRad protein assay reagent. Graph Pad Prism software was used for data representation.

2.13 Glutathione (GSH) Assay

The GSH assay was performed using a slight modification of a previously published method (Baker *et al.* 1990). Briefly, 2 μl of the homogenate and 100 μl of reaction mixture (containing 1 mM NADPH and 100 units GSH reductase in distilled water) was added in a 96-well plate and then incubated for 20 minutes at 37°C. Following incubation, 100μl of 1 mM DTNB (dissolved in 0.1 M PO_4 buffer, pH=8.0) was incubated for 15 min at 37°C. Absorbance was measured at 412 nm using a Bio-tek ELx 808ru Ultra Micro plate Reader. GSH levels were determined from the standard curve constructed by using 1 mM GSH. Results were expressed per microgram of protein. Protein concentration was measured using the BioRad protein assay reagent and bovine serum albumin was used as a standard as above. Graph Pad Prism software was used for data representation.

2.14 Lipid Peroxidation Assay

The lipid peroxidation assay was performed as previously described (Cereser *et al.* 2001). Briefly, 100 µl of the homogenate obtained from the midbrain was added to 1ml of thiobarbituric acid (7 g trichloroacetic acid, 0.185 g thiobarbituric acid, 12.5 ml 0.25 N HCl in 37.5 ml dH₂O) and incubated at 100°C on a heat block for 20 min. After cooling the tubes, absorbance was measured at 535 nm using a Genesys spectrophotometer. Lipid peroxidation levels were determined from a standard curve prepared using 100 µM malondialdehyde (MDA). Results were calculated per microgram of protein. Protein concentration was measured using the BioRad protein assay reagent with bovine serum albumin as a standard. Graph Pad Prism software was used for data representation.

2.15 ATP Assay

ATP content was measured as previously described (Sandhu *et al.* 2003). Briefly, 100 µl of tissue homogenate was mixed with 100 µl luciferin-luciferase solution in a black 96-well plate and incubated for 30 minutes at 37°C and the bioluminescence was using a plate reader using a Spectra Max Gemini XS spectrophotometer at 560 nm. The amount of ATP produced was determined from internal standard curve prepared by using 100 µM ATP standard solution. The final results were expressed per microgram protein.

2.16 SDS-PAGE and Western Blotting

The SDS-PAGE and western blot protocols were slightly modified from the original protocol (Sambrook *et al.* 1989). All the western blots were conducted used the following protocol with variations in the primary and secondary antibody as needed [monoclonal anti-TH (1:1000), polyclonal anti-GFAP (1:1000), polyclonal anti-PARKIN (1:1000), or polyclonal anti-synuclein (1;1000), polyclonal anti-iNOS (1:500)]. Protein samples were resolved using a 10% SDS-PAGE. 20 μg of protein (per well) was mixed with 4 X loading buffer (50 mM Tris-HCl, pH=6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol), heated for 2 minutes on a heat block at 95°C and then centrifuged before being loaded onto the gel. The protein was transferred from the gel onto a nitrocellulose membrane which was subsequently blocked using a non-fat 5% milk solution in TBS with 0.2% Tween (TBST) for 1 hour on a shaker at room temperature. Then blots were then stained with Ponceau S and the blots were scanned and recorded to ensure equal loading. The membrane was then incubated with the required primary antibody in 2% non-fat milk solution overnight at 4°C for 12-18hours. After this incubation period, the membrane was washed with TBST for 15 minutes, followed by three more five minute washes and finally incubated with secondary antibody (anti-mouse IgG (whole molecule) or anti-rabbit (whole molecule) peroxidase conjugate) at a dilution of 1:3000 in 2% milk solution and incubated at room temperature on a shaker for 1h. Finally, the blots were then washed with TBST for 30 minutes (1x15 minutes, 3x5 minutes) and developed using a ChemiGlow West kit according to manufacturer's protocol (Sigma Aldrich) and recorded using an Alpha Innotech Corporation Imaging

System. All the Western Blots were performed twice from tissue homogenates obtained from one animal in each group.

2.17 Cytokine Array

In order to assess the relative levels of multiple cytokines in rats undergoing various treatments, a cytokine assay was performed. Tissue homogenates were prepared according to a protocol provided with the kit from R&D Systems. Briefly, the mid brain was homogenized in PBS (pH=7.6) with protease inhibitors (1 μM leupeptin, 1 μM pepstatin A and 10 μM PMSF) and 0.1% Triton-X-100. The samples (100 μg protein) were incubated with detection antibody cocktail for 1 hour at room temperature on a rocking platform. Meanwhile, the membranes were incubated with blocking solution for 1 hour at room temperature, after which the sample/detection antibody mixture was applied onto the membranes and they were incubated on a rocking platform overnight at 2-8°C. The membranes were washed with Wash buffer for 30 minutes (3x10 minutes).

After blotting excess wash buffer the membranes were incubated with Streptavidin-HRP and incubated for 30 minutes. The membranes were then washed for 30 minutes and incubated with chemiluminescent reagent in accordance with the manufacturer's protocol. The images were recorded with an Alpha Innotech Corporation Imaging System. Data was obtained from two rats in each group belonging to the five injection regime.

2.18 Behavioural Assessment

2.18.1 Rotorod Apparatus

We measured rats' ability to maintain their balance on a rotorod as per a previously described protocol (Winer, 1978). This rotorod apparatus consisted of a paper-covered (80 grid) 15 cm long, 7 cm diameter wooden dowel attached to a variable speed motor. Clockwise revolutions of the rotorod could be adjusted from 6 to 12 R.P.M. The rod was separated from the motor by a vertical 30 x 48 cm grey wooden panel that was scored with black vertical and horizontal lines to form 12 x 12 cm squares as shown in Figure 3.7B. The apparatus rested on a small table so that the dowel was 27 cm above its surface. A digital video camera (Orbyx Electronics Inc, CA) was positioned 1 m in front of and level with the rod. The apparatus was illuminated by regular fluorescent ceiling lighting and a 60-W lamp approximately 3 m in front of it.

Experiment 2 setup consisted of a two week pre-injection phase followed by an injection phase of three weeks and a two-week post-injection phase. Rats received three rotorod sessions during the pre-injection phase, spaced approximately two days apart over the two-week period, one rotorod session five days after the second injection (mid-injection test), and one session in the post-injection phase given one week after their last injection. The first two pre-injection sessions served to train the rats to maintain their balance on the rotorod and gradually its rotation speed from 6 to 12 R.P.M was increased. From the third test on, the rotorod's rotation speed was maintained at 12 R.P.M. Rats' performance on the third pre-injection test provided baseline performance for comparison with the next two tests. Rats were always placed in the forward position (facing left) as the rod rotated clock-wise.

2.18.2 Dependent Behavioural Measures and Statistical Analyses

Each rat's video recording was converted to 5 frames per second still pictures from which we recorded its movements over the last 100 seconds (500 frames) with a software tracking program (7 Software, Inc, Montana, U.S.A.). Figure 3.7B illustrates a picture frame superimposed on the tracking frame composed of 450 vertical and 450 horizontal pixels. It was noted that although rats were initially oriented to walk forward on the slowly rotating rotorod, they also turned around and walked backward during some portions of these sessions. Therefore, we calculated the amount of time each rat actually spent (proportion of frames) walking backward as well as its average horizontal and vertical nose locations as it walked in each direction.

Each rat's proportion of time spent walking backward was transformed into an arcs in $2\sqrt{X}$ measure to enhance homogeneity of variance for parametric statistical analysis of proportion scores (Whishaw *et al.* 2003). Each dependent measure was analyzed by 2 (Injection: PQ vs. Saline) by 2 (Liquid Supplement: CoQ_{10} vs. Placebo) by 3 (Rotorod Test Session: pre-, mid-, post-injection) analysis of variance (ANOVA) with repeated measures on the last factor. We conducted statistical analyses of the behavioural data with SPSS (version 17) program and report significant effects at $p < 0.05$.

2.19 Statistical Analysis

Unless otherwise stated, all the biochemical data are represented as the mean ± SEM of three independent animals; each assayed in triplicate. For the statistical analysis of the histochemical data, three animals have been used unless stated otherwise. A value of $P < 0.05$ is considered as statistically significant. Two way ANOVA tests followed by

post-hoc Bonferroni's multiple comparisons test was used to assess statistical differences were performed using S.P.S.S software version 17 for three injection regime of PQ. Since the five injection studies were preliminary, no ANOVA was performed.

T-tests were performed using Statistica 7.0 software for all the *in vitro* studies.

2.20 Experiments Designed to Study the Mechanism of Neuroprotection by WS-CoQ$_{10}$

2.20.1 Cell Culture

2.20.1.1 Human Neuroblastoma Cells or (SH-SY5Y cells)

SH-SY5Y cells were grown as per manufacturer's protocol (ATCC) in Ham's F12 medium (Gibco) with 2 mM L-Glutamine that was modified to contain 1.5 g/L sodium carbonate, 10% Fetal Bovine Serum and 10 mg/ml gentamycin at 37°C at 5% CO_2. The cells were sub cultured by splitting 1:5 once a week.

2.20.1.2 Transformed Human Embryonic Kidney Cells (HEK 293 cells)

Cells were grown according to manufacturer's protocol (ATCC) using DMEM/F-12 media (Sigma). The media were supplemented with 15% (v/v) Fetal Bovine Serum (FBS) in tissue culture flasks and/or Petri dishes and incubated in 5% CO_2 at 37°C and 95% humidity.

2.20.2 Measurement of ROS Production from Isolated Mitochondria of SHSY-5Y cells and HEK 293 cells

SH-SY5Y cells were grown, and intact mitochondria were isolated as previously described (Li *et al.* 2003). Cells were harvested by mechanical dislodging using a rubber policeman and centrifuged at 500 x g for 5 minutes. They were washed twice with ice cold PBS and centrifuged at 250 x g, the cells were re suspended in isolation buffer containing 1 mM EDTA, 10 mM HEPES (pH= 7.4) and 250 mM sucrose. Cells were homogenized by a Dounce homogenizer with 20 strokes. The disrupted cells were centrifuged for 5 minutes at 600 x g at 4°C. The supernatant was collected and centrifuged at 15,000 x g at 4°C for 5 minutes and the resulting pellet was mitochondria. The pellet was re-suspended in buffer without EDTA. Mitochondrial suspensions were kept on ice and all the experiments were performed within 5 hours. For rotenone or PQ-induced mitochondrial dysfunction, mitochondrial hydrogen peroxide was measured using p-hydroxy phenyl acetic acid (PHPA) by an increase in the fluorescence due to oxidized p-hydroxyphenylacetate fluorescence by horseradish peroxidase as previously described (Li *et al.* 2003). Total mitochondrial protein was estimated using BioRad with BSA as a standard. The reaction began by adding 50 μg of mitochondria to a 100 μl reaction mixture containing 0.25 M Sucrose,10 mM HEPES 10 μg/ml PHPA and 0.4 units of HRP per well. In order to study the effects of WS-CoQ$_{10}$, 50 μg/ml CoQ$_{10}$ was incubated with mitochondria for 30 minutes prior to addition of 10 μM rotenone, or 200 μM PQ. 10 mM succinate was added as a substrate and the contents were mixed. After 30 minutes of incubation, the fluorescence of oxidized PHPA (excitation 320 nm, emission 400 nm) was measured in a 96 well micro-titer plate using the Spectra Max Gemini XS.

Graph Pad prism 4.0 was used for data representation. This experiment was done in triplicate and repeated three times.

For Bax-induced mitochondrial dysfunction, mitochondria were isolated from HEK 293 cells. The mitochondrial pellet, isolated as described above, was re-suspended in a reaction buffer consisting of 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM HEPES, 100 mM succinate and 10 mg/mL PHPA. Mitochondria, were either untreated or pre-treated for 30 min with 0.5 mg/mL WS-CoQ_{10} formulation. Following the 30 minute incubation with WS-CoQ_{10}, they were incubated with 5 µg/mL of recombinant Bax for 5 minutes at room temperature. Control mitochondria were not treated with Bax. The mitochondrial suspensions (100 µL per well) were placed in a 96 well plate and the wells were subsequently treated with 0.4 units of HRP and fluorescence was measured starting immediately after the addition of HRP for 10 min in 30-s intervals. The fluorescence readings were performed using the Spectra Max Gemini XS at an excitation wavelength of 320 nm and an emission wavelength of 400 nm. Microsoft excel was used for data representation. This experiment was done in triplicates and repeated thrice.

Table 3. Summary of immunohistochemistry experiments during different injection regimes.

	3-injection	5-injection (Prophylactic vs. Therapeutic effects of WS-CoQ$_{10}$)	5-injection (Old vs. Young)	8-injection (Used for standardization)
Number of rats (per group)	3	2	1	2
Type of Staining	DAB	DAB & IF	IF	DAB &IF
Stained for	TH	TH, GFAP & NeuN	TH	TH, GFAP, Parkin, VMAT, 4-HNE & α-synuclein

Table 4. Summary of the biochemical assays performed during different injection regimes.

	3-injection	5-injection (Prophylactic vs. Therapeutic effects of WS-CoQ$_{10}$)	5-injection (Old vs. Young)
Brain Region Assayed	Midbrain & Striatum	Midbrain	Midbrain
Type of tissue used	Fresh	Frozen	Frozen
Type of Assay	GSH, mitochondrial and total ATP, Lipid Peroxidation	GSH, total ATP & Lipid Peroxidation	GSH

Chapter 3

Results

This chapter is divided into three parts. The first part of the chapter focuses the results obtained from the initial studies with three injections of paraquat. The second part focuses on the specificity of paraquat treatment and evaluation of neuroprotection offered by WS-CoQ$_{10}$ at higher doses of paraquat. The final segment focuses on deciphering the probable mechanism of neuroprotection offered by WS-CoQ$_{10}$.

3.1. Initial Studies with Three Injections of Paraquat

This study was performed to evaluate:

1. The toxicity of paraquat to the dopaminergic neurons.

2. Neuroprotection offered by WS-CoQ$_{10}$ against paraquat mediated toxicity.

3.1.1. Effects of PQ on DA Neurons in the Midbrain and Neuroprotection by WS-CoQ$_{10}$

Many studies have used tyrosine hydroxylase (TH) immunostaining to estimate the number of dopaminergic neurons (McCormack *et al.* 2002, Peng *et al.* 2007). TH is an enzyme which converts the amino acid tyrosine into dihydroxyphenylalanine (DOPA). This reaction is the first step in the production of dopamine (Figure 3.1).

Tyrosine $\xrightarrow{\text{TH}}$ DOPA $\longrightarrow$ Dopamine $\begin{array}{c} \nearrow \text{Norepinephrine} \\ \searrow \text{Epinephrine} \end{array}$
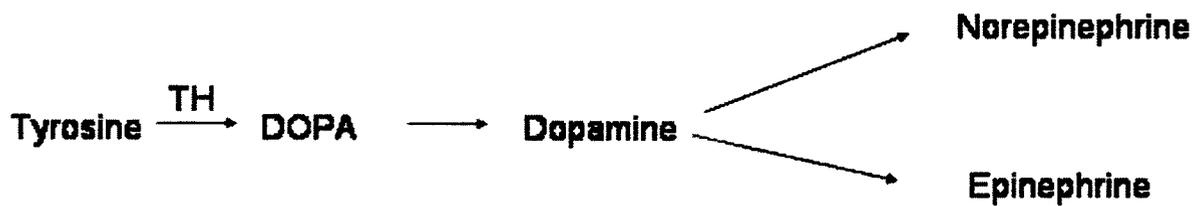
Figure 3.1 Conversion of tyrosine to dopamine. Tyrosine is converted to DOPA by tyrosine hydroxylase (TH), which is the rate limiting enzyme.

TH-immunostaining is used as a marker of choice since TH is the limiting enzyme for dopamine synthesis and is clearly reduced after dopaminergic insults (Emborg 2004).

Examination of midbrain sections revealed a reduced TH-immunostaining in the PQ-injected rats (for Water-fed and Placebo-fed groups, Figure 3.2B and 3.2C respectively) as compared to the control Saline-injected rats (Figure 3.2A). There also was diminished staining of TH-positive fibres, indicating neuronal loss and reduced fibre density (compare Figures 3.2B-b'and 3.2C-c' to 3.2A-a').

In contrast, the images of the PQ-WS-CoQ$_{10}$ midbrain sections showed strong TH-immunostaining of both the cell bodies and the fibres (Figure 3.2D and 3.2D-d'). This group of rats was given the WS-CoQ$_{10}$ supplemented drinking water, which might have counteracted the effects of PQ. The immunostaining data was confirmed by counting the TH-immunopositive neurons in the entire SN region (Figure 3.2E).

The counts revealed approximately 60% reduction of the number of cells in the PQ-injected rats receiving regular drinking water (from 313.7 ± 6.4 in the saline group to 91.3 ± 3.4 in the PQ-Placebo group, P<0.001) and about 60% reduction in the placebo fed group (down to 143.7 ± 4.3) compared to saline injected group. The cell counts also

confirmed the neuroprotective effects of WS-CoQ$_{10}$. Although the approximate number of TH-immunopositive neurons in the PQ-WS-CoQ$_{10}$ group was lower than the control saline group, total TH-positive neuronal number was still 2.5 fold higher than in the PQ-Water group ($P<0.001$) and 1.5 fold higher than the PQ-WS-placebo group ($P<0.001$). Interestingly, the counts also revealed a higher number of TH-immunopositive neurons in the PQ-Placebo group in comparison to the PQ-Water group ($P<0.001$), although this difference was not as clear in the microscopic images (Figure 3.2C). It should be noted, that the placebo solution contained PTS, a derivatised form of vitamin E, which could be responsible for the observed neuroprotection.
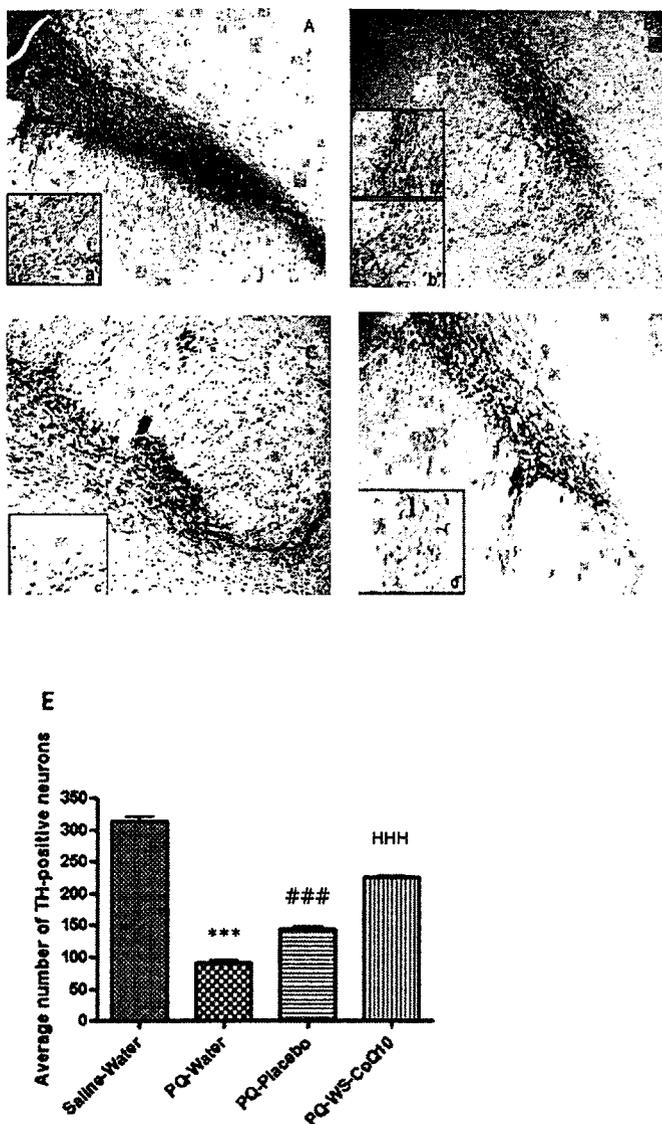
Figure 3.2 Immunohistochemical evaluation of midbrain damage. Rats were sacrificed, following the completion of experiment #1; brains were fixed, paraffin embedded and processed for immunohistochemistry. Images were captured using a Carl Zeiss Axiovert 200M microscope. Representative photomicrographs of midbrain sections showing TH-immunopositive neurons from saline injected rats (A), PQ injected water fed rats (B), PQ injected placebo fed rats (C) and PQ injected WS-CoQ$_{10}$ fed rats (D). Magnification: 10X. Insets (a' to d') show the density of TH-immunopositive neurons and fibres in the respective groups. Magnification: 20X. The number of TH-immunopositive neurons was counted manually (E). Data is shown as mean ± SEM. Statistically significant differences are indicated as: ***, between saline injected group and PQ injected water fed group, $P< 0.001$; ###, between PQ injected water fed group and both PQ injected placebo fed group and PQ injected WS-CoQ$_{10}$ fed group, $P< 0.001$; HHH between PQ injected placebo fed group and PQ injected WS-CoQ$_{10}$ fed group, $P< 0.001$, n = 3.

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3.1.2 Biochemical Analysis

The pro-oxidant effects of PQ such as oxidative damage to lipids and other cellular macromolecules, have been studied in a number of experimental systems and tissues other than the brain (Di Monte *et al.* 1986, Tamarit *et al.* 1998, Cheng *et al.* 1999). It has been proposed that oxidative stress may initiate PQ-induced apoptotic cell death in cultures of cerebellar granule cells (Gonzalez-Polo *et al.* 2004) and may underlie tissue damage when PQ is directly infused into the SN of rats (Mollace *et al.* 2003). *In vitro* studies in our lab also indicated a number of biochemical changes induced by PQ treatment including oxidative stress, activation of caspases and loss of mitochondrial membrane potential (McCarthy *et al.* 2004).

The most common tests used to analyse the damage caused by ROS include measuring the levels of oxidatively modified proteins, DNA and lipids. Also, the levels of antioxidants such as reduced form of glutathione are indicators of oxidative stress levels.

3.1.2.1 Evaluation of Levels Oxidative Stress Markers

3.1.2.1.1 Assessment of Reduced Glutathione after PQ Administration

In order to assess PQ-induced oxidative damage in different regions of the brain, and to assess if WS-CoQ$_{10}$ could this prevent this damage, I measured GSH content (Figure 3.3) in the midbrain of the rats following the three injection regime. These rats received 10 mg/kg of PQ once a week for three weeks. GSH is an abundant anti-oxidant, found in many tissues and is known to protect cells against oxidative damage (Dringen and Hirrlinger 2003). Depletion in GSH levels have been observed in the MPTP model of PD (Liang *et al.* 2007).

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In my study, animals from experiment #2 were sacrificed, brain tissues were harvested and then dissected as well as homogenised and the DTNB assay was performed as described in Materials and Methods. I compared the levels of GSH in the brains of PQ-injected groups (Placebo-fed and WS-CoQ$_{10}$ fed groups) to the saline injected groups (Placebo-fed and WS-CoQ$_{10}$- fed groups).

Results of GSH content analysis was expressed as percent control; the placebo-saline group was considered the control group. In the midbrain, the GSH content in the PQ-Placebo group (8.11 ± 0.7) was reduced by half as compared with the saline placebo (16.34 ± 5.7) and saline WS-CoQ$_{10}$ (13.19 ± 1.8) groups ($P<0.001$), consistent with it being utilized to combat the effects of PQ in the midbrain (Figure 3.3B). However, the GSH level was maintained at nearly control level in the PQ-WS-CoQ$_{10}$ group (11.05 ± 1.1) compared with both PQ injected groups, $P<0.001$).

A similar outcome was seen in the striatum (Figure 3.3A), where GSH levels in the PQ-Placebo (6.9 ± 0.69) group was reduced to half compared to the saline WS-CoQ$_{10}$ (11.39 ± 1.86) group and saline placebo (15.46 ± 1.21) group. Furthermore, WS-CoQ$_{10}$ offered protection against PQ-induced loss in GSH levels (10.66 ± 2.54). These results suggest that the anti-oxidant content of WS-CoQ$_{10}$ formulation offset PQ effects, sparing the GSH system. Two way ANOVA revealed a statistical significance in the GSH levels (comparing saline vs. PQ-injected groups, as well as placebo and WS-CoQ$_{10}$ groups, $p<0.05$).
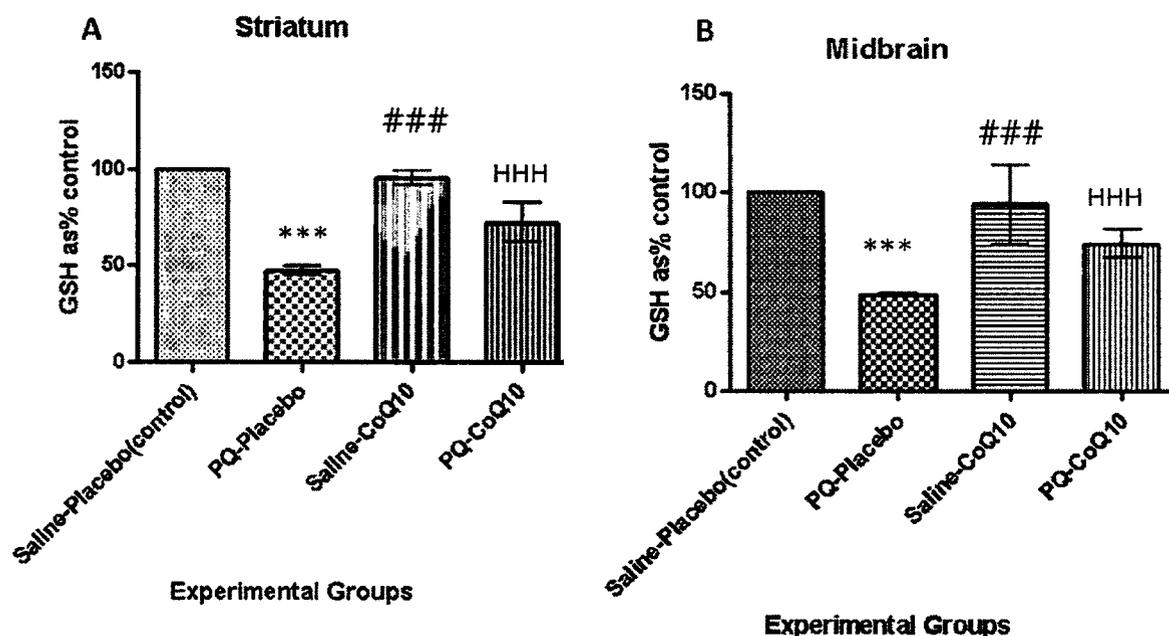
Figure 3.3 Levels of reduced GSH in the striatum and midbrain. Following the completion of experiment #2 rats were sacrificed and midbrain tissue was homogenized and assayed for GSH in the striatum (A) and midbrain (B). Data (mean ± SEM) was normalized to that of saline injected placebo fed group, which was taken as 100%. A) Statistically significant differences are indicated as: *** between saline injected placebo fed group and PQ injected placebo fed group, $P < 0.001$; ###, between PQ injected placebo fed group and saline injected WS-CoQ$_{10}$ fed group, $P < 0.001$; HHH between PQ injected placebo fed group and PQ injected WS-CoQ$_{10}$ fed group, $P < 0.001$, (n = 3).

3.1.2.1.2. Assessment of Levels of Lipid Peroxidation. Another marker of

oxidative stress is lipid peroxidation. Recent studies report a higher malondialdehyde

(MDA) level (Schmuck *et al.* 2002) and increased lipid peroxidation in the substantia

nigra region of PD brains (McCormack *et al.* 2006). I measured the content of MDA, the

product of lipid peroxidation, to assess the degree of PQ-induced lipid damage in the

midbrain (Figure 3.4). There was particularly evident damage in PQ-Placebo (139.34 ±

20.34) group as indicated by a significantly increased MDA level in comparison to

groups injected with PQ and protected with WS-CoQ$_{10}$ ($P<0.05$). Again, the MDA levels

in PQ-WS-CoQ$_{10}$ (93.90 ± 13.19) was nearly the same as the saline placebo (100 ± 0) and

saline WS-CoQ$_{10}$ (73.70 ± 23.4) groups and were significantly lower than in PQ-Placebo

group ($P<0.05$). Thus, these results indicated that PQ injections lead to increased lipid

peroxidation, which was mitigated by WS-CoQ$_{10}$.
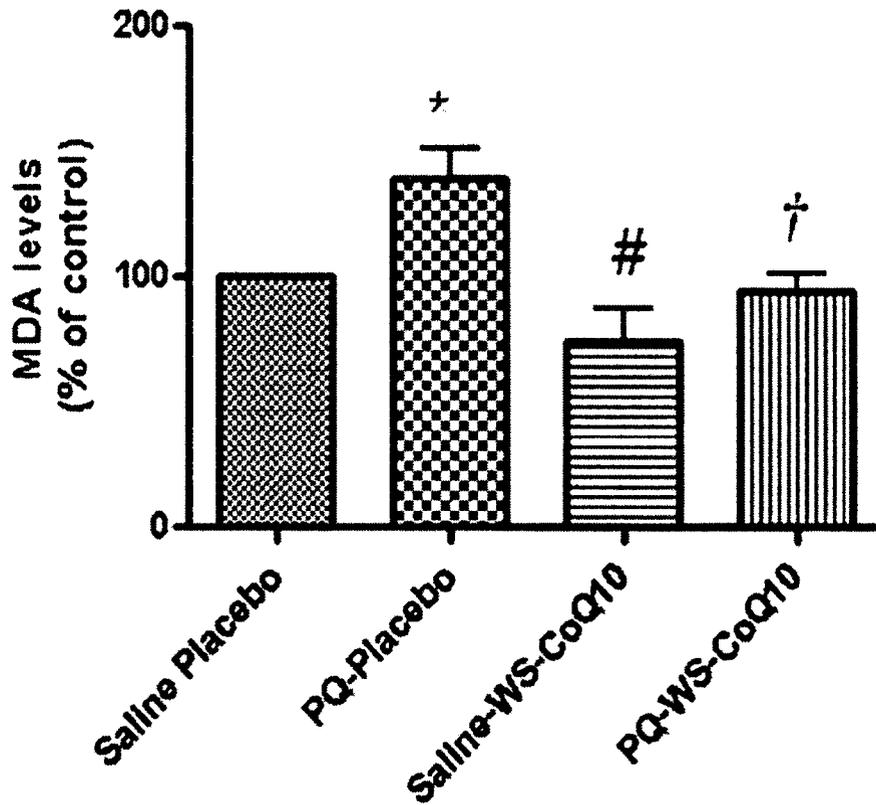
Figure 3.4 Levels of lipid peroxidation in the midbrain. Following experiment 2, rats were sacrificed; midbrain tissue was homogenized and assayed for MDA levels. Data (Mean ± SEM) was normalized to that of saline injected placebo fed group, which was taken as 100 %. Statistically significant differences are indicated as: [*], between saline injected placebo fed group and PQ injected placebo fed group, $P < 0.05$; [#] between PQ injected placebo fed group and saline injected WS-CoQ$_{10}$ fed group; [†], between PQ injected placebo fed group and PQ injected WS-CoQ$_{10}$ fed group, $P < 0.05$ (n = 2).

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3.1.2.2. Assessment of ATP Levels in the Striatum and Midbrain

3.1.2.2.1 Evaluating the Levels of Mitochondrial ATP Levels after PQ Administration

Studies have shown that toxins such as MPTP once accumulated in neurons, may cause damage by impairing mitochondrial function. As a consequence, cellular energy supplies in the form of ATP would be rapidly consumed, leaving cells unable to perform functions critical for their survival (Scotcher *et al.* 1990). Cell death after exposure to MPTP and MPP^+ has been linked to ATP depletion in studies using hepatocytes and PC 12 cells as *in vitro* model systems (Di Monte *et al.* 1986, Denton and Howard 1987, Singh *et al.* 1988). It has been shown that PQ causes mitochondrial dysfunction by inhibiting the electron transport chain, resulting in increased ROS production (Dinis-Oliveira *et al.* 2006). *In vitro* studies in our lab indicated that PQ treatment caused depletion in ATP levels. To analyse if PQ could induce ATP depletion and accordingly, whether WS-CoQ$_{10}$ could prevent ATP depletion, a luciferin-luciferase assay was performed using the midbrain and striatum to measure mitochondrial ATP production. The results were expressed as percent control where the placebo saline group was used as the control group.

As shown in figure 3.5, I observed that both PQ-injected groups significantly decreased their ATP levels compared to that maintained by the control saline-placebo group in both the midbrain and striatum. In the midbrain (Figure 3.5A), the PQ-CoQ$_{10}$ rats showed far less of a decline than the PQ-Placebo rats (67% vs. 52%), which missed significance (p=0.086). Unexpectedly, the saline-CoQ$_{10}$ group slightly (78%) but significantly had reduced ATP levels.

Similar results were observed in the striatum region (Figure 3.5B), where the PQ-Placebo rats showed about 30% decline in the ATP levels as compared to the saline-placebo and the PQ-WS-CoQ$_{10}$ animals. The ATP levels in the PQ-WS-CoQ$_{10}$ group were near control (saline-placebo group). Therefore, our results indicate that WS-CoQ$_{10}$ moderated mitochondrial dysfunction caused by PQ.
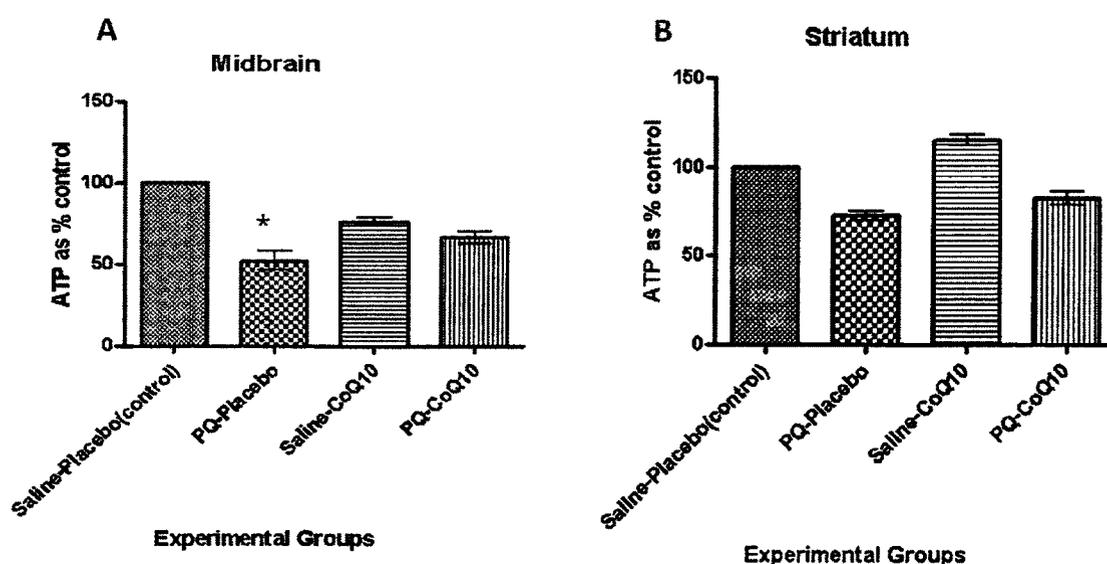
Figure 3.5 Levels of mitochondrial ATP in the midbrain(A) and the striatum(B). Following experiment 2, animals were sacrificed and fresh midbrain and striatum homogenates were obtained. Mitochondrial fractions were separated and then subjected to a luciferin-luciferase assay to detect mitochondrial ATP levels. In both regions, the results were normalised to saline–placebo group (control group). * reveals statistical significance, $p < 0.05$, (n=3).

3.1.2.2.2. Measurement of Total Cellular ATP Contents After PQ Exposure

In the previous experiment, only the mitochondrial ATP was measured. However, these results do not reflect total ATP levels. I performed a luciferin-luciferase assay on the midbrain extracts to establish a steady-state content of ATP in the PQ-injected rats (Figure 3.6). The data revealed a nearly 50% drop in the ATP level in PQ-Water when compared to the saline injected group (41.4% of control, $P<0.001$). The ATP levels in the PQ-injected animals were also lower than the saline group. The ATP levels were significantly higher, especially in the PQ-WS-CoQ$_{10}$ group than in unprotected PQ-injected group compared to the PQ-Water group ($P<0.05$).
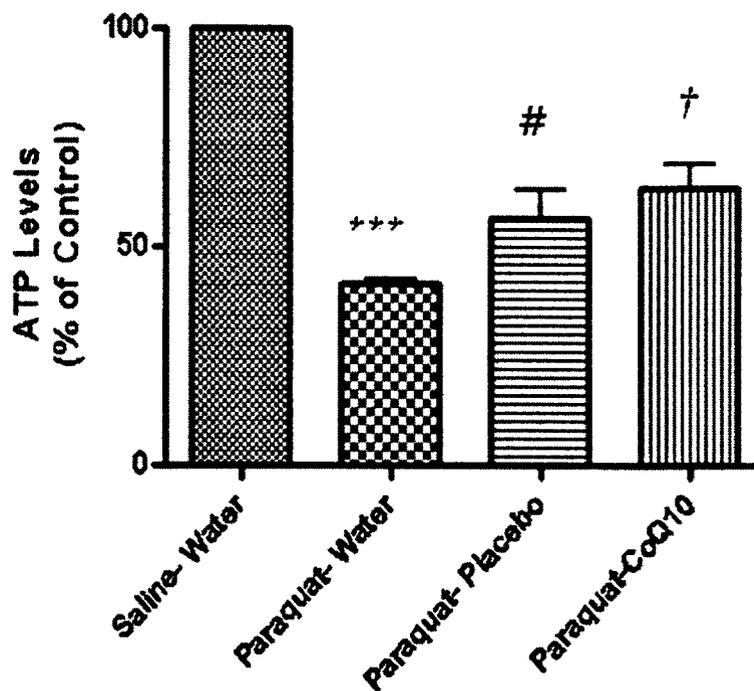
Figure 3.6 Levels of total cellular ATP in the midbrain. Following experiment 1, rats were sacrificed; midbrain tissue was homogenized and assayed for net ATP content using the luciferin–luciferase assay as described in Materials and Methods. Data (mean ± SEM) was expressed as percent of control, where Saline-Water group was used as the control group. Statistically significant differences are shown as: ***, between saline injected and PQ injected water fed group, $P< 0.001$; #, between PQ injected water fed group and PQ injected placebo fed group, $P< 0.05$; H, between PQ injected water fed group and PQ injected WS-CoQ$_{10}$ fed group, $P< 0.05$ (n = 2).

3.1.3 Behavioural Assessments

To determine the effects of PQ on Long Evans hooded rats and the potential neuroprotective effects of WS-CoQ$_{10}$, biochemical, histochemical as well as behavioural parameters were examined. Specific behavioural changes are attributed to PD, and analysis of these changes is fundamental when using an animal model of PD. For this purpose, the rats were subjected to a series of rotorod tests over the course of the three injection regime (experiment 2) to measure spontaneous changes in their balancing behaviour. These neuroprotective effects were accompanied by maintenance or even improved balance on the rotorod (Figure 3.7A). In fact, animals of the PQ injected WS-CoQ$_{10}$ group behaved the same on the rotorod tests as animals in both saline injected groups.

3.1.3.1. Proportion of Time Spent in Walking Backwards

Changes in the amount of time that rats in each group spent walking backward over the three test sessions (pre-injection, mid-injection, post-injection) are shown in figure 3.7A. Although all groups spent a greater proportion of their time walking forward, they spent 30% to 40% of their time walking backwards on their first (pre-injection) test. Rats in the PQ-Placebo group were the only ones that steadily reduced the amount of time spent spontaneously walking backward. In fact, during the post-injection test they almost exclusively maintained a forward walking gait. The three-way ANOVA on the transformed data uncovered a significant injection type among all three factors, p=0.015. One-way within-subjects ANOVAs for each group revealed that only rats in the PQ-

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placebo group significantly reduced their proportion of walking backwards, $p<0.01$. There were no statistically significant effects for any changes in the other three groups.

3.1.3.2. Vertical and Horizontal Nose Positions

Separate analyses of rats' vertical and horizontal nose positions while on the rotorod did not uncover any significant changes reflecting the experimental treatments (i.e., PQ vs. saline injections or placebo vs. CoQ_{10} supplementation). As shown in figure 3.7B, rats tended to walk forward with their noses slightly below the top of the rotorod (below the horizontal line at 210 pixels) and extended well away from it (177 pixels from the left vertical line). When rats did walk backwards, they also tended to extend their noses slightly below the top of and away from the rotorod.

In summary, I found clear evidence of a motor balance dysfunction in rats that had suffered PQ-induced neuronal loss in the SNc (PQ-Placebo group). These rats drastically reduced their tendency to spontaneously turn around and walk backwards during and following their series of injections. On the other hand, PQ-injected rats whose SNc area was protected by WS-CoQ_{10} (PQ-CoQ_{10} group) retained their pre-injection levels of the backward gait on the final post-injection. Thus this group did not differ statistically from either of the saline-injected groups.
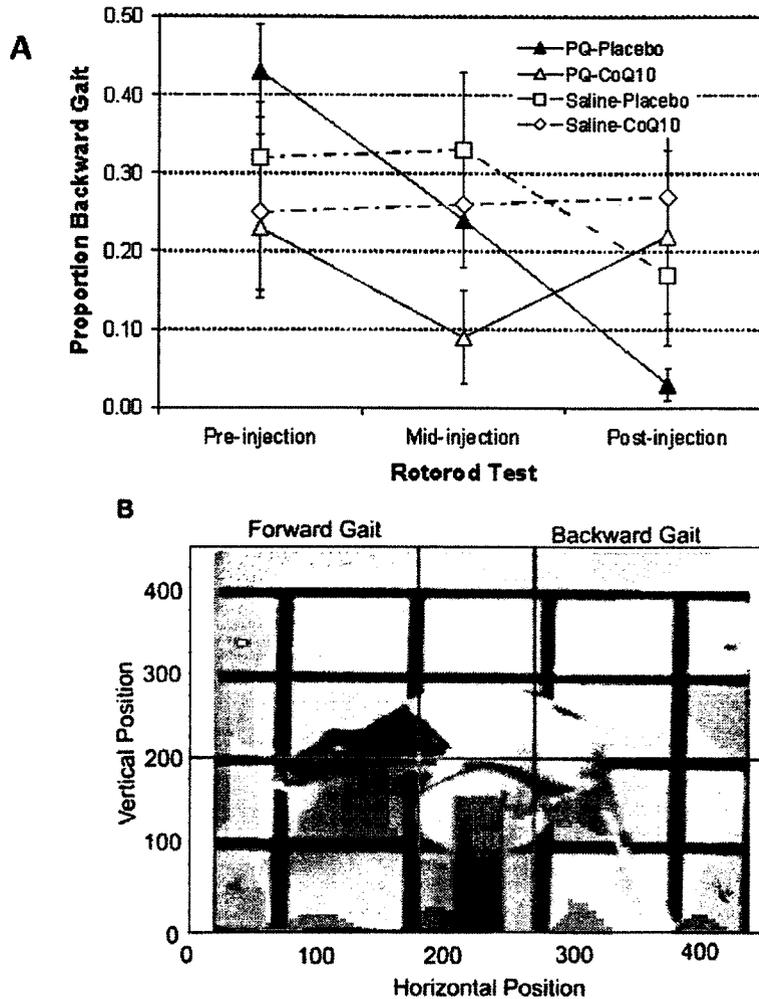
Figure 3.7 Behavioural assessments. Rats were trained on the rotorod and behavioural testing was carried out. (A) Rats were placed on a moving rotorod and the time spent walking backwards on the rotorod was monitored. Measurements were made before (pre-injection), during (mid-injection) and after (post-injections) the PQ-injections. The data points represent the mean proportion of time spent walking backwards during each testing session (n=6). (B) Representative image of the tracking format used for determining forward/backward walking posture as well as the location of nose on the rotorod. The horizontal line is positioned at the top of the rotorod and the two vertical lines determine the forward and backward walking positions. Any nose locations between the left and right vertical lines were not counted.

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Thus treating animals with three injections of 10 mg/kg PQ not only induced neuronal loss in the SNc but also caused behavioural deficits and increased oxidative stress levels and decreased ATP levels in the midbrain and striatum. Interestingly, pre-feeding with WS-CoQ$_{10}$ substantially prevented loss of DA neurons, decreased oxidative stress and maintained and ATP levels near control. Moreover, behavioural deficits were also significantly reduced by WS-CoQ$_{10}$ pre-feeding.

3.2 Specificity of Paraquat Neurotoxicity and Evaluation of Neuroprotection by WS-CoQ$_{10}$ at Higher Doses of Paraquat

This study was designed to evaluate:

1. If higher doses of PQ could lead to a greater loss of DA neurons.

2. Specificity of PQ-induced neuronal cell death

3. Effects of prophylactic and therapeutic use of WS-CoQ$_{10}$ (pre- and post- injection feeding) on neuroprotection

4. Effects of age on PQ-induced toxicity

I wanted to evaluate the effects of higher doses of PQ and examine if a higher dose would cause increased death of DA neurons. While trying to improve the existing model, I came across the advantages of using multiple insults to cause death of DA neurons, in the literature. Reportedly, eight injections of 10 mg/kg PQ alone or 10 mg/kg PQ in combination with another fungicide, maneb (30 mg/kg) were used to induce death of DA neurons (Thiruchelvam *et al.* 2000a, Thiruchelvam *et al.* 2000b, Cicchetti *et al.* 2005, Saint-Pierre *et al.* 2006). Therefore I began a study involving a combination of 10

mg/kg PQ and 30 mg/kg maneb injected twice a week for four weeks. This combination proved to be fatal to a number of rats and was therefore discontinued. I decided use eight injections of 10 mg/kg PQ alone (twice a week for four weeks) and analyze if more neuronal damage could occur with this regime than with only three injections. However, this regime also proved to be fatal Long Evans hooded rats.

Since the eight injection PQ regime was highly toxic to the rats, I decided to use five injections of 10 mg/kg PQ, and evaluate if this regime could increase the loss of DA neurons compared to three injections. The five injection PQ regime did not cause any fatalities. Therefore preliminary studies were carried out to evaluate the nigral loss and biochemical parameters.

3.2.1 Evaluating the Effects of the Five Injections of PQ and the Efficacy of WS-CoQ$_{10}$ as a Prophylactic and Therapeutic Agent on Nigral Cells

Our studies with three injections of PQ clearly revealed the neuroprotective effect of WS-CoQ$_{10}$ as a prophylactic agent. However, the ability of this compound as a therapeutic agent needed to be studied. To study the effects of WS-CoQ$_{10}$ as a prophylactic agent, WS-CoQ$_{10}$ feeding was started before the commencement of PQ injections; to investigate the therapeutic abilities of WS-CoQ$_{10}$, WS-CoQ$_{10}$ feeding began only after the last injection. For comparison, the DA neuronal numbers were counted using TH-immunostaining.

The number of DA neurons was estimated by counting TH-immunopositive neurons in the substantia nigra pars compacta. Assessment of midbrain sections showed a clear decrease in the density of TH-immunopositive neurons in rats injected five times

with 10 mg/kg PQ. In this preliminary study, I compared the prophylactic effects of WS-CoQ$_{10}$ (pre-fed) to its therapeutic effects (post-fed). As shown in figure 3.8(A-B) rats belonging to the PQ-Water group were vulnerable to PQ, as it caused about 30% reduction of TH-positive neurons compared to the saline injected groups. Rats belonging to the PQ-Placebo pre-fed group showed a decline of about 26%, while rats of the PQ-CoQ$_{10}$ pre-fed group showed only about 14% loss in the number of TH-positive neurons, compared to the control group. When WS-CoQ$_{10}$ is fed after PQ injections, it still offered neuroprotection against PQ toxicity and prevented further loss of DA neurons (28% decline in TH-immunopositive cells in PQ-CoQ$_{10}$ post-fed rats compared to about 40% decline in PQ-Placebo post-fed rats) as indicated in figure 3.8A-B.
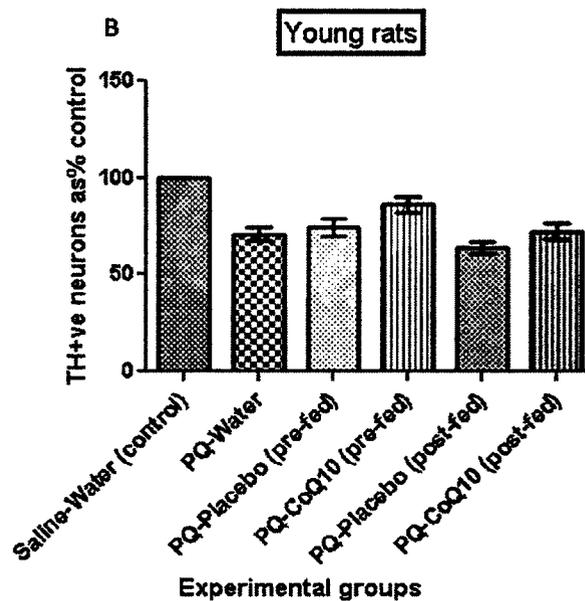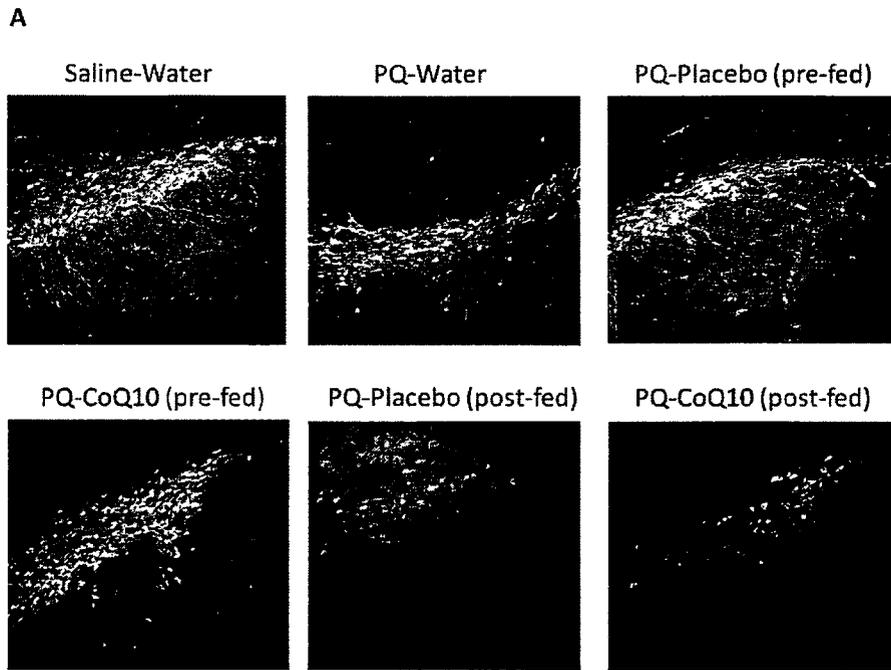
Figure 3.8 Evaluation of neuroprotective effects of WS-CoQ$_{10}$ as a prophylactic and therapeutic agent. (A) Pictures of TH-immunostained sections were obtained by using a Nikon ECLIPSE E800 microscope. Original magnification 10X. (B) TH-positive neurons were counted manually in the substantia nigra pars compacta of rats treated with five injections of 10 mg/kg PQ. Data is represented as percentage of control. Mean ±SEM, n = 2.

3.2.2 Assessment of Oxidative Stress Levels

3.2.2.1 Evaluating Levels of Reduced Glutathione after PQ Treatment

As mentioned earlier, GSH is a very important anti-oxidant in the brain. In this study, the GSH content was measured from the midbrain. The results were normalised to the Saline–Water (control) group. The effects of prophylactic versus therapeutic applications of WS-CoQ$_{10}$ on levels of GSH after PQ treatment were analysed. There was a loss of about 40% in GSH content in the PQ-Water group as compared to the Saline-Water (control) group (Figure 3.9). Although the PQ-Placebo pre-fed group showed higher GSH levels than PQ-Water group, the GSH levels were maintained at a near control level in the PQ-WS-CoQ$_{10}$ pre-fed group. Interestingly, the amount of GSH in the PQ-CoQ$_{10}$ post-fed group was maintained at a slightly higher level as compared to PQ-Water and PQ-Placebo post-fed groups.
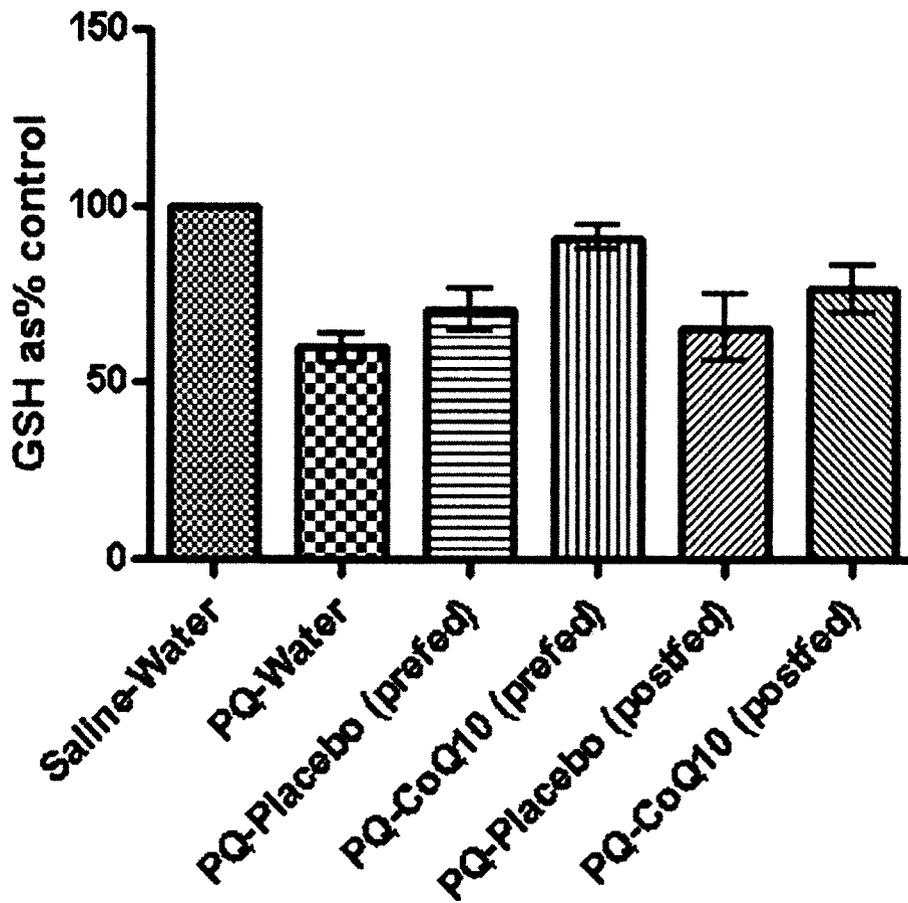
Midbrain

Figure 3.9 Evaluating levels of reduced GSH in the midbrain. After the completion of the experiment, rats were sacrificed, midbrain tissue was homogenized and assayed for GSH Data (Mean ± SEM) was normalized to that of Saline-Water group, which was taken as 100 %. n = 2.

3.2.2.2 Evaluating the Levels of Lipid Peroxidation in the Midbrain

As mentioned earlier, levels of oxidative stress can be monitored by evaluating the levels of lipid peroxidation. I measured the content of MDA, the product of lipid peroxidation, to assess the degree of PQ-induced lipid damage in the midbrain. The results were normalised to the Saline–Water or the control group. The effects of prophylactic versus therapeutic applications of WS-CoQ$_{10}$ on levels of lipid peroxidation after PQ treatment were analysed. There was an increase of about 34% in lipid peroxidation levels in the PQ-Water group as compared to the Saline-Water group (Figure 3.10). Although the PQ-Placebo pre-fed group showed lower lipid peroxidation levels than PQ-Water group, the lipid peroxidation levels were maintained at a slightly lower level in the PQ-WS-CoQ$_{10}$ pre-fed group. Furthermore, the lipid peroxidation levels in the PQ-WS-CoQ$_{10}$ post-fed group were maintained at a near control level as compared to PQ-Water and PQ-Placebo post-fed groups. Thus WS-CoQ$_{10}$ does offer protection against PQ-induced oxidative stress.
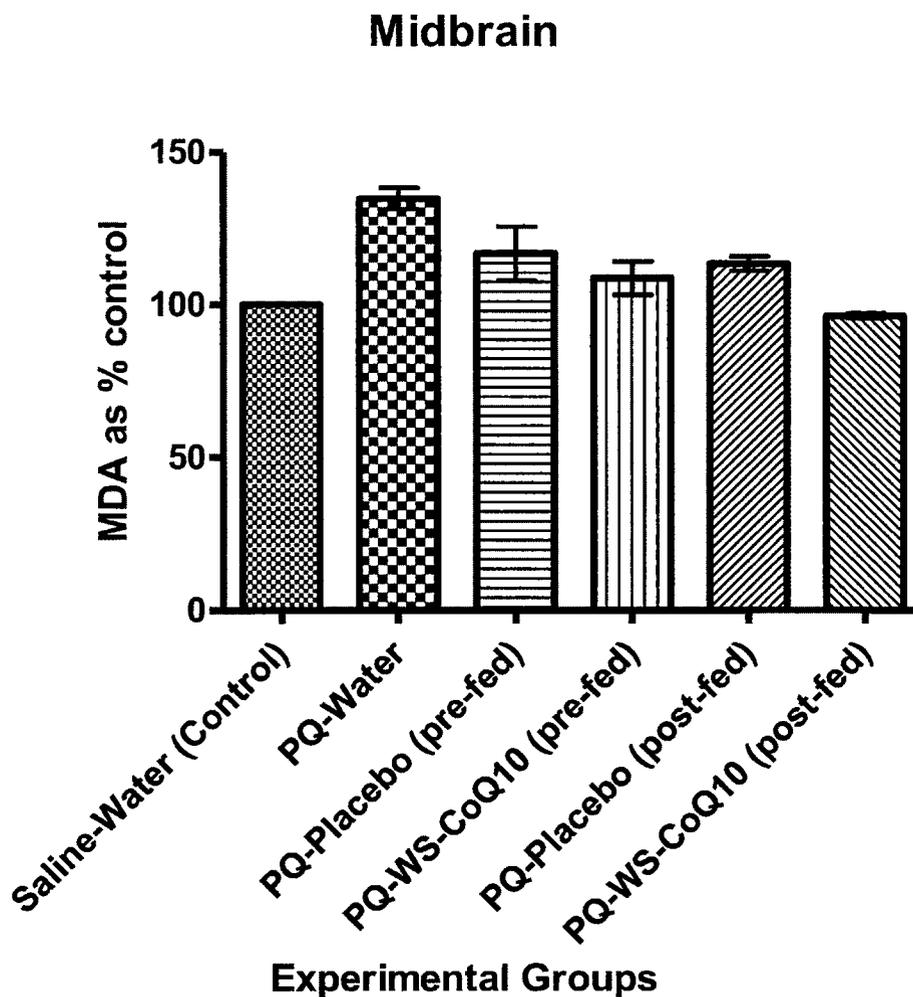
Midbrain

Figure 3.10 Evaluating the levels of lipid peroxidation in the midbrain. After the completion of experiment, rats were sacrificed, midbrain tissue was homogenized and assayed for lipid peroxidation. Data (Mean ± SEM) was normalized to that of Saline-Water group, which was taken as 100 %. n = 2.

3.2.3 Analyzing the Levels of Total ATP in the Midbrain

The net cellular ATP content, however, reflects a balance between energy production and consumption, in which an increase would be expected under oxidative stress conditions since ROS detoxifying enzymes are energy dependent. I performed a luciferin-luciferase assay on the midbrain extracts to establish a steady-state content of ATP in the PQ-injected rats (Figure 3.11). The results were normalised to the saline–water or the control group. The effects of prophylactic versus therapeutic applications of WS-CoQ$_{10}$ on levels of total ATP after PQ treatment were analysed. The data revealed a 44% loss in the ATP levels in PQ-Water group when compared to the Saline-Water group. The ATP levels in the all the PQ-injected rats were also lower than the Saline-Water (control) group. Although the PQ-Placebo pre-fed group showed higher ATP levels than PQ-Water group, the ATP levels were maintained even higher in the PQ-WS-CoQ$_{10}$ pre-fed group. Interestingly, the amount of ATP in the PQ-CoQ$_{10}$ post fed was maintained at a slightly higher level as compared to PQ-Water and PQ-Placebo post-fed groups.
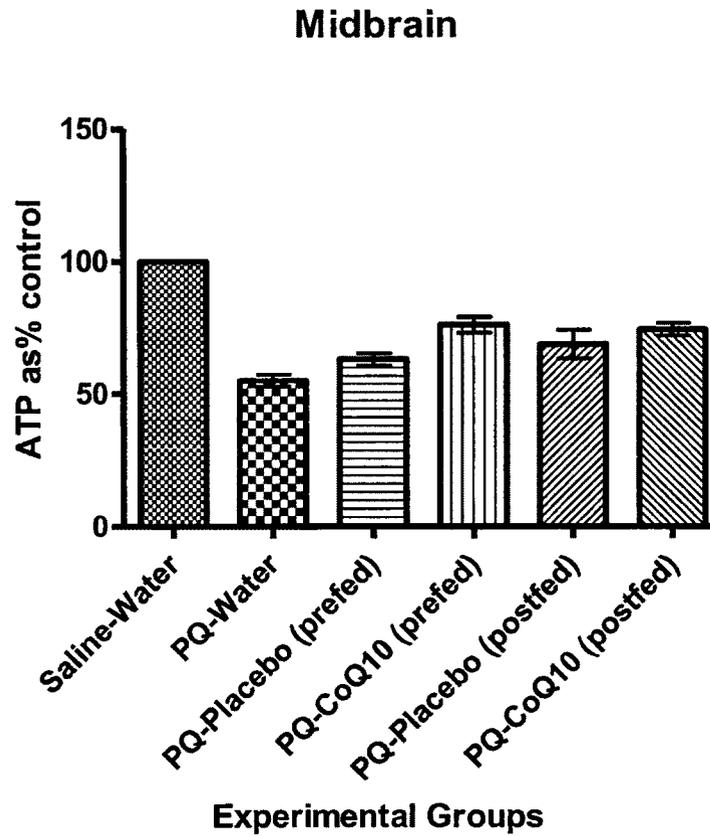
Midbrain

Figure 3.11 Evaluating the levels of total ATP in the midbrain. After the completion of experiment, rats were sacrificed, midbrain tissue was homogenized and assayed for ATP levels using luciferin-luciferase method. Data (Mean ± SEM) was normalized to that of Saline-Water group, which was taken as 100 %. n = 2.

3.2.4 Evaluating the Effect of PQ on Protein Expression. Western blot analysis was performed on the midbrain homogenates to evaluate PQ induced response on the level of proteins such as tyrosine hydroxylase, parkin, α-synuclein and GFAP. As mentioned earlier, TH is a marker for DA neurons. Preliminary results indicate a decrease in the levels of TH in all the rats subjected to PQ treatment as compared to their saline injected counterparts, correlating to the loss of DA neurons. α-synuclein expression is increased in midbrains of rats challenged by PQ (Bove *et al.* 2005). Up regulation of this protein has been observed in MPTP-treated squirrel monkeys (McCormack *et al.* 2008). Our preliminary results indicate no change in the expression levels of α-synuclein post PQ injections. Parkin may be critical for protecting DA neurons from toxic insults by targeting misfolded or oxidatively damaged proteins for proteasomal degradation (Perez *et al.* 2005). When I examined parkin levels, they remained unchanged between saline and PQ-injected rats (Figure 3.12). These are preliminary results and no conclusions can be drawn from this data.
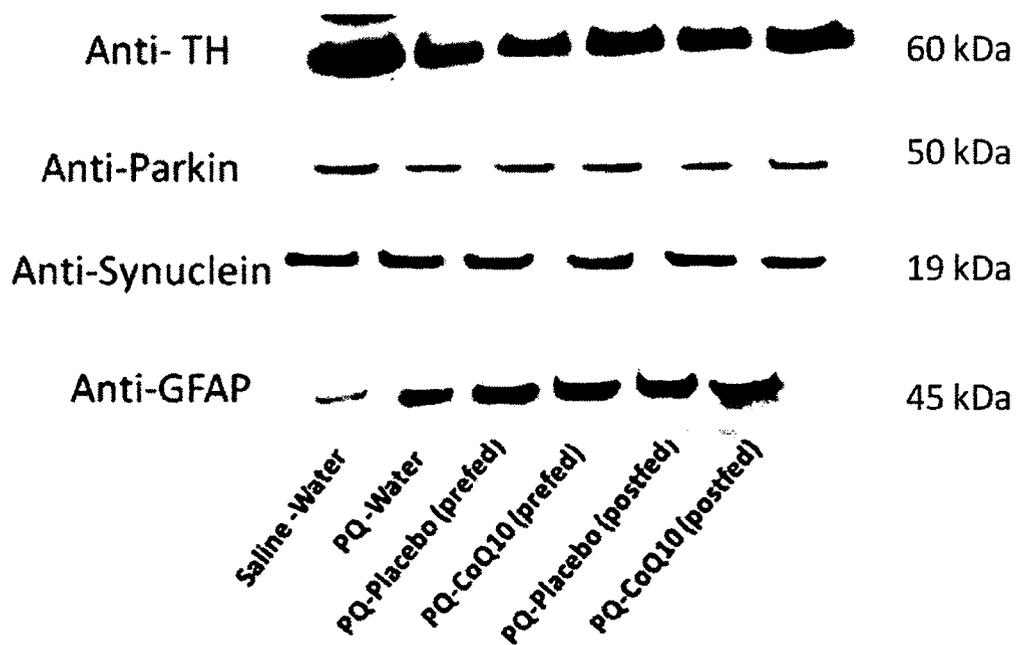
Figure 3.12 Evaluating the levels of protein expression. Western blots of TH, parkin, α-synuclein and GFAP proteins in the midbrain of rats treated with 10 mg/kg PQ once a week for five weeks. Midbrain homogenates were subjected to western blot analysis for analyzing the expression levels of different proteins. Ponceau S staining was performed to confirm equal loading in all wells.

3.2.5 PQ-induced Selective Damage of DA Neurons in the SNc

3.2.5.1 Selectivity of PQ-induced Neuronal Loss

Results have indicated a loss in DA neurons after PQ injections. The selectivity of PQ toxicity was further evaluated by immunohistochemical examination of neurons in the hippocampus region, cortex and the cerebellum. I stained the sections (coronal sections across the substantia nigra region) with anti-NeuN, as well as hematoxylin and eosin (H&E).

H&E staining has been widely used to label viable and damaged cells in the brain (Schmued *et al.* 1997). Hematoxylin stains nuclei blue and eosin stains cytoplasm pink. NeuN is a neuronal-specific protein (Mullen *et al.* 1992) that is frequently used as a specific marker for viable and intact neurons (Mullen *et al.* 1992, Xu *et al.* 2002, Hassen *et al.* 2004). Immunohistochemistry of NeuN shows that this protein is visible both in nuclei and in portions of the cytoplasm of neurons, such as the cell body, axons and dendrite processes (Mullen *et al.* 1992).

Neurons in the hippocampus are vulnerable to a variety of toxins (Stoltenburgdidinger 1994, McCormack *et al.* 2002) and therefore were identified as a cell population that could be a potential, albeit non-intended target of PQ toxicity. NeuN staining of the hippocampus region in the saline and PQ-injected rats did not reveal any significant decrease in the numbers of neurons as shown in figure 3.13A. A similar result was observed for NeuN staining of the cortex (Figure 3.13B). However, a decrease in the number of NeuN positive cells was observed in the midbrain region of rats treated with PQ. Moreover, H& E staining of saline and PQ treated rats did not reveal any damage to cells in the hippocampus region and cerebellum. The gross morphology of the midbrain

also did not seem to be affected by PQ treatment (Figure 3.13D). These studies were performed by a neurologist (Dr. T.S. Sridhar) who interpreted the results (in a blind study). These results need to be quantified and also confirmed.

D

Saline water | PQ water

Hippocampus region

Saline water | PQ water

Midbrain

Saline-Water | PQ-Water

Cerebellum
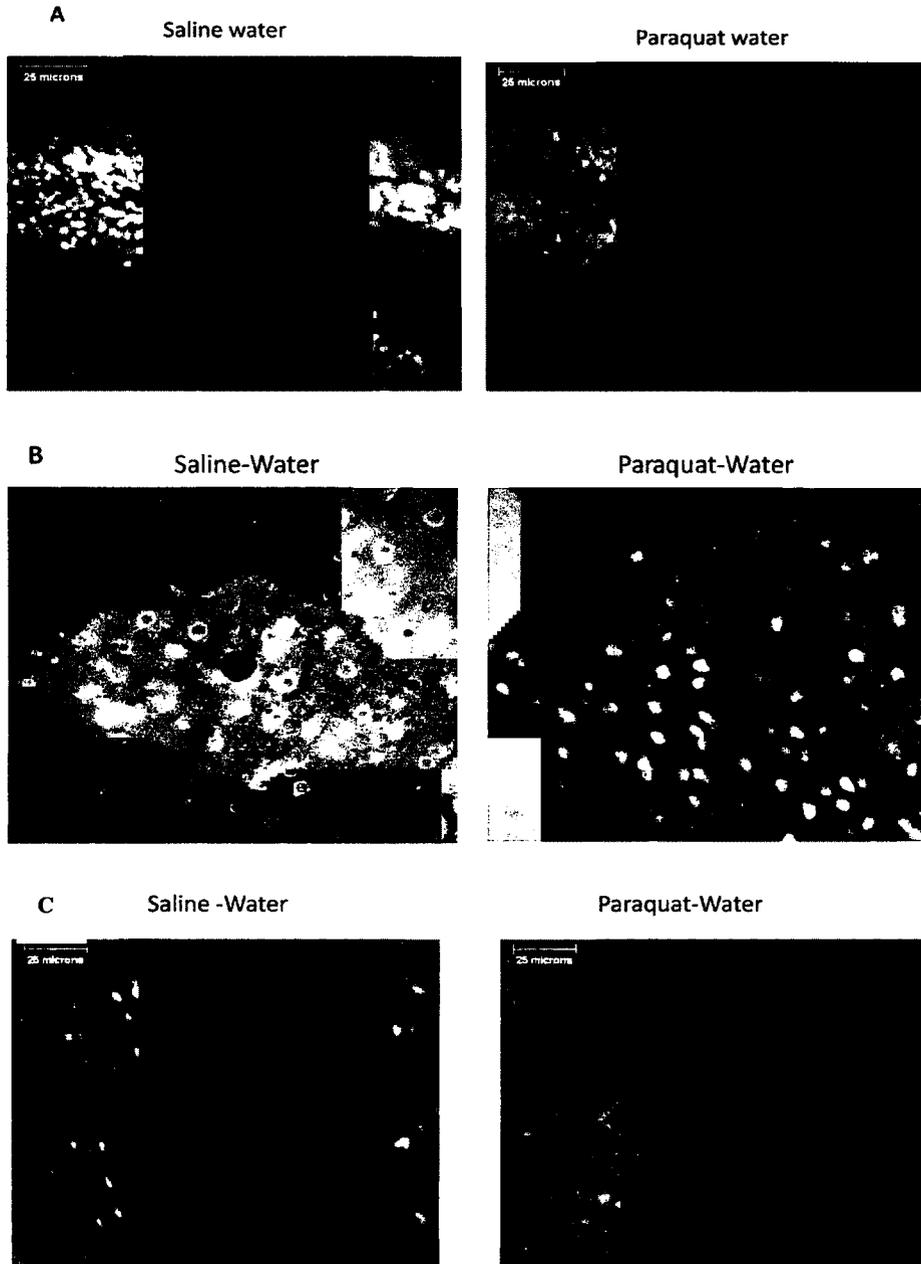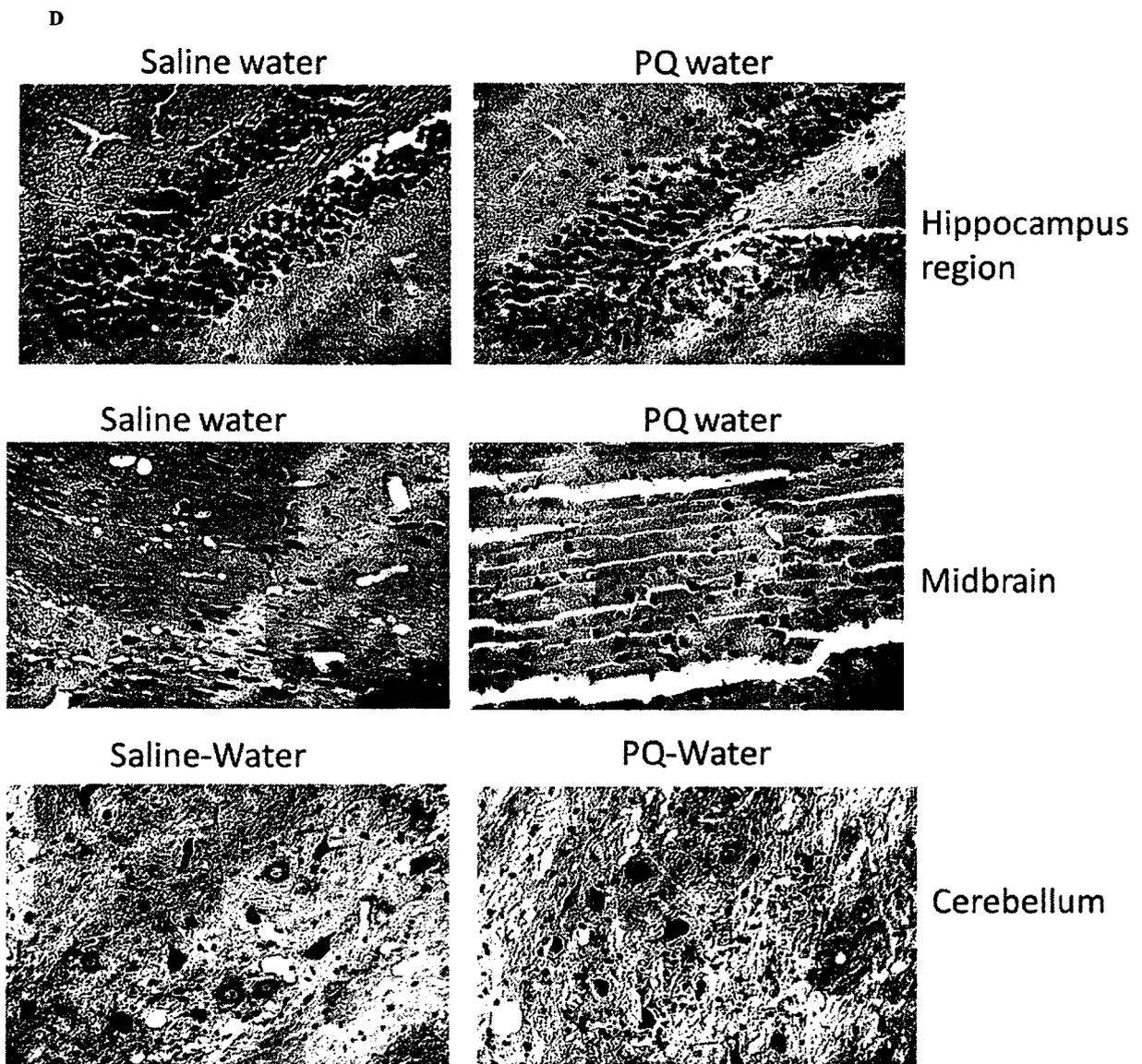
Figure 3.13 Effects of PQ on neuronal cells in different areas of the brain. Coronal brain sections were immuno-stained for NeuN as well as stained with Hemtoxylin and Eosin (H&E). Pictures were obtained using a Leica DM IRB microscope. NeuN immuno-staining in hippocampus region (A), the cortex (B) and the midbrain region (C). H&E staining of the hippocampus, midbrain and cerebellum (D). Pictures were taken at 40 X magnification.

3.2.5.2 PQ Treatment does not Cause any Damage to Peripheral Tissue

Most of the rats treated with PQ gained weight progressively, albeit a little slower as compared to their saline counterparts especially during the weeks of injections. No significant weight loss was observed between the saline and PQ-treated rats (Figure 3.14). More importantly, there were no fatalities after five PQ injections.

Average Cage Weights with 5 injections of Paraquat

Legend:
Saline-Water
Paraquat-CoQ10(prefed)
—▲—Paraquat-placebo(prefed)
—●—Paraquat-Water
—◆—Paraquat-placebo(postfed)
—■—paraquat-CoQ10(postfed)

Y-axis: Weights (g)
X-axis: Days
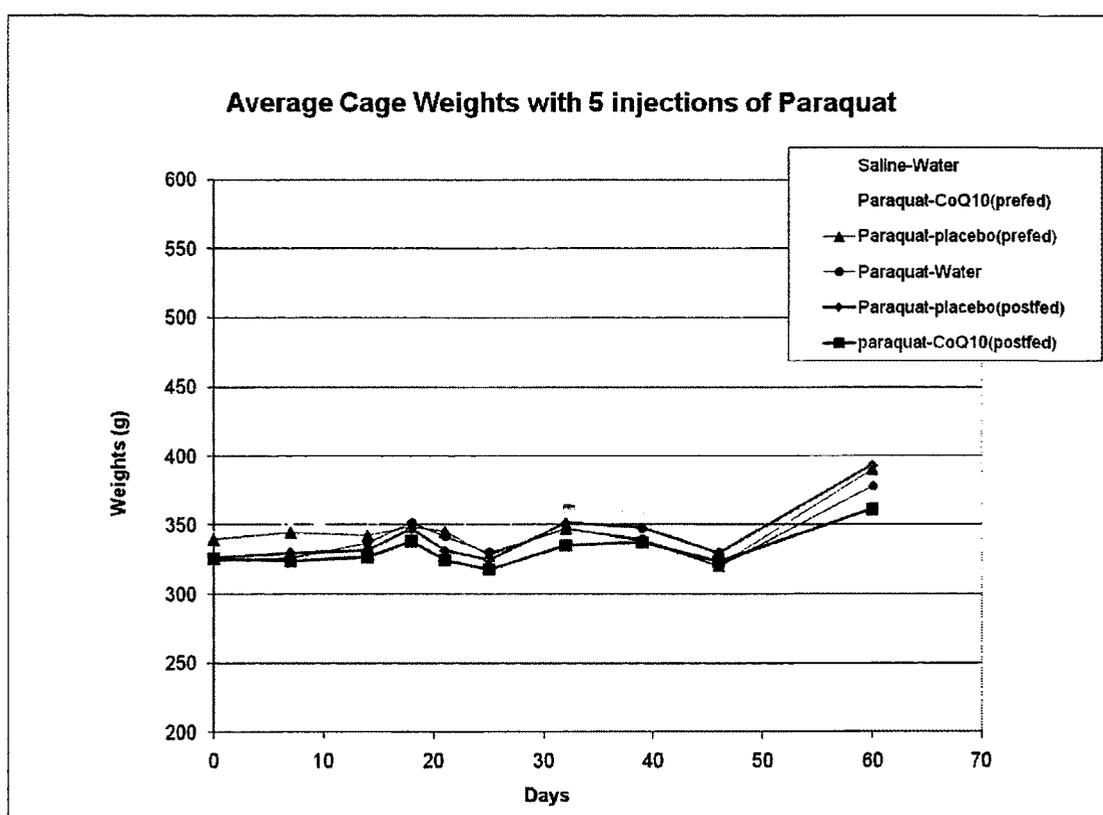
Figure 3.14 Weights of rats for the five injection PQ regime. Weights were recorded for each animal every week. An average weight of the four animals in a cage is shown here (n=4).

Since the herbicide was injected intraperitoneally, a general pathological analysis of the liver, kidneys and lungs was carried out. The liver and kidneys are responsible for elimination of toxins from the body. H&E stained sections of these tissues were evaluated by a pathologist (Dr. Jyothi S. Prabhu) in a blind study, without the knowledge of treatment groups. Results revealed that there was no damage to the liver or kidneys (Figure 3.15). Acute PQ exposure has been shown to cause severe damage to the lungs (Dinis-Oliveira *et al.* 2006). The analysis of the lung tissue revealed that there was blood present in the tissue making it difficult to draw any conclusions. The arrows indicate the presence of blood cells.
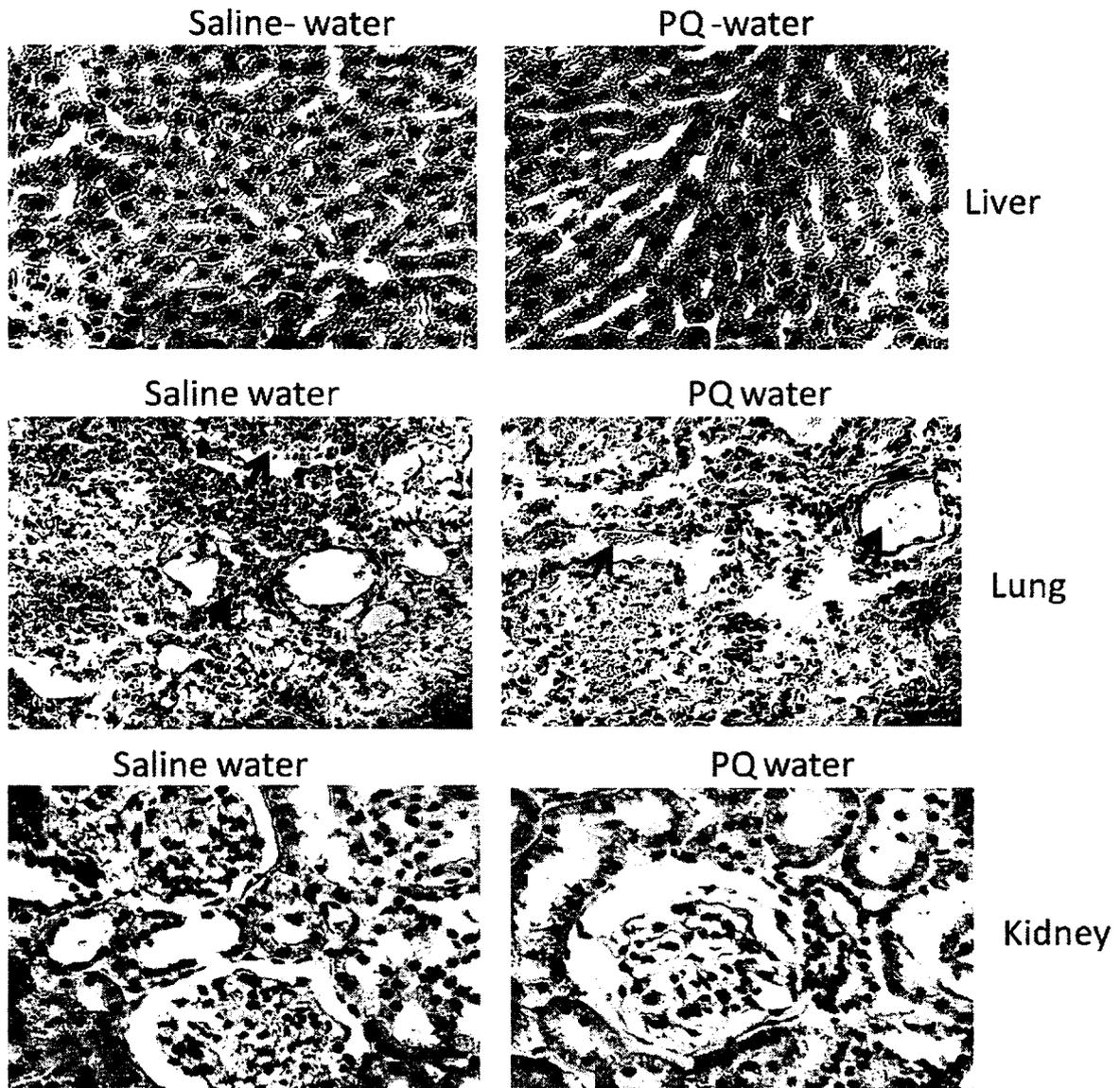
Figure 3.15 Analysis of peripheral organs after PQ exposure. The lungs, liver and kidneys were fixed and 8 μ sections were obtained from different parts of the organs and stained with H&E. The pictures of the lungs and liver were taken at 40X magnification while the pictures of the kidney were taken at 63X magnification.

3.2.6 PQ Induces Astroglia Response in the Midbrain

Several studies have documented that glial activation and neuroinflammatory processes play an important role in the pathogenesis of PD (Hirsch et al. *et al.* 2005, Wersinger and Sidhu 2006, Rogers 2008). Glial fibrillary acidic protein (GFAP), which is abundantly expressed by astrocytes in the central nervous system (CNS), is widely used as a marker of astrocytic activation (Eng *et al.* 2000). It has shown in mice that PQ exposure does lead to inflammation (McCormack *et al.* 2002). To evaluate the effects of PQ on inflammation in Long Evans Hooded rats, I immunostained midbrain sections for GFAP. In order to do so, rats were injected with 10 mg/kg PQ (once a week for five weeks), sacrificed and perfused. The brains were fixed in 10% buffered formaldehyde, sectioned and stained with anti-GFAP antibody as in Materials and Methods. The staining revealed increased astrocyte expression of GFAP in all PQ-injected groups (Figure 3.16B-D) when compared to the saline-injected rats (Figure 3.16A).The strongest GFAP response, however, was seen in the brains of the rats that drank the WS-CoQ$_{10}$ supplemented water. Under a higher magnification, the astrocyte processes appeared to be thicker and fibrous (hypertrophic), typical of reactive astrocytes, which were rarely seen in the saline-injected rats receiving regular drinking water (Figure 3.16 b' c' d' as compared to a'). The quantitative cell counts confirmed the increased presence of reactive astrocytes in the PQ-injected groups, but especially in the WS-CoQ$_{10}$ groups (Figure 3.16E). The number of counted astrocytes in the WS-CoQ$_{10}$ groups was nearly double that in the saline group and even the PQ-Placebo fed and PQ-WS-CoQ$_{10}$ fed groups. The astrocytic presence in the later two groups was also significantly higher than in the control group (Saline-Water). Thus, the neuroprotective effects of WS-CoQ$_{10}$ were

clearly associated with the significantly induced astroglia response (PQ-WS-CoQ$_{10}$ fed group vs. saline injected group, $P<0.001$; PQ-WS-CoQ$_{10}$ fed group vs. PQ-Placebo fed group, $P<0.001$).
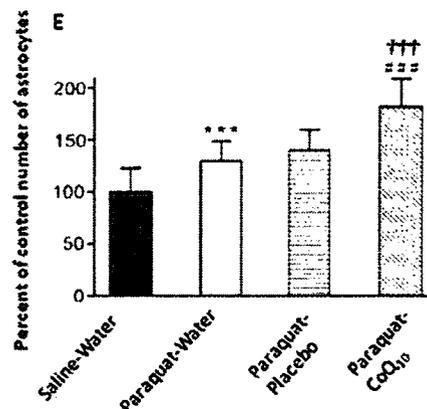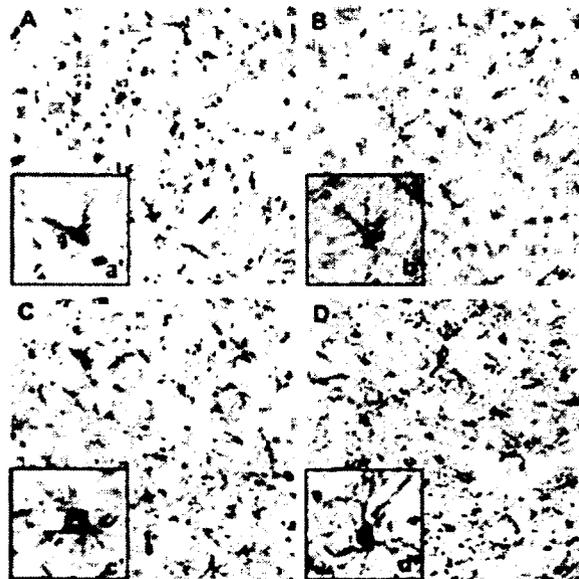
Figure 3.16 Midbrain astroglia responses. Brains from rats were fixed, paraffin embedded and processed for immunohistochemistry. Representative photomicrographs of midbrain sections showing GFAP-immunopositive astrocytes from saline-injected group (A), Saline-injected Water-fed group (B), PQ-injected Water-fed group (C) PQ-injected Placebo-fed group and (D) PQ-injected WS-CoQ$_{10}$-fed group. Magnification: 20X. Insets (a' to d') show a single cell at higher magnification from the respective groups. (E) The number of astrocytes with increased GFAP expression was scored; at least 300-500 cells were counted at 40X magnification in 2 separate experiments. The number of astrocytes in control (Saline-Water group) was taken as 100% and the treated groups were expressed as percentage of control. Statistically significant differences are indicated as: *** between saline-injected group and PQ-injected Water-fed group, $P<$ 0.001; ###, between PQ injected Water-fed group and both PQ-injected Placebo-fed group and PQ injected WS-CoQ$_{10}$ fed group, $P<$ 0.001; HHH, between PQ-injected Placebo-fed group and PQ-injected WS-CoQ$_{10}$-fed group, $P<$ 0.001, (n=2).

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Furthermore, I observed astrocyte activation in the brain upon PQ treatment, especially in the hippocampus region. Preliminary results revealed that increased numbers of activated astrocytes were seen in the PQ-injected rats as compared to the saline-injected rats (Figure 3.17A). Interestingly, I observed that the orientation of astrocytes found above the hippocampus was unique in both the saline and PQ injected rats and there was more activation of these cells after PQ treatment (Figure 3.17B).

A

Saline- Water

PQ-Water

PQ-Placebo

PQ-CoQ10
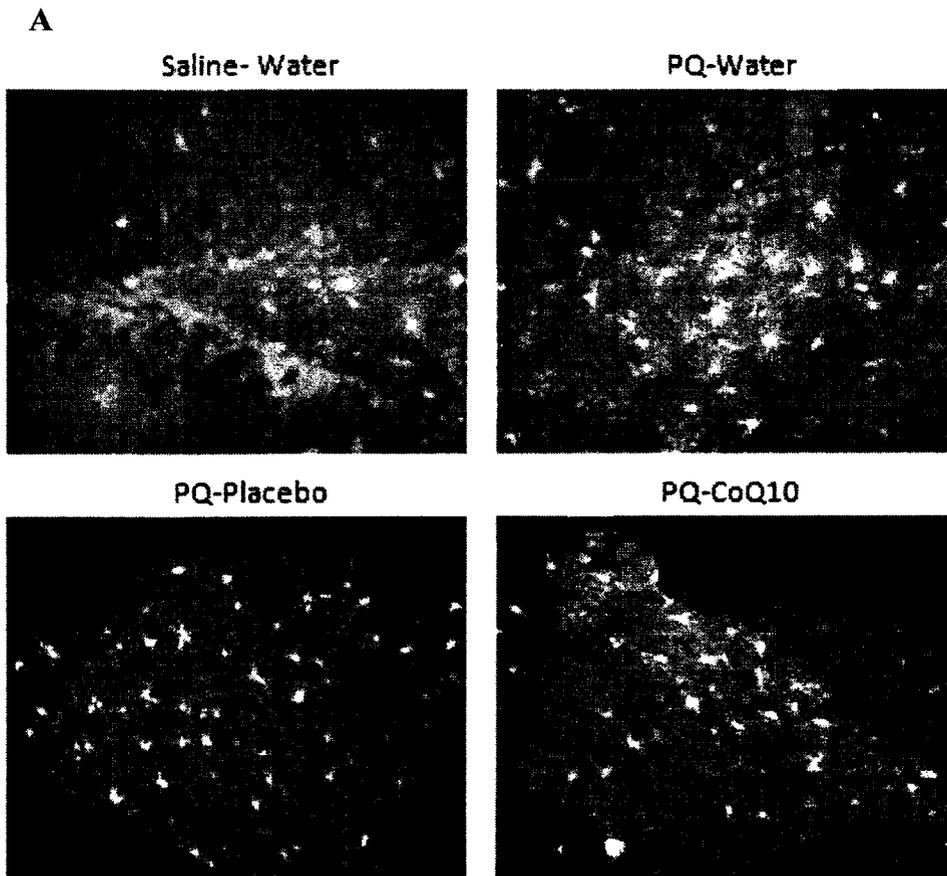
(Please refer to the figure legend on the next page)

B

Saline-Water

PQ-Water

PQ-Placebo

PQ-CoQ10
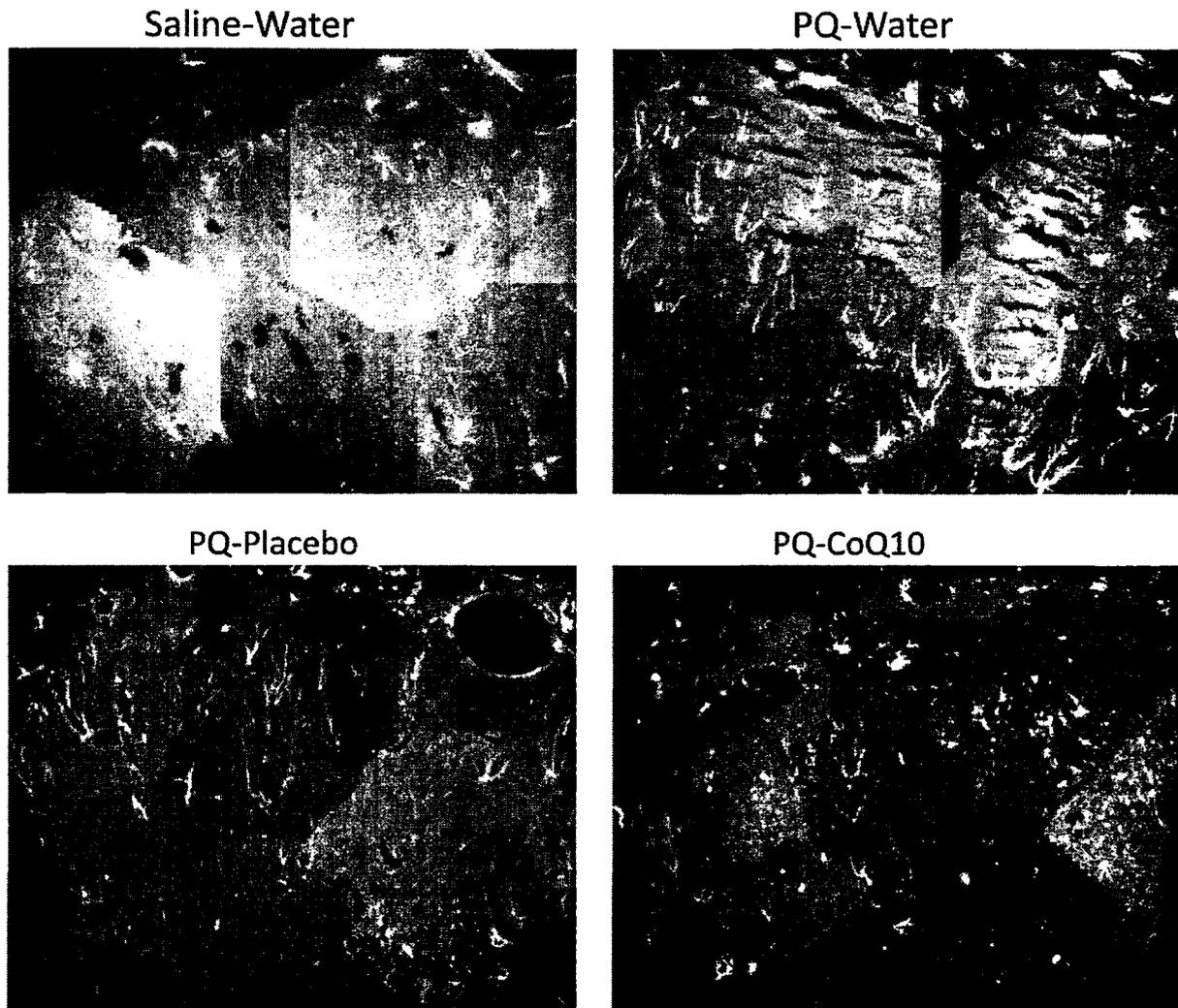
Figure 3.17 Astroglia responses in other brain areas. Brains from rats were fixed, paraffin embedded and processed for immunohistochemistry as described in the Materials and Methods. Representative immunofluorescence images of GFAP–immunoreactive astrocytes from hippocampus region (A), and above the hippocampus region (B) of rats at 40X magnification on a Leica DM IRB microscope. (n=1)

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3.2.6.1 Evaluating the Levels of Different Chemokines and Cytokines after PQ Administration

Cytokines and chemokines are extracellular signaling molecules that mediate communication between cells. They are released from cells and play pivotal roles in many biological processes such as cellular growth, differentiation, migration, gene expression, immunity and inflammation (Borish and Steinke 2003). I observed activation of astroglia in the midbrain and hippocampus after PQ exposure. Since the main communication signals used by microglia are cytokines and chemokines (Rock and Peterson 2006) I then measured the levels of various cytokines, using a cytokine array kit. This assay allowed us to determine the levels of multiple cytokines in a single experiment and to understand if the cytokines being over expressed are neuroprotective, or if they mediate neurotoxicity. Results showed that after injections with PQ, the CNTF, Fractalkine or CX3CL1, sICAM or CD54, LIX, L-Selectin or CD62L / LECAM-1 and Thymus cytokine or CXCL-7 are over expressed. In the PQ-CoQ$_{10}$ group, there was over expression of the cytokine L-Selectin or CD62L / LECAM-1 indicated by the red circle (Figure 3.18). Our results are preliminary and need to be confirmed by further experiments.

	12 34 56 78 910 1112 1314 1516 1718 1920	
A B C D		Rat#1-4 Saline Water
		Rat#2-4 PQ Water
		Rat#3-4 PQ placebo
		Rat#4-4 PQ CoQ10
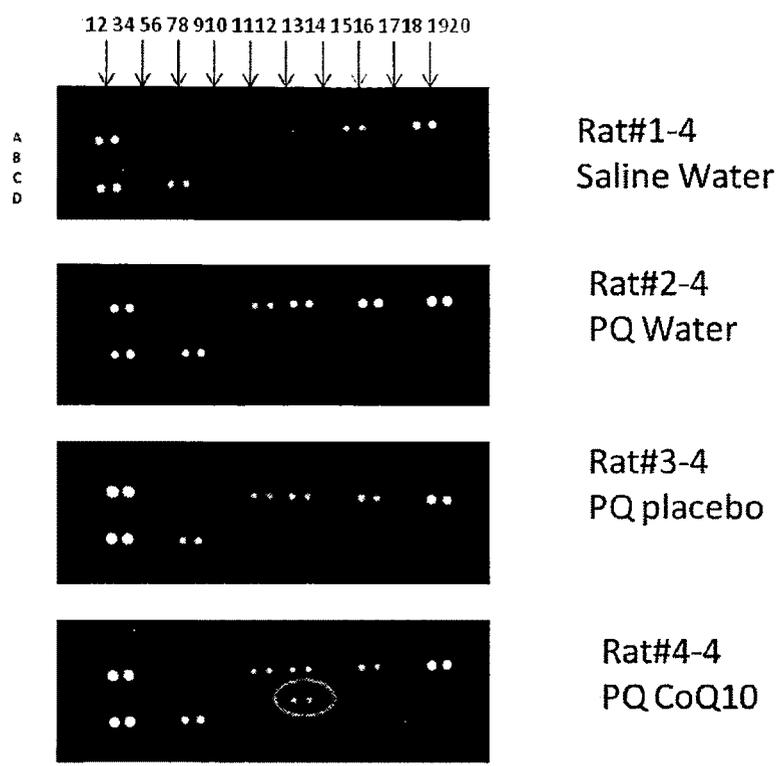
Positive controls	A1,2, A19,20 and D1,2
CNTF	A9,10
Fractalkine or CX3CL1	A11,12
sICAM or CD54	A15,16
LIX	C 9, 10
L-Selectin or CD62L / LECAM-1	C11,12
Negative controls	D19,20
Thymus cytokine or CXCL-7	D5,6

Figure 3.18 Evaluating the levels of cytokines/chemokines. The midbrain homogenates from saline and PQ- injected rats were subjected to a cytokine array. The results show the expression profiles of different cytokines/chemokines.

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3.2.7 Effects of Age on PQ-Induced Toxicity

3.2.7.1 Effect of PQ on Nigral Cells

Microscopic assessment of midbrain sections stained with an antibody against TH showed a clear decrease in the density of TH-positive neurons after the last of the five 10 mg/kg PQ injections. The number of DA neurons was estimated by counting TH-immunopositive cells in the SNc manually. In this preliminary study, I compared the effects of PQ on old rats. The data were normalised to the Saline-Water group which was considered the control group. As shown in figure 3.19(A-B), rats belonging to the PQ-Water group were vulnerable to PQ which caused about 50% reduction of TH-positive neurons when compared to the saline injected counterparts. Rats belonging to the PQ-Placebo showed a decline of about 35%, while those belonging to the PQ-CoQ$_{10}$ group showed only about a 26% loss in the number of TH-positive neurons when compared to control.

A
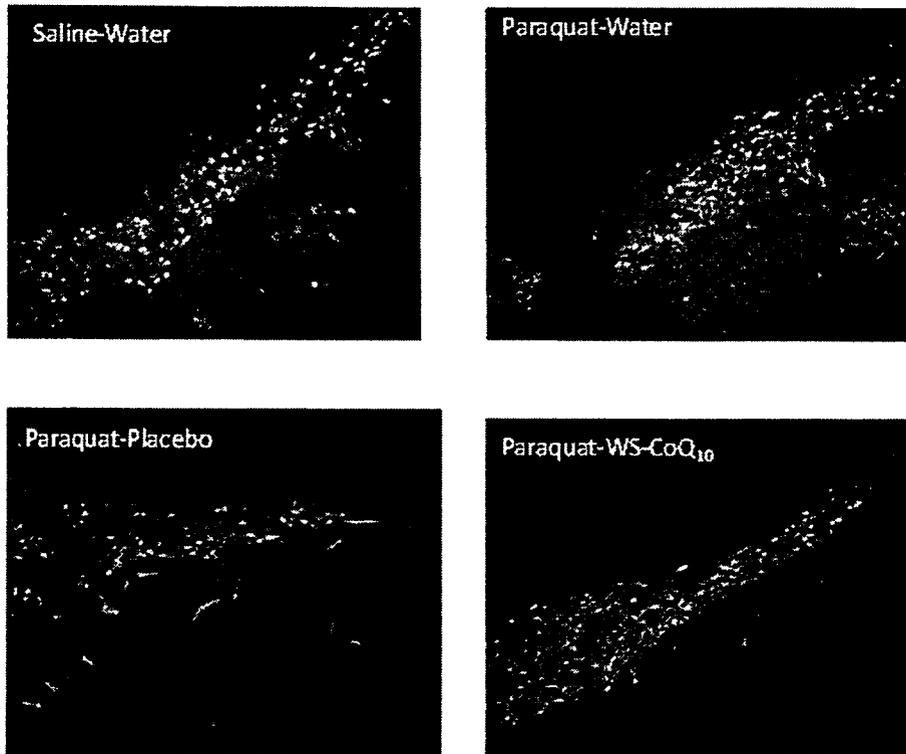
B
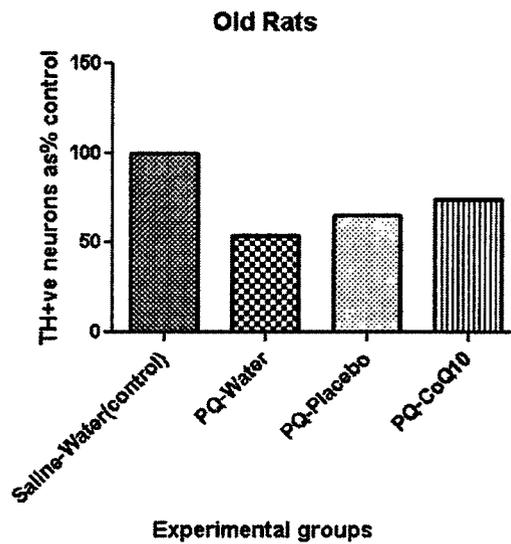
Figure 3.19 Effects of PQ on nigral cells in old rats. (A) Pictures of TH-immuno-stained sections from old rats using the Leica DM IRB microscope. Original magnification 10X. (B) TH-positive neurons were counted manually in the SNc of rats treated with five injections of 10 mg/kg PQ. The data (Mean ±SEM) were normalised to the Saline-Water group which was considered as the control group (n=1).

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3.2.7.2 Biochemical Analysis

3.2.7.2.1 Analysis of GSH Levels

In this study, GSH content was measured from the midbrain. The effect of PQ on levels of GSH in old and young rats was analysed. In the young rats, a loss of about 40% in GSH content in the PQ-Water group as compared to the Saline-Water (control) group was observed (Figure 3.20). In the PQ-CoQ$_{10}$ group, higher levels of GSH were observed. However, in the case of the old rats, the saline-injected rat had a lower level of GSH as compared to the young counterparts. There was a loss of about 60% GSH content in the PQ-Water group when compared to the saline–water rat.
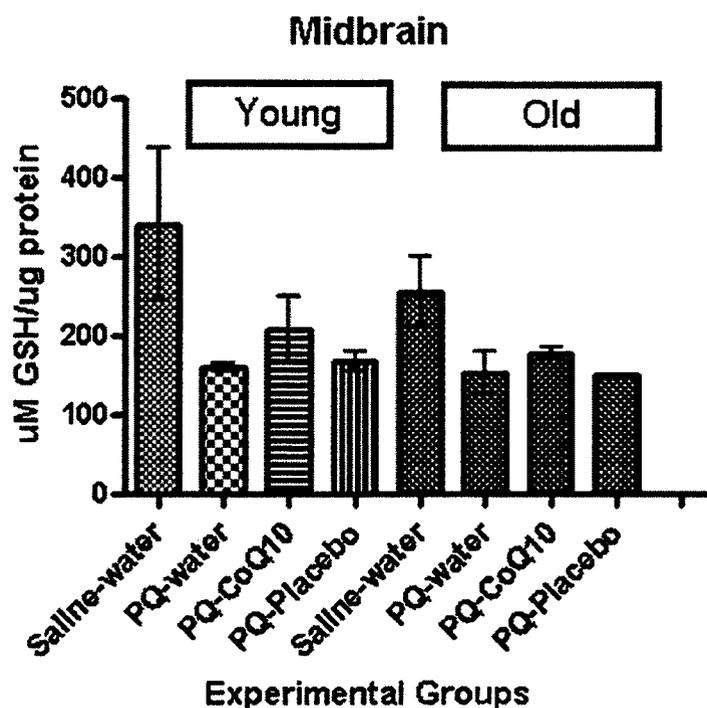
Figure 3.20 Evaluating GSH levels in old and young rats. Rats were sacrificed and midbrain homogenates were subjected to GSH assay. Data (mean ±SEM) is represented as percentage of control, n =2.

3.3 Evaluation of the Probable Mechanism of CoQ$_{10}$ Neuroprotection

A portion of this study to evaluate the mechanism of neuroprotection by WS-CoQ10 was done with Dr. Jafar Naderi.

Studies in our lab have shown that WS-CoQ$_{10}$ can prevent HEK 293 cells from undergoing apoptosis when exposed to hydrogen peroxide-induced oxidative stress (Naderi et al. 2006). H$_2$O$_2$-induced mitochondrial membrane collapse and increased ROS generation is involved in apoptosis (Naderi et al. 2006). Studies in our lab have shown that oxidative stress induced by either H$_2$O$_2$ or paraquat (PQ) leads to mitochondrial dysfunction and loss of mitochondrial membrane potential (Somayajulu et al. 2005, McCarthy et al. 2004). Our studies have also indicated that pre-treatment with WS-CoQ$_{10}$ prevents the loss of mitochondrial membrane potential (Somayajulu et al. 2005, McCarthy et al. 2004).

CoQ$_{10}$ (ubiquinone) is found in inner mitochondrial membrane where it shuttles electrons from complex-I and I to complex-III. I wanted to investigate if protection by WS-CoQ$_{10}$ is based on its ability to act as an anti-oxidant, or if this compound is capable of stabilizing mitochondria under oxidative stress.

In order to examine the role of WS-CoQ$_{10}$ as an anti-oxidant as well as a stabilizer of the mitochondria, I used compounds such as complex-I blockers and Bax protein, which are known to cause damage to mitochondria and then observe the effects of this formulation.

3.3.1 WS-CoQ$_{10}$ Prevents Mitochondrial ROS Production Induced by Complex-I Blockers Such as Rotenone and Paraquat

Mitochondrial dysfunction has been associated with increased production of reactive oxygen species (ROS) (Li *et al.* 2003). Several studies have shown that complex–I inhibitors such as rotenone can induce mitochondrial ROS production (Sousa *et al.* 2003). I was interested in studying the protective effects of WS-CoQ$_{10}$ against oxidative stress induced mitochondrial damage. In order to study the effects of WS-CoQ$_{10}$, isolated mitochondria were pre-treated with WS-CoQ$_{10}$ for 20 min and then treated with 10 µM rotenone. ROS production was measured using a horseradish peroxidase-based PHPA oxidation method. This method is used to detect H$_2$O$_2$ that is produced from intact mitochondria when they are incubated with different mitochondrial chain substrates and inhibitors.

Results from this study indicate that rotenone-treated mitochondria isolated from SH-SY5Y cells show a significant increase in the production ($p<0.05$) of ROS as compared to control (Figure 3.21A). The mitochondria pre-treated with WS-CoQ$_{10}$ and exposed to rotenone showed a significantly lower ROS production compared to those treated with rotenone alone ($p<0.05$). Therefore WS-CoQ$_{10}$ functions at the mitochondrial level and inhibits mitochondrial ROS production induced by rotenone.

A similar study using PQ was also performed. Our results clearly indicated there was an increase in mitochondrial ROS production upon PQ treatment. A significant reduction ($p<0.05$) in ROS production was observed in mitochondria pre-treated with WS-CoQ$_{10}$ and exposed to rotenone (Figure 3.21B). In conclusion, WS-CoQ$_{10}$ reduces mitochondrial ROS production induced by complex I inhibitors.
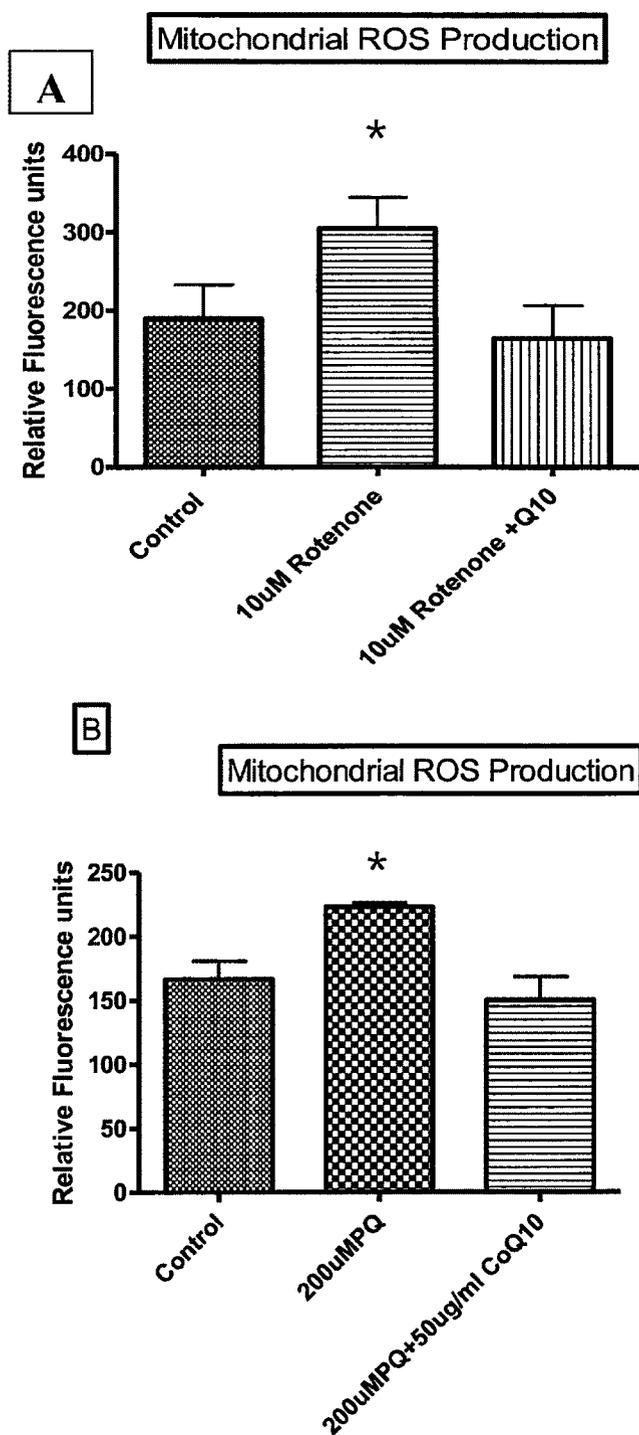
Figure 3.21 Evaluating ROS production in isolated mitochondria. SH-SY5Y cells were grown, lysed,mitochondria was isolated and treated with either 10 μM Rotenone (A) and 200 μM PQ (B) in the presence and absence of WS-CoQ$_{10}$. ROS production was measured using PHPA as described in the Materials and Methods. Statistical significance is denoted by *, $p < 0.05$.

3.3.2 WS-CoQ$_{10}$ Inhibits ROS Generation Induced by Bax in Isolated Mitochondria

Bax is a pro-apoptotic protein of the Bcl-2 family of proteins that translocates to the mitochondria during apoptosis, inducing release of mitochondrial components such as cytochrome c and apoptosis inducing factor (AIF), which activate downstream events that culminate as apoptosis. Previous studies in our lab have shown that incubating Bax with isolated mitochondria leads to the production of reactive oxygen species (ROS) and release of cytochrome c (Gueorguieva *et al.* 2006). In this study I evaluated the neuroprotective effects of WS-CoQ$_{10}$ on Bax-induced mitochondrial damage. Isolated mitochondria from normal HEK293 cells were treated with purified Bax protein to compare the protection offered by WS-CoQ$_{10}$. Our results indicate that ROS production from isolated mitochondria was increased in the presence of Bax, implying that indeed Bax causes mitochondrial dysfunction; however, mitochondria pre-treated with WS-CoQ$_{10}$ had significantly inhibited ROS production (Figure 3.22). These results clearly indicate that WS-CoQ$_{10}$ is capable of directly inhibiting Bax–induced mitochondrial dysfunction in isolated mitochondria.
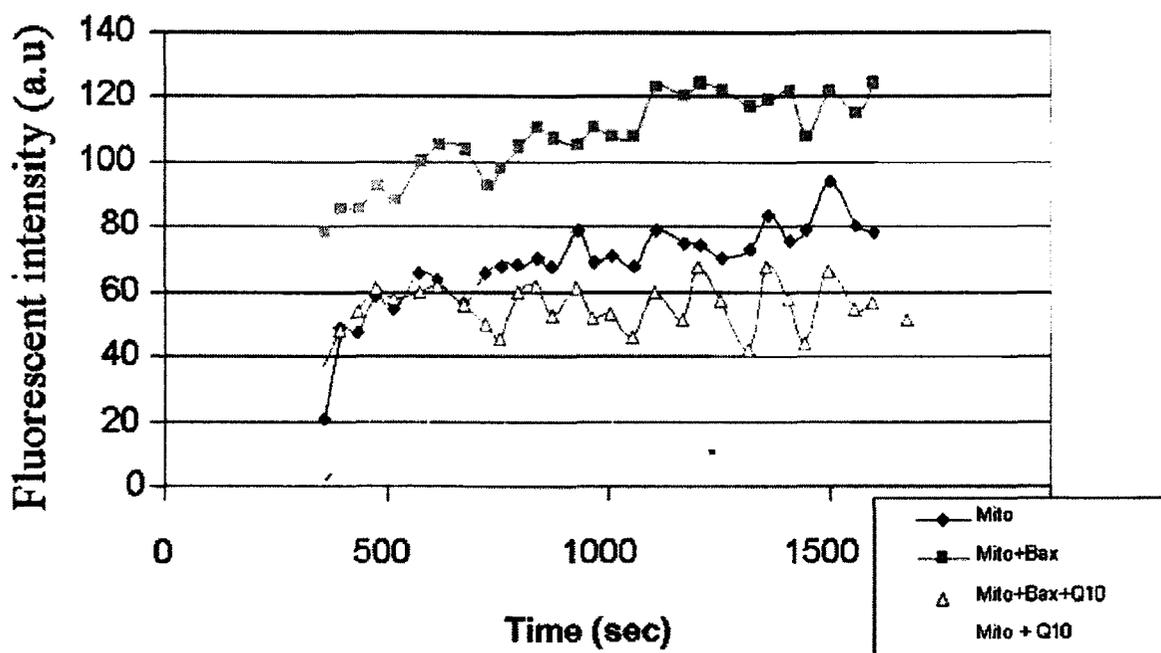
Figure 3.22 Evaluating ROS generation in mitochondria isolated from HEK 293 cells treated with Bax in the presence or absence of WS-CoQ$_{10}$. Isolated mitochondria from 293 cells were incubated with Bax in the presence and absence of WS-CoQ$_{10}$ and ROS production was measured using PHPA as described in the Materials and Methods.

Chapter 4

Discussion

In this study, I have successfully induced DA neuronal loss specific to the SNc in Long Evan's hooded rats by administering PQ. Studies have revealed that PQ induces oxidative stress, mitochondrial dysfunction as well as astrocyte activation. More importantly, I observed neuroprotection by WS-CoQ$_{10}$ against PQ-mediated toxicity. This formulation not only prevented neuronal loss but also offered protection against oxidative stress and mitochondrial dysfunction both as a prophylactic and therapeutic agent. Interestingly, I also observed protection against mitochondrial damage induced by rotenone, PQ and Bax in *in vitro* studies.

4.1 Animal Models used for Studying PD

In order to better understand the pathogenesis, and develop neuroprotective strategies, a good and reliable model of PD is pivotal. Until recently, very little was known about how and why neurodegeneration ensues and progresses in PD. Much of information regarding the etiology and pathogenesis of PD is obtained from clinical studies, post-mortem studies, and epidemiological studies. Accessibility to a human brain is limited because of ethical approval, availability of postmortem brain tissue, and difficulty in drawing definitive conclusions due to individual variability and differences in tissue quality (Emborg 2004). The main characteristic of a PD model should be the loss in DA neurons in the SNc. Although *in vitro* studies have proved to be a tool in deciphering many mechanisms, cell culture systems cannot closely replicate the complex environment in the human brain. Animal models can help to bridge this gap. Compared to

cell culture studies, animal models can evaluate the degree of impairment in motor function observed in PD (Emborg 2004). Rodents are a common choice of animals used to study PD. Rats have been the model choice to study several human diseases. In the context of neurological sciences, they have proved to be an important tool to study processes like neural development, diseases, neurodegeneration, addiction and behavior. There are many advantages of using rats: (1) availability of extensive baseline data for a meaningful interpretation of experimental data, (2) larger size makes live operations feasible and (3) certain physiological similarities of rats to humans (Conn 2007).

Recent studies suggest that PD may arise from a combination of genetic susceptibility and exposure to environmental toxins (McCormack *et al.* 2002, Di Monte 2003, Dinis-Oliveira *et al.* 2006). Indeed, several environmental risk factors such as metals and herbicides have been linked to the incidence and progression of PD (Dinis-Oliveira *et al.* 2006). Amongst these factors, the herbicide paraquat (PQ) shows a clear neurotoxicity in the CNS. PQ was chosen as the toxin to induce Parkinsonism in our studies because PQ is an environmental toxin and also its chemical structure is similar to that of MPP^+ (Figure 6). Moreover, there is strong evidence of correlation between long term PQ exposure and increased risk of PD (Uversky 2004). PQ can enter the CNS through neutral amino acid transporters associated with the blood brain barrier system (McCormack *et al.* 2002) and into dopaminergic neurons via DAT (Ossowska *et al.* 2005a) and damages the SNc neurons in mouse models (McCormack and Di Monte2003). It has also been reported that a prolonged exposure to non-pneumotoxic levels of PQ causes the damage to basal ganglia (Dinis-Oliveira *et al.* 2006).

4.2 Challenges Associated in Finding a Neuroprotective Therapy for PD

There is no perfect model of PD to test strategies for neuroprotection, yet the models in existence do portray the basic characteristics required. Specific requirements need to be met by an animal model of PD in order to enable the testing of neuroprotective strategies. These include: (1) induction a replicable nigral lesion; (2) the loss of DA neurons should occur stably over time without any spontaneous recovery and (3) there should be a window of opportunity in order to design a neuroprotective strategy. A successful neuroprotective agent should be able to offer protection from behavioural impairment, pathological degeneration and neurochemical deficits (Emborg 2004).

4.2.1 Limitations of Current Models used for Neuroprotection

Cell culture systems are very essential tools to screen compounds. Most putative neuroprotective drugs are tested first on cultured neurons to determine whether they can protect cells from a variety of toxins. However, cell culture models do not provide insight into how a drug will behave in a complex organism. In order to evaluate the capacity of a drug to protect DA neurons from toxic insults, its side effects and treatment-associated complications, *in vivo* models are indispensable.

4.2.1.1 Drawbacks of Animal Models used to Study PD

PD a complex disease occurs primarily in the SNc but degenerative changes also occur in the central and peripheral nervous system (Lang 2004a). Several models currently in use, exhibit the characteristic features of PD, but none of them closely mimic the complex features of human PD.

Rodent models (mice, rats) are advantageous because they are easily accessible , their reproduction rates are high and are they are easy to house (only a small living space, simple feeding and drinking schedules is needed) (Emborg 2004). A varied susceptibility exists between species as to how they respond to neurotoxins. For example, mice are more susceptible to MPTP than rats. Besides this, different strains of mice and some even within a given strain exhibit different sensitivity to MPTP (Sedelis *et al.* 2003). Furthermore, age, gender and body weight also play an important role in determining the sensitivity of an animal to neurotoxins (Przedborski and Vila 2001). Similar observations have been made in terms of rats. For example, Lewis rats require two fold higher dosing of 6-OHDA than Fischer or Sprague-Dawley rats. Interestingly, when rotenone is administered in Lewis rats, there is less variability and more consistency compared to Sprague-Dawley rats (Betarbet *et al.* 2002). Evidence of neuroprotection in rodents does not guarantee similar results in non-human primate models, given the larger volume and complexity of a monkey brain. However, translation to human subjects may prove to be even more intricate (Emborg 2004). The advantage of using an animal model is to obtain a phylogenetic perspective about the odds of success when translating a therapy to humans. In summary, due to the several advantages of utilizing animal models, they have been developed to test neuroprotective strategies in neurodegenerative disorders. However, these models are ongoing efforts of improvising and improving existing techniques to evaluate neuroprotective compounds.

4.2.1.2 The Precise Causative Factors of PD are Unknown

One of the biggest challenges in developing neuroprotective strategies is that the exact causative factors of PD remain elusive. While some familial cases of PD are linked with a particular gene mutation, most cases occur sporadically and are idiopathic. If the precise mechanisms underlying cell death in PD could be determined, this may allow the identification of probable targets for the development of putative neuroprotective agents (Olanow *et al.* 2008). Current drugs have focused on blocking oxidative stress, excitotoxicity, mitochondrial dysfunction, and signaling pathways that might be connected with apoptosis. Nonetheless, it remains vague which, if any, of the proposed pathogenic mechanisms that have been implicated in PD is primary and initiates the apoptotic program. It is possible that neuronal cell death in PD occurs as a consequence of an overlap of multiple pathogenic factors that interact in a complex network, contributing to neuronal cell death in different individuals. Interestingly, studies have shown that patients carrying the same gene mutation, and who are family members may exhibit different clinical symptoms and different pathology (Olanow *et al.* 2008). This theory would imply that a combination of various (neuroprotective) agents acting simultaneously on different mechanisms might be required in order to achieve neuroprotection.

4.3 PQ-induced Loss of DA Neurons and Dopamine Deficiency in the Striatum

Since the dopaminergic neurons are densely packed in the SNc, loss of these neurons can be measured by histochemical methods. I have immunostained for TH because this enzyme is often used as a phenotypical marker to determine neuronal loss.

The rate of dopaminergic neuronal loss in the SNc may be accelerated by local factors to which these neurons may be uniquely vulnerable. These factors include free radical formation, reduced protection from oxidative stress, high levels of DAT, increased iron concentrations, increased glutamate input and neuromelanin formation (Lang 2004b).

Our data clearly confirmed the toxicity of systemically administered PQ, especially towards DA neurons of the SNc. Thus, in the first phase of this study, this herbicide effectively killed approximately 65% DA neurons in adult male Long-Evans hooded rats and induced PD-like symptoms (Figure 3.2). Similar results have been shown by (Brooks *et al.* 1999). They used C57BL/6J strain of mice and used 3 injections of 10 mg/kg paraquat. Their study revealed significant losses of TH positive neurons in the substantia nigra pars compacta (~61% loss) and their terminals in the striatum (~90% loss) after treatment with paraquat at the same dosage of the neurotoxin used in our study. Furthermore, low dose PQ-treated mice (5 mg/kg) showed a 36% loss of dopaminergic cells (Brooks *et al.* 1999).

Furthermore, in the second phase of this study, our preliminary results with older animals showed a loss of about 50% neurons in the substantia nigra pars compacta (Figure 3.19). This is certainly higher than the 35% loss seen in younger animals (Figure 22). A loss in TH expression levels was clearly observed in all the animals treated with PQ when compared to saline injected counterparts (Figure 3.12). Older animals, like humans, develop age-related changes in the nigrostriatal system, which resemble the early stages of nigrostraital degeneration. The extents of these changes vary from species to species (Emborg 2004). McCormack *et al.* showed a loss of about 25% in the dopaminergic neuronal numbers in C57BL/6 mice which were exposed to 10 mg/kg paraquat for three weeks (McCormack *et al.* 2002). Furthermore, their study revealed an

increased loss of dopaminergic neurons in the SNc of older animals when compared to their younger counterparts following PQ injections. Eighteen month-old animals exposed to PQ lost about 35% DA neurons, while five month-old animals showed a loss of about 25% dopaminergic neurons. Similar results have been obtained from paraquat maneb (PQ/MB) studies in rats (Sprague-Dawleys), where the authors have revealed that older animals are more susceptible to brain toxicity from PQ/MB exposure (Thiruchelvam *et al.* 2003, Saint-Pierre *et al.* 2006). However, there exists a lot of discrepancy in data regarding the effects of age on paraquat toxicity. A recent study in mice has shown that paraquat toxicity is age independent and demonstrated that young animals are as susceptible as old rats to this particular neurotoxin (Peng *et al.* 2007). Studies with six to eight rats (per group) will be needed to establish statistical significance and confirm preliminary results.

Given the fact that there is variability in the way animals respond to paraquat treatment, discrepancy in our data is not surprising. There are conflicting reports about the effects of paraquat following systemic exposure in mice (Bové *et al.* 2005). Also there is a strong indication of the variability in the response to toxins within different species of rats (Emborg 2004). Studies with rotenone have indicated that there is variability within a batch of animals as to how they respond to rotenone treatment. It has been reported that while some animals were sensitive to rotenone treatment, others did not respond to treatment at all (Betarbet *et al.* 2002).

I noticed that eight injections of 10 mg/kg PQ alone and 10 mg/kg PQ along with 30 mg/kg maneb (Thiruchelvam *et al.* 2002) proved to be fatal for Long Evans hooded rats. Animals treated with eight injections of 10mg/kg paraquat alone (twice a week for four weeks) regime in our lab suffered fatalities before completion of eight injections.

However, in the first batch, 3 animals (1 from PQ-Water, 1 from Saline-CoQ$_{10}$, 1 from PQ-Placebo) did not complete the injection regime and died halfway. Therefore I fixed 2 animals from each group in formaldehyde and froze 1 animal from each group to perform biochemical analysis. In the second batch, 8 animals (4 from PQ-Placebo, 3 PQ-CoQ$_{10}$, 1 from saline- water) did not complete the treatment and died about halfway through the treatment. I fixed all the animals with 10% buffered formaldehyde and used them for histochemistry. There were no drastic reductions in weights between the saline and PQ injected animals (data not shown). Since we did not have statistically sufficient animals, this study was used for standardising the protocols for immunohistochemistry. However, this dose seems to be well tolerated by Sprague Dawley rats and C57BL/6 mice (Thiruchelvam *et al.* 2000a,b, Cicchetti *et al.* 2005). This indicates that Long Evan's hooded rats may be more sensitive to toxins.

Assessment of catecholamine contents in the nigrostriatal system provides a profile of the production as well as metabolism of dopamine. Previous reports have indicated that systemic administration of PQ significantly decreased the level of DA and its metabolites in the striatum. PQ decreased DA level by 20%, DOPAC and HVA decreased by 35% and 52%, respectively, in mice treated with PQ (Chen *et al.* 2008). A loss of about 30% in dopamine levels has been reported in mice after exposure to PQ/MB (Thiruchelvam *et al.* 2000a,b). Future experiments will focus on measuring dopamine and its metabolites.

4.4 Specificity of PQ-induced Loss of Neurons

Our results have indicated that PQ administration in rats causes the loss of DA neurons in the SNc. However, immunostaining to count serotonergic and cholinergic neurons has to performed to ensure specificity of PQ. Studies have shown that PQ enters DA neurons via DAT present on these neurons (Ossowska *et al.* 2005b). However, toxicity to other areas of the brain has not been very well documented. For this reason, I decided to use a general marker for neurons, namely NeuN, to stain various areas of the brain during the five injection PQ regime. NeuN is a neuronal-specific protein (Mullen *et al.* 1992) that is frequently used as a neuronal marker for viable and intact neurons (Xu *et al.* 2002, Hassen *et al.* 2004). Immunohistochemistry of NeuN shows that the marker is visible both in nuclei and in portions of the cytoplasm of neurons, such as the cell body, axons and dendrite processes (Mullen *et al.* 1992). Data from the second phase of study clearly revealed that even with a higher dose of PQ, damage was caused specifically to the DA neurons in the SNc. Results indicated a decrease in NeuN-immunopositive neurons in the SNc following PQ injections, but no difference in NeuN immunostaining in other areas of the brain; e.g. hippocampal region and cortex (Figure 3.13 A,B) between the saline and PQ groups was observed. McCormack *et al.* (2002) also reported the specificity of PQ-induced DA loss in mice. Their study revealed that PQ does not target the hippocampus or the neurons in the substantia nigra *pars* reticulata (SNr) which are anatomically adjacent to the SNc where PQ causes damage. Future experiments will examine the neuronal counts in the ventral tagmental area (VTA) as well as the SNr.

I also stained the brain sections with H&E and pathologists confirmed that no gross changes in morphology occurred in the brain of rats treated with PQ (Figure

3.13D). Furthermore, the intraperitoneal treatment did not cause any damage to the liver or the kidneys (as assessed by pathologists) (Figure 3.15). Damage to lungs due to acute dosing of PQ has been shown (Dinis-Oliveira *et al.* 2006). However, due to technical difficulties, the analysis of the lungs could not be performed.

4.5 The Mechanism of PQ Toxicity

The mechanism of PQ neurotoxicity appears to be mediated by oxidative stress. Superoxide anion radicals are generated through redox cycling reactions of PQ with oxygen as well as with NADH-oxidoreductases (Peng *et al.* 2007) These superoxide anion radicals can then lead to the formation of ROS (Figures 1.7, 1.8). The damage inflicted by ROS in tissues can be evaluated by measuring the modifications to biomolecules such as proteins, DNA and lipids.

Studies have shown that there is a loss in the GSH during oxidative stress. Again, results revealed a reduced content of GSH in the brains of PQ-treated rats, consistent with its utilization to combat the effects of PQ (Figures 3.3, 3.9, 3.20). Recent studies have pointed out that PQ induces alternation of GSH levels in the SNc in mice. Levels of GSH in the SNc have decreased more for the PQ group (10 mg/kg) than for the control group, while levels of oxidized glutathione (GSSG) increased in the SNc region (Kang *et al.* 2009).

Lipid peroxidation is another method to monitor the oxidative damage caused by PQ. The end products of lipid peroxidation are usually 4-hydroxy nonenal (4-HNE) and malondialdehyde (MDA). I also attempted to measure the levels of lipid peroxidation by measuring these end products. Immunostaining procedures for 4-HNE have been

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standardized and will be used to complement the data. I have successfully measured the levels of MDA using thiobarbituric acid assay. Our results show increased levels of lipid peroxidation in the SNc in PQ treated animals (Figures 3.5, 3.10). Similar results have been shown in mice treated with PQ exposure caused an increase in the number of midbrain cells positive for 4-hydroxy-2-nonenal, a marker of lipid peroxidation (McCormack et al. 2006). Increased lipid peroxidation in the striatum and midbrain regions of mice exposed to PQ (Thiruchelvam et al. 2005). Increased lipid peroxidation could lead to the changes in the membrane properties and affect cellular homeostasis (Dinis-Oliveira et al. 2006).

Oxidative stress also leads to mitochondrial dysfunction. Production of ROS leads to increased formation of superoxide anion radical which can then form peroxynitrite by reacting with nitric oxide. This peroxynitrite can impair the mitochondrial respiratory chain and lead to decreased ATP synthesis (Ebadi and Sharma 2003). Consistent with these phenomena, I also found reduced ATP levels in rat brains following PQ exposure (Figures 3.6, 3.11). Postmortem degradation of ATP is known to occur during tissue extraction (Delaney and Geiger 1996). Since, the experimental conditions were the same for all the rats dissected, the postmortem degradation of ATP is relatively the same for all animals. Moreover, I have measured the relative levels of ATP and compared the results between different groups. The solution to this issue is using a high-energy focused microwave irradiation method, which involves rapid inactivation of enzymes and allows for the measurement of endogenous ATP (Delaney and Geiger 1996). Using in vitro assays, I have shown that PQ can destabilize the mitochondria from differentiated human neuroblastoma cells (McCarthy et al. 2004). Therefore, there is strong evidence

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suggesting that dysfunctional mitochondria and oxidative stress are most likely the significant contributors to the PQ-induced neurotoxicity.

Systemic exposure of rodents to PQ, alone or in combination with the maneb, mimics the pathological features of PD, such as the intra-neuronal deposition of α-synuclein as well the selective degeneration of DA neurons in the SNc (Manning-Bog *et al*. 2002, McCormack *et al*. 2002, Peng *et al*. 2004). In order to evaluate the levels of α-synuclein in the frontal cortex and midbrain, western blot analysis was performed. However, our results did not show any increase in levels of α-synuclein (Figure 3.12). Although preliminary, this study needs to be repeated with more animals. Similarly no increase in the levels of Parkin was observed in our study (Figure 3.12).

I also tried to estimate the levels of iNOS but was not successful. Once activated, iNOS produces nitric oxide which can react with superoxide anion radical to form peroxynitrite. One potential target of peroxynitrite is tyrosine hydroxylase (TH). Inactivation by nitration of TH by peroxynitrite appears to be a key process in the development of PD in humans (Symeyne and Jackson-Lewis 2005).

Dopamine is also subjected to free radical attack especially by hydroxyl radical and autooxidation in the extracellular space. Moreover, dopamine can also be nitrated intracellularly and therefore may cause degeneration of neurons containing this neurotransmitter (Symeyne and Jackson-Lewis 2005).

Oxidative stress, c-Jun N-terminal kinase activation, and α -synuclein aggregation are each induced by PQ, but details of the cell death mechanisms involved remain unclear. Recently, a Bak-dependent cell death mechanism for PQ-induced neurotoxicity has been reported. PQ-induced biochemical features that were consistent with apoptosis,

included cytochrome *c* release, with subsequent caspase-3 and poly (ADP-ribose) polymerase cleavage (Fei *et al.* 2008).

In order to make a model of neurodegeneration functionally relevant, a behavioural component is necessary. Consideration of PQ as a candidate neurotoxicant requires that systemic delivery not only produces DA neuron loss in the SNc, but also exhibits the resultant neurobehavioral syndrome reflecting depletion of dopamine terminals within the striatum. Decreased motor activity has been reported following PQ exposure in mice (Brooks *et al.* 1999). Stepping impairments can be assessed using a rotorod. Stepping impairments are assessed by counting the number of adjusting steps taken by a rat while moving on a rotating rod. While the animals move on the rotorod, they are videorecorded from the front, lateral and posterior views. The recorded behaviour is then analysed frame by frame (Whishaw *et al.* 2003). I was interested in evaluating the translational effects of loss in DA neurons to change in behaviour. In the first phase of our study, PQ affected rats showed clear signs of deficiency in fine motor control as indicated by a reduced tendency to turn around and walk backwards on the rotorod (Figure 3.7). The data for the second phase is currently being analysed.

4.6 Neuroprotective Effects of WS-CoQ$_{10}$

Our results indicate that PQ causes a loss in DA neurons in the SNc, induces oxidative stress, mitochondrial dysfunction as well as behavioural deficits. Furthermore, I have established that the brain damage and the performance deficits could be minimized by giving the animals WS-CoQ$_{10}$, which contained WS-CoQ$_{10}$ and derivatised vitamin E and was provided throughout the duration of the experiment. I eliminated the

neurotoxicity of PQ by providing the animals with drinking water containing WS-CoQ$_{10}$, administered three weeks prior to PQ exposure and throughout the duration of the experiment. Data have also shown that placebo does offer some protection against oxidative stress; however placebo alone is not sufficient to protect the neurons from undergoing neuronal death induced by PQ. The placebo formulation consists of vitamin-E and poly ethylene glycol. Coenzyme Q$_{10}$ (CoQ$_{10}$) on the other hand, works at the mitochondrial level and does prevent neuronal cell death induced by PQ. The preliminary studies using WS-CoQ$_{10}$ as a therapeutic agent also showed the ability of this compound to offer protection after inducing damage to the nigral cells with PQ. Despite statistical lack of significance due to a small group of rats used in this study, this formulation has shown that it can protect the rats against PQ toxicity.

My data adds to the mounting evidence that antioxidants, especially CoQ$_{10}$ and vitamin E, are important for the management of neurodegenerative diseases (McDonald *et al.* 2005). Vitamin E has been tested for its potential as a neuroprotectant, however, it proved to be a failure (Beal 2001). Neuroprotective effects of CoQ$_{10}$ in the CNS has been extensively evaluated (Beal and Matthews, 1997, Cleren *et al.* 2008) and numerous *in vivo* studies demonstrate its protective role against experimental ischemia, sparing the levels of GSH and ATP (Beal 2003, Sikorska *et al.* 2003). CoQ$_{10}$ is a highly hydrophobic, naturally occurring compound that primarily functions in mitochondrial membranes as a diffusible electron carrier of the mitochondrial respiratory chain complexes. It is also a powerful antioxidant readily scavenging for free radicals (Beal 2003). Its pharmaceutical applications, however, seem to suffer from the lack of solubility and low bioavailability, both which are needed to achieve therapeutic effect. An open-label phase I clinical trial of CoQ$_{10}$ in PD patients reveal good absorption and

tolerance of CoQ_{10}, however, very high dosages were required (up to 1200 mg per day) to achieve some beneficial effects. Although very encouraging results were acquired from this study, the authors express a concern that PD patients would have to take such a high dosage of CoQ_{10} (Shults et al. 2002, Shults et al. 2004).

A recent study has shown that oil soluble Tishcon CoQ_{10} formula provided significant protection against MPTP toxicity in a mouse model (Cleren et al. 2008). However the effective doses used in this work were 200 mg/kg/day -1600 mg/kg/day (equivalent to 14 g/day-114 g/day for a 70 kg patient). This dose is extremely high and unlikely to be used in human patients. On the other hand the effective daily dose of water soluble formulation used in my study that offered significant neuroprotection is 5 mg/kg/day in rats, roughly one fourth of the dose used in the aforementioned clinical trial and 40 times less than the dose used by (Cleren et al. 2008) in mice. The more recently developed water soluble formulation of CoQ_{10} (WS-CoQ_{10}) has been reported to be more effective as it combines two potent antioxidants, i.e., derivatised vitamin E (PTS) and CoQ_{10} (Sikorska et al. 2003, Borowy-Borowski et al. 2004, www.Zymes.com). PTS is a pro-drug form of vitamin E (a-tocopherol), which was chemically derivatised by sebacic acid and polyethyleneglycol (PEG) and used a component (carrier) in WS-CoQ_{10} formulation (Sikorska et al. 2003). These two compounds form a stable and water soluble complex thereby causing them to be easy to deliver and test (i.e., in drinking water). The effective daily dose of this formulation that offered significant neuroprotection in our study will translate to 350 mg/day for a 70 kg human subject (roughly one fourth of the dose used in the aforementioned clinical trial). Furthermore, rats fed with the WS-CoQ_{10} containing diet have shown elevated plasma levels of CoQ_{10} (Sikorska et al. 2003). Due to the water-soluble nature of our formulation, I have done extensive work on its effect as

neuroprotective agent in neuronal cell cultures (Sandhu *et al.* 2003, McCarthy *et al.* 2004, Somayajulu *et al.* 2005). In my previous *in vitro* studies I have shown that this formulation of CoQ_{10} protects differentiated SHSY-5Y cells against PQ toxicity by stabilizing mitochondrial membranes, maintaining mitochondrial membrane potential and sustaining ATP production (McCarthy *et al.* 2004). More recently, I established that it inhibits Bax activity and prevents Bax-induced destabilization of mitochondria in mammalian cells (Naderi *et al.* 2006). Thus this formulation has advantages over the current formulations being used.

4.7 Elucidating the Probable Mechanism of Neuroprotection by WS-CoQ10.

Studies using PQ have shown that WS-CoQ_{10} can offer neuroprotection *in vivo*. This formulation decreased the numbers of DA neurons undergoing cell death as well as protected against PQ-induced oxidative stress and subsequent mitochondrial dysfunction. *In vitro* studies have shown that WS-CoQ_{10} can offer neuroprotection against glutamate toxicity, PQ toxicity and also hydrogen peroxide induced toxicity (Sandhu *et al.* 2003, McCarthy *et al.* 2004, Somayajulu *et al.* 2005).

This raises the question: Is the neuroprotective ability of WS-CoQ_{10} solely based on the fact that it is acts as an anti-oxidant? However, this is not the case. It has been observed that when only the reduced formulation of CoQ_{10} is used as a neuroprotective agent, it is toxic to the cells and could not protect them against oxidative stress (Sikorska *et al.* personal communication). Interestingly, results using equimolar concentrations of reduced and oxidized forms of CoQ_{10} offered neuroprotection against oxidative stress and other apoptotic inducing agents. Vitamin-E and polyethylene glycol are used as carriers

to solubilize CoQ_{10} and help in the uptake of this compound by the cells. Studies aimed at evaluating the bioavailability of this formulation established that cells were capable of internalizing WS-CoQ_{10} when it was added to the media as an aqueous solution (Borowy-Borowski et al. 2004). A 3-day exposure to10 µg/ml of WS-CoQ_{10}, caused an increase in cellular mitochondrial membranes from 13.3 ng/10^6 cells to 27.5 ng/10^6 cells and an increase in membranes from 0.7 to 6.0 ng/10^6 cells. The treatment also caused an increase in total cellular levels of ATP as compared to untreated cells (Sandhu et al. 2003). This formulation also further resulted in the prevention of mitochondrial collapse under oxidative stress induced by PQ in vitro (McCarthy et al. 2004). These studies have indicated that this formulation does enter the mitochondrial membrane and stabilize the mitochondrial membrane by increasing the efficiency of the electron transport chain (ETC). If the electrons are transferred efficiently via ETC, then the conversion of oxygen to superoxide anion radical can be prevented. Our studies using isolated mitochondria have shown that WS-CoQ_{10} can offer neuroprotection against rotenone and PQ-induced toxicity (Figure 3.21).

More interestingly, in vitro studies have indicated that WS-CoQ_{10} can prevent cells from Bax-induced mitochondrial damage (Figure 3.22) (Naderi et al. 2006). Recent studies have reported the involvement of Bak in mediating the toxicity of PQ (Fei et al. 2008). Bax belongs to the family of pro-apoptotic factors which also include Bad, Bax, Bid, Bak and Bim amongst others and can permeabilize the outer mitochondrial membrane or bind to the voltage dependent anionic channel (VDAC) and cause the opening of this channel. VDAC is a component of the mitochondrial permeability transition pore (PTP). Bax binding therefore can lead to the leakage of cytochrome c, and other factors from the mitochondria to the cytosol, which can induce apoptosis (Naderi et

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al. 2006). Two mechanisms of neuroprotection by WS-CoQ$_{10}$ are possible in this scenario: (1) WS-CoQ$_{10}$ can associate with Bax and prevent its binding to mitochondria and (2) WS-CoQ$_{10}$ can bind to the VDAC and prevent the binding of Bax to the channel. Previous studies have shown that there are ubiquinone binding sites on the PTP. Furthermore, binding of ubiquinone to PTP can inhibit the opening of the pore (Fontaine *et al.* 1998).

Results from my study indicate that PQ as well as PQ-WS-CoQ$_{10}$ may cause the activation of astroglia, a feature of neuroinflammation. Therefore it is speculated that WS-CoQ$_{10}$ can cause astrocyte activation, which could be that be neuroprotective. However, I do not have sufficient data for extrapolation currently.

4.8 Role of Astroglial Activation in Neuronal Cell Death and Neuroprotection

There appears to be a great amount of communication between neurons and non neuronal milieu. Astrocytes are the most abundant cell type in the CNS and provide an essential metabolic support to neurons, including regulation of ion homeostasis, GSH metabolism, free radical scavenging and regulation of the blood-brain barrier. GSH acts as an important intracellular antioxidant due to its ability to detoxify lipid and organic peroxides including peroxynitrite. Activation of glial cells in the CNS in response to injuries or neuropathological processes is well known and documented phenomenon, although not always linked to neuroprotection (Eddleston and Mucke 1993, Drukarch *et al.* 1998, Hailer *et al.* 2001, Liberto *et al.* 2004, Hanisch 2007) Although preliminary, I have observed in this study, the activation of astrocytes in all the PQ-treated groups after GFAP-immunostaining (Figure 3.17) as well as an increased expression of this protein in

western blot analysis (Figure 3.12). Results from my study are intriguing because astrocyte activation was also increased in PQ-WS-CoQ$_{10}$ treated animals. Surprisingly, the levels of activated astrocytes were not reduced in the presence of WS-CoQ$_{10}$. This suggests that WS-CoQ$_{10}$ may cause the release of neuroprotective cytokines and this is novel (Figure 31). I observed an increase in the expression of the cytokine L-Selectin or CD26L/LECAM-1. Further studies are needed to completely understand the neuroprotective role of inflammation in PD, while using WS-CoQ$_{10}$ as a neuroprotectant.

Activated astrocytes produce neurotrophic factors (i.e., Nerve growth factor (NGF), Glial cell derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), cytokine ciliary neurotrophic factor or CNTF), scavenge excess of neurotoxic glutamate and may provide energy substrates for neurons and perform the neuroprotective function. For example, astrocytes store and subsequently metabolize glycogen to sustain their own energy requirements. They can also export glucose and/or lactate to support energy demands of neighbouring neurons (Dringen et al. 1993, Brucklacher et al. 2002). My data is consistent with this scenario as the brain steady-state ATP content remained unchanged in PQ-treated rats under the neuroprotective conditions. In addition, astrocytes are equipped with a robust antioxidant system and play a major role in GSH metabolism and neuroprotection (Peuchen et al. 1997, Wilson 1997, Dringen et al. 2000, Drigen and Hirrlinger 2003). PQ exposure causes a time-dependent increase in the number of cells with immunohistochemical and morphological features of activated microglia (Purisai et al. 2007). Multiple treatments with PQ or PQ and maneb have been associated with activation of microglia, although the relationship between this effect and neurodegeneration has yet to be demonstrated (McCormack et al. 2002, Saint-Pierre et al. 2006).

4.9 Conclusions

In this study, I have successfully induced neuronal loss specific to the SNc in Long Evan's hooded rats by 10 mg/kg PQ treatment. Results have revealed that PQ induces oxidative stress, mitochondrial dysfunction, astrocyte activation as well as reduced motor function. More significantly, I have observed that WS-CoQ$_{10}$ offers protection against PQ-mediated toxicity by not only preventing neuronal loss but also, offering defense against oxidative stress, mitochondrial dysfunction and improves motor function both as a prophylactic and therapeutic agent. Furthermore, *in vitro* studies revealed protection against mitochondrial damage induced by rotenone, PQ and Bax. PD is diagnosed usually after a substantial loss of DA neurons has occurred. The aim of this study is to protect the remaining neurons from undergoing cell death and thus improving the quality of life of PD patients. This study is, in fact, the first pre-clinical evaluation of WS-CoQ$_{10}$ as a neuroprotective (prophylactic) agent as well as therapeutic (restorative) agent to prevent the loss of DA neurons.

4.10 Future work

Majority of the time and effort was spent on standardizing the protocols for immunohistochemistry, behavioural studies and the biochemical studies. Results obtained from the second phase of the study were performed using fewer numbers of rats. These preliminary studies need to be repeated in order to obtain statistically significant data. Also, using a larger sample of rats will be beneficial to minimize the issue of variability in how the rats respond to PQ treatment.

This work has significance for finding neuroprotective strategies to arrest the progressive loss of DA neurons in PD. Interestingly, under all circumstances; I have observed the neuroprotective ability of WS-CoQ$_{10}$. I have initiated the experiments to evaluate the role of this formulation as a therapeutic agent. Therapy for PD begins after diagnosis, by which time a large number of DA neurons have already been lost. For a candidate to be considered as a good therapeutic/ restorative agent, it should be able to arrest the further loss of neurons and thereby prevent the progression of this disease. Further experiments will be needed to confirm our studies aimed at evaluating the potential effects of WS-CoQ$_{10}$ as a therapeutic intervention in PD (post-injection feeding). Also, the experiments analyzing the effects of age on neuronal loss as well as neuroprotection by WS-CoQ$_{10}$ need to be thoroughly investigated. Many of the biochemical and immunohistochemical assays need to be repeated due to small sample size used in the current experiments.

Bioavailablity of WS-CoQ$_{10}$ is very important because it is essential to know how much of WS-CoQ$_{10}$ can cross the blood brain barrier. Therefore the bioavailability of WS-CoQ$_{10}$ in the brain tissue as well as the mitochondria in neuronal cells will be evaluated. The next set of experiments will include determining levels of WS-CoQ$_{10}$ in the brain and neuronal mitochondria by HPLC. Toxicopathology of WS-CoQ$_{10}$ needs to be performed to determine if it is safe for human consumption as this compound is a potential candidate for clinical trials.

Of equal importance is to determine the levels of striatal dopamine and its metabolites in PQ treated rats because one of the neurochemical features of PD is the loss of dopamine in the striatum. HPLC will be used to determine the levels of striatal dopamine and its metabolites.

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Further studies need to be performed to reveal the role of neuroinflammation in development of this disease. Preliminary studies have revealed astrocyte activation in PQ-WS-CoQ$_{10}$ treated groups. There is a significant implication, and to analyze under what circumstances inflammation is protective or toxic is very important.

Behavioural data for the second phase of the study is currently being compiled. Furthermore, new tests are being standardised to assess behavioural impairment. Several clinical studies have reported that exercise and balance training in the early stages of PD improves overall muscle strength, balance, motor performance, and ambulation in patients (Miyai *et al.* 2000, Bergen *et al.* 2002, Hassen *et al.* 2004). A recent study on mouse model of PD has shown that endurance exercise training effectively reversed PD-like behavioral deficits related to regular movement and balance (Konstantinos *et al.* 2009). Therefore, studies will be aimed at testing if endurance exercise training might enhance the effectiveness of WS-CoQ10 treatment.

Finally, the mechanism of protection of WS-CoQ$_{10}$ needs to be elucidated.

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Contributions to this project

<u>Honours' Students 2004 - present</u>:

2004-2005

Jennifer Vergel de Dios   helped with biochemical analysis for phase one studies.

2006-2007

Spencer Hills   helped standardizing protocols for histochemistry, involved in sectioning as well as staining.

2007-2008

Anca Matei   performed the biochemical analysis for one batch of the 5 injection (therapeutic and prophylactic effects of WS-CoQ$_{10}$) regime.

Edward Schwartzenberger   helped with injections, dissections and performed the immunohistochemistry for 8 injection regime, standardized (DAB), 4-HNE, alpha-synuclein, and GFAP staining. He also performed TH immuno-staining for 5 injection regime (therapeutic and prophylactic effects of WS-CoQ$_{10}$). He worked with us for 2 years and so has helped immensely with homogenizing tissues, dissections, and performing Bradford assays.

2008-2009

Kristen Church – Performed TH- immunohistochemistry, counted TH-positive neurons and also performed GFAP and NeuN immuno-staining, as well as H&E staining for the different tissues in the 5 injection regime. Performed all the western blots for Anca's thesis in 2007-8 when she was a volunteer.

<u>Other students involved:</u>

1. Carly Griffin: Graduate student (cancer project). Helped with dissections.

2. Sudipa June Chatterjee: Graduate Student (cancer project). Helped with dissections and flash freezing tissues for 5 injection animals.

3. Natasha Rafo: 4th year Honour's student for the year 2009-2010. Has helped with injections, dissections, perfusions as well as flash freezing tissues for the 5 injection animals.

4. Parvati Dadwal: Volunteer 2008-current. Undergoing training for injections, dissections, perfusions, flash freezing tissues. Actively involved in processing tissues for histochemistry, sectioned brain tissues for a couple of animals from the 5 injection animals (old vs. young study and the current tissue processing expert). Performed several Bradford assays and helped with the GSH Assays and standard curves for the lipid peroxidation assay.

5. Joe Barkho: Holds the animals during injections.

6. Cynthia Tran: Helped with dissections for the 5 injection animals. Performed Bradford assays for many experiments.

7. Dennis Ma: Helped in homogenizing tissues for phase 2 experiments.

8. Justin Kale: Cast the 10% gels for the western blots and helped with using the Photoshop software to layer images.

9. Pamela Ovadje: Helped with the chemdraw software to draw structures for my thesis.

10. Ren Xiang: Former graduate student from Dr. Zielinski's lab. Trained me to perform the perfusions, dissections and separating the different brain parts.

11. Varakini Parameswaran: Worked for Dr. Cohen and helped with identifying animals for injection and dissection periods during phase one studies.

Other people involved:

1. Dr. Cohen: Collaborator, studies the behaviuoral component of this project. The rotorod data for phase one has been performed by his team.

2. Dr. Pardeep Jasra: Helped with dissections (2006-2007) and also helped with the protocols for cryosectioning.

3. Dr. T.S. Sridhar: Analyzed all the slides for H&E staining for the brain. Valuable suggestions and critique for improvising the biochemical and histochemical methodology used in the Pandey lab.

4. Dr. Jyothi S. Prabhu: Analyzed all the slides for H&E staining for the liver, lung, and kidneys.

5. Ms. Elaine Rupke: Teaching me how to use the CO_2 chamber, how to draw blood from animals and also injecting animals.

6. Technicians from the University of Geulph (Department of Pathology): Provided me with protocols and tips for paraffin embedding and tissue sectioning.

7. Ms. Patricia Lanthier and Dr. JK Sandhu: NRC Ottawa: Provided me with protocols and trained me for sectioning and paraffin embedding of brain tissues.

Appendix A

Rat Injections

Animals are injected once a week with either saline or 10mg/kg paraquat. Injection regimes usually begin a month after the animals arrive and have been handled and put on different water regimens. Injections are performed in the Biology building (basement: room adjacent to 72). Solutions and supplies are taken to the Biology building from Essex Hall. Two people are required for injections: one for holding the animal (properly and firmly), and the other for injecting the animal. Mr. Jouseph Barkho is currently the person who holds the animals during injections. Efforts are made to ensure that animals experience minimal discomfort, pain and distress during the injection process. Ms. Elaine Rupke can train/ show a person how to inject intraperitonially in the proper way without causing pain or stress to the animals. Following are the steps taken to make the process convenient.

1. Make up a schedule and choose a preferable day and time for injections.

2. Make 10 mg/ml paraquat in phosphate buffered saline (PBS) pH= 7.6 (recipe in materials and methods) and carry 5 ml of PBS pH=7.6. Make sure to label tubes correctly.

3. Weigh the animals and record weights.

4. Use a syringe with a $25G^{5/8}$ needle and draw up the required volume.

5. Ensure that there are no air bubbles.

6. Place the animal in the position favourable for injection. Joe always flips the animals upside down and holds the base of the chin and the hind legs.

7. Inject the animal on the right side (in the area lower than the rib cage and above the hind leg). Inject saline animals first.

NOTE: <u>PQ is a very toxic chemical and needs to be handled with extreme caution. Wear a labcoat, facemask and gloves while using PQ and discard the gloves after use in the biohazard bag. Also, wash your hands thoroughly with soap and water.</u>

Perfusion and Dissections

All dissections are performed in the dissection room in the animal facility (room 72) located in the basement of the Biology building.

Equipment Preparation

1. Gloves, labcoat and protective eyewear must be worn during this procedure.

2. A uume hood with sufficient room for equipment and working exhaust system.

3. Perfusion rack, isofurane (anaesthetic) and oxygen cylinder (make sure it is not empty).

4. Perfusion kit consisting of:

   - 4 pairs scissors (2 blunt and 2 sharp), 5 sets of forceps: toothed, serrated, coverglass forceps bent, smooth jaw forceps, bone cutting forceps.

   - Needles (20G1, with short bevel work best for most rats).

   - Perfusion apparatus (2 clean bottles: one labelled tyrodes and other formaldehyde, clean tubes and clasps).

5. Labelled tubes (50 ml), 70% ethanol solution in a spray bottle, 2L containers for storing fixative solution as well as Tyrode's solution with heparin

Fixatives: 10% Formaldehyde in 0.1 M PBS solution pH=7.6.

Before you start, check the checklist.

Checklist:

<u>Things to bring from Essex Hall:</u>

1. Tyrode's stock solution

2. Heparin (added) ?

3. 10% buffered Formaldehyde stock

4. Labelled Tubes (rat numbers and cage numbers, date, organ type and batch), forceps, scissors, isofurane, ethanol, paper towels, biohazard bags (orange coloured)

<u>In the animal facility:</u>

- Oxygen cylinder, garbage bag for collecting carcasses and the bottles for perfusion.

- Animal care technician informed

Procedure

- Fill the two bottles with corresponding liquids, adjust the tubing and the flow rate to 30 ml/minute and ensure no air bubbles are present through the tubing. No formalin should be present in the tubing when you start. Rinse the tubing with Tyrode's.

- Weigh the rat and place it into the plastic chamber used for anaesthetising animals. Turn on the anaesthetic pump and the oxygen cylinder. Anaesthetize the rat into a deep surgical plane. As this is a terminal procedure, it is important that the rat be very deeply anaesthetized, but the heart must still be beating. This

process takes upto 7-8 minutes, after which the rat will stop moving in the chamber.

- Put the rat into the dissecting tray. Quickly, put the nozzle on the rat's nose and hold it in place.

- Two people are required for this step: One person is responsible for dissections while the other person assists (holds the nozzle, helps switch liquids, hands equipment needed to the person dissecting).

- Check to make sure the rat is deeply anaesthetized (usually by pinching one of the hind legs with a pair of forceps). If the animal retracts its foot, then put it back in the chamber for an additional 5-7 minutes.

- Spray animal with 70 % ethanol so that the fur doesn't stick to the insides after cutting and it is easy to clean fur from the scissors and forceps.

- Make vertical surgical cut along the chest midline to below the rib cage by pulling up flesh with a pair of toothed forceps and cutting with blunt scissors.

- Using a pair of toothed forceps pinch the sternum and pull up (white cartilage flap) and cut through the diaphragm.

The rest of this procedure must be done quickly to make sure the heart continues to pump the blood.

- Whilst holding the sternum with toothed forceps the in one hand, make lateral cuts bisecting ribs on both sides of animal towards armpits.

- Pull up the rib cage exposing the liver, stomach and heart.

- Introduce the needle into the left ventricle (on your right as you look down), making sure not to pierce through to the left atrium all the way into the aorta. This will be the entrance site for the saline and formaldehyde.

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- Start pumping the Tyrode's solution and make a small cut on the lobe of the liver to allow the blood, saline and the formaldehyde to leave the body during perfusion.

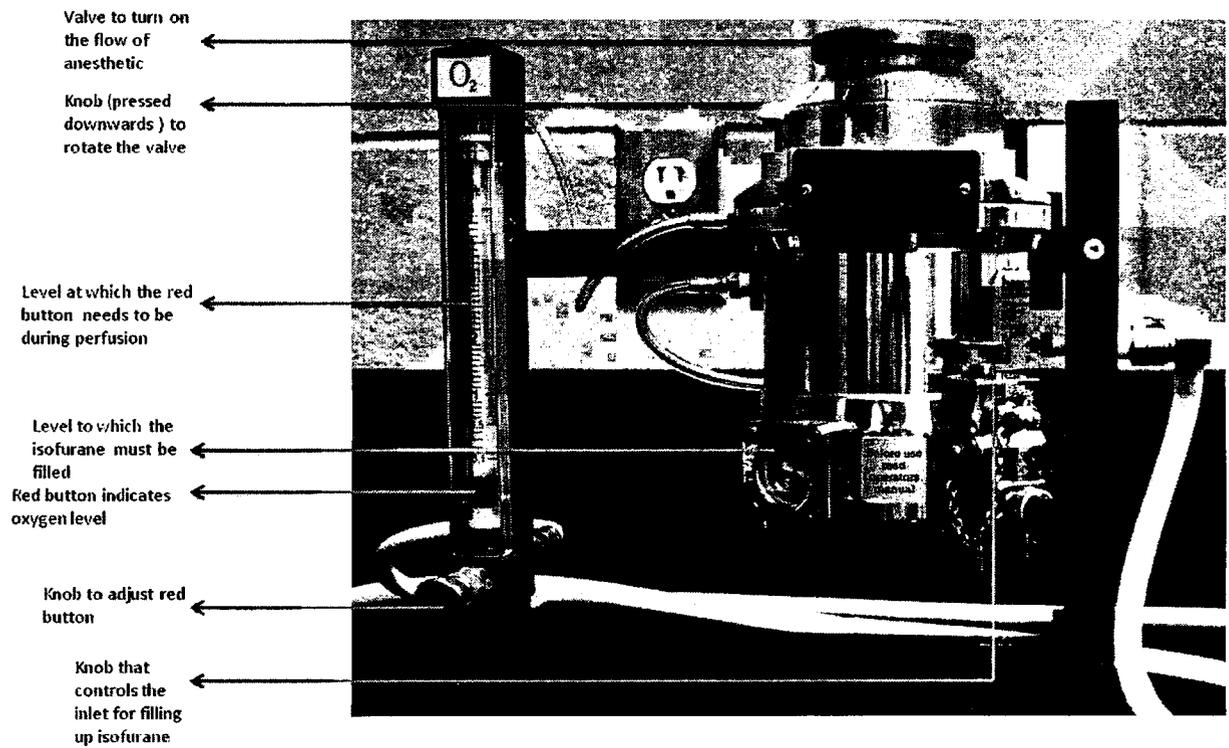- The liver should become pale as the blood drains. Within 30 seconds there should be some muscle twitching. Be careful to keep the needle in place all while this occurs. The person assisting can hold the tail and the legs to help keep the animal in place.

- Turn of the isofurane and remove the nozzle from the nose at this point.

- Allow blood to drain; the liquid as it is pumped through will change from dark red to clear if the procedure is done properly.

- Switch the solutions and run the fixative through the animal. Once the front paws are stiff (150-200ml of fixative), then turn off the fixative. Remove needle from heart. Pour out any liquid left in the rat's chest cavity.

- Place the rat in the guillotine and remove head for the brain to be harvested.

- Fix the brain after surgery. For immunocytochemistry for 24 hours then switch to 70% ethanol and store in 2-8°C.

- Clean the apparatus thoroughly with antibacterial reagent and wipe down the work area. Turn off the O_2 cylinder. Shut down the fumehood. Place the carcasses into the cold room (biology basement, just opposite Ms. Rupke's office). Autoclave the biohazard bag in Essex Hall before disposing the waste.

The different apparatus used in this experiment is shown below.

1. Perfusion Apparatus

- 10% Formalin
- Flap to keep bottles secure
- Tyrode's Solution
- Clasp to regulate the flow of tyrode's solution
- Clasp to regulate the flow of formalin
- Needle for perfusion
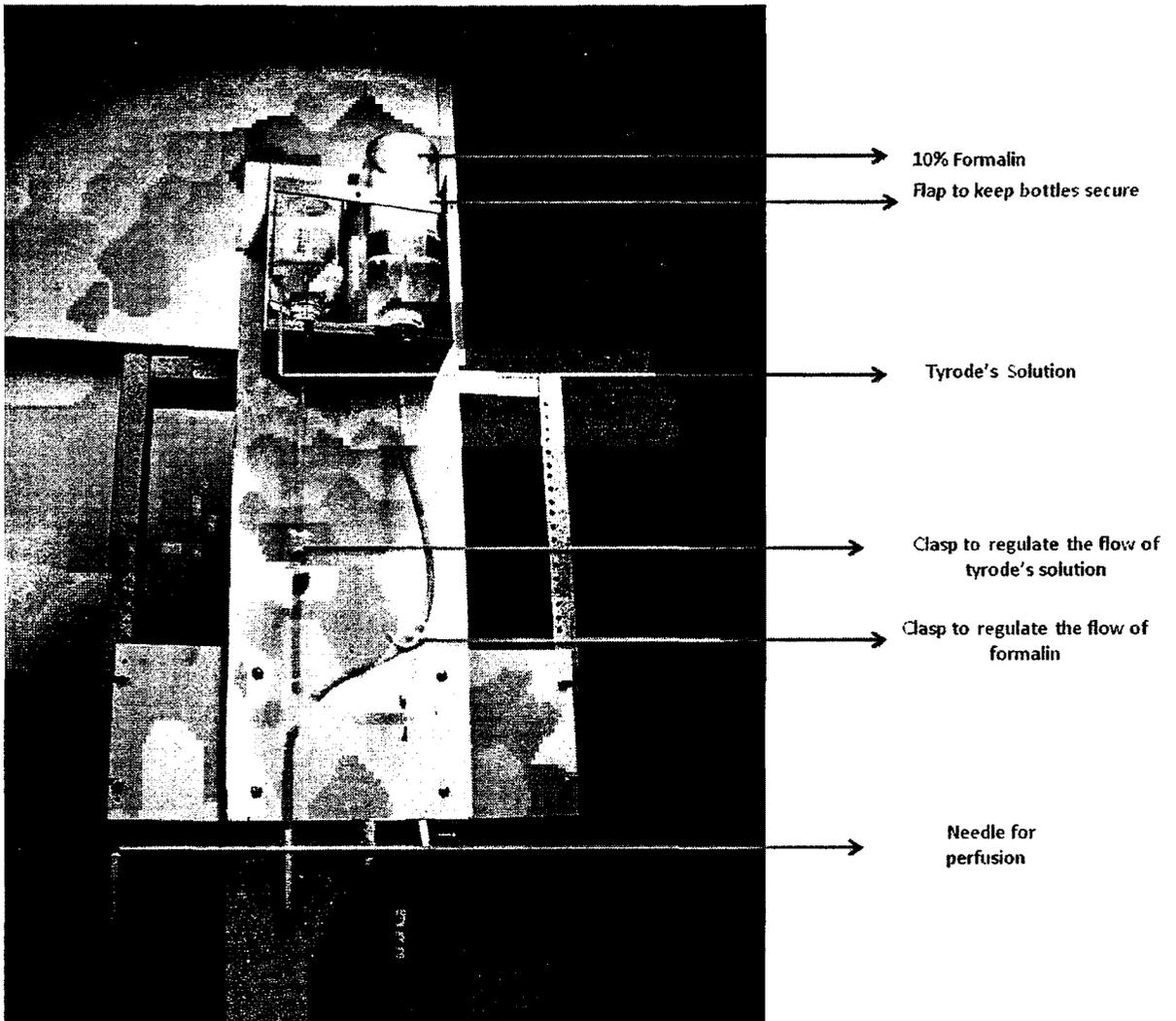
2. Vapourizer for Isoflurane

Valve to turn on the flow of anesthetic

Knob (pressed downwards) to rotate the valve

Level at which the red button needs to be during perfusion

Level to which the isofurane must be filled

Red button indicates oxygen level

Knob to adjust red button

Knob that controls the inlet for filling up isofurane

Rat Tissue Snap Freezing

All dissections are performed in the dissection room in the animal facility (room 72) located in the basement of the biology building.

Equipment Preparation

- Gloves, lab coat and protective eyewear should be worn during this procedure

- A fume hood with sufficient room for equipment and working exhaust system

- Dry ice, liquid nitrogen and carbon dioxide cylinder (make sure it is not empty)

- Dissection kit consisting of :

    4 pairs scissors (2 blunt and 2 sharp), 5 sets of forceps: toothed, serrated, coverglass forceps bent, large forceps bent, bone cutting forceps

- Guillotine

- Clean labelled tubes

Before you start, check the checklist.

Checklist:

Things to bring from Essex Hall:

- Dry ice

- Liquid Nitrogen

- Labelled Tubes, forceps, scissors, ethanol, paper towels, bags for collecting carcasses and biohazard bags (orange coloured)

<u>In the animal facility:</u>

- Carbon dioxide cylinder, garbage bag for collecting carcasses

- Animal care technician should be informed about the dissections

Procedure

- Weigh the rat and place it into the gas chamber. Turn the nozzle on the CO_2 cylinder so that the gas flows into the chamber. The first rat will take about 5-6 to minutes to "fall asleep." (Once the chamber is filled with CO_2, the other rats take less time) Wait till the rat stops moving in the chamber. Make sure that the rat is "asleep" by checking the heart beat and by giving an obnoxious stimulus to the hind leg. Make sure that the exhaust tube for the chamber is in the fumehood and the fan is on.

- Put the rat into the guillotine and swiftly decapitate.

- Two people are required for this step: One person is responsible for dissecting the brain and flash freezing tissues while the other person is responsible for dissecting the other organs i.e. liver, lungs, heart, spleen and kidneys and passing them onto the person responsible for flash freezing.

- Spray animal with 70 % ethanol so that the fur doesn't stick to the insides after cutting and it is easy to clean fur from the scissors and forceps.

- Make vertical surgical cut along the chest midline to below the rib cage by pulling up flesh with a pair of toothed forceps and cutting with blunt scissors.

- Using a pair of toothed forceps pinch the sternum and pull up (white cartilage flap) and cut through the diaphragm.

- Whilst holding the sternum with toothed forceps the in one hand, make lateral cuts bisecting ribs on both sides of animal towards armpits.

- Pull up the rib cage exposing the liver, stomach and heart.

- Cut a small piece of the liver, mince it fine with the scalpel and flash freeze (make sure that the tissue is frozen and there is no liquid nitrogen in the tube when you place the cap on it. Follow these steps inside the fumehood and lower the barrier while dealing with liquid nitrogen. These steps require caution)

- Follow the same with the heart, lungs, kidneys and spleen.

- Meanwhile dissect the brain and make a coronal cut just at the base of the optic chiasm and remove the cerebellum. This procedure has been followed for the 5 injection regime (for 3 injection animals, the striatum was separated from the substantia nigra region before freezing).

- Freeze each part separately.

- Place the tissues on dry ice. Bring the tissues back to Essex Hall. Place the tubes in labelled Ziploc bags and place them in -80°C in Dr. Mutus' lab in the shelf allotted to the Pandey lab.

- Clean the apparatus thoroughly with antibacterial reagent and wipe down the place. Turn off the CO_2 cylinder. Shut down the fumehood. Place the carcasses into the cold room (Biology basement, just opposite Ms. Rupke's office). Autoclave the biohazard bag in Essex Hall before disposing the waste.

The different apparatus used in this experiment is shown below.

1. CO_2 chamber
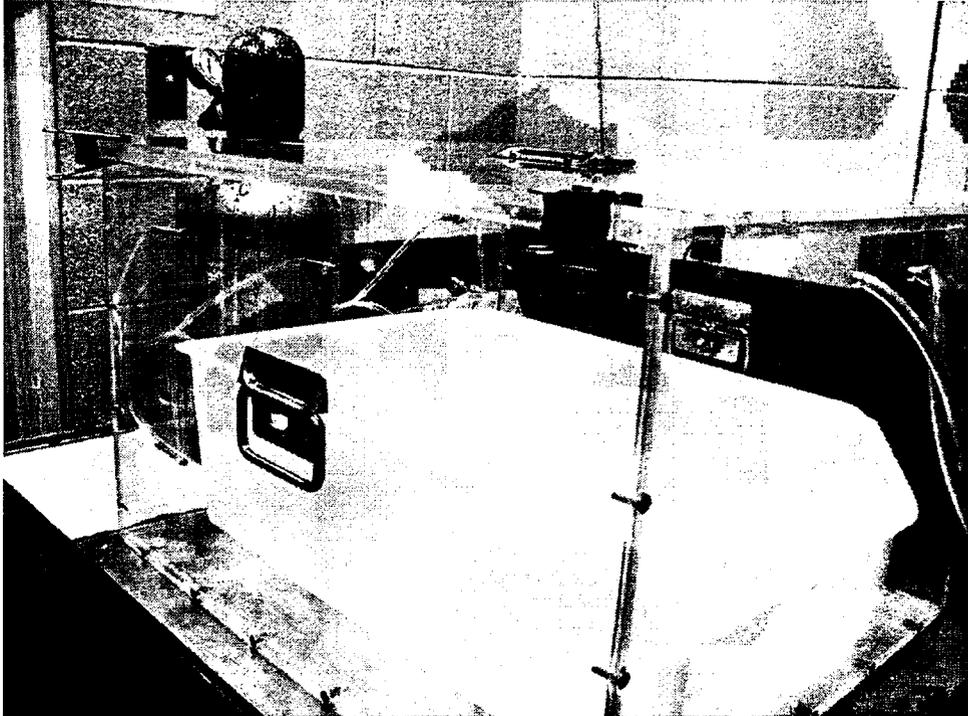
2. Guillotine
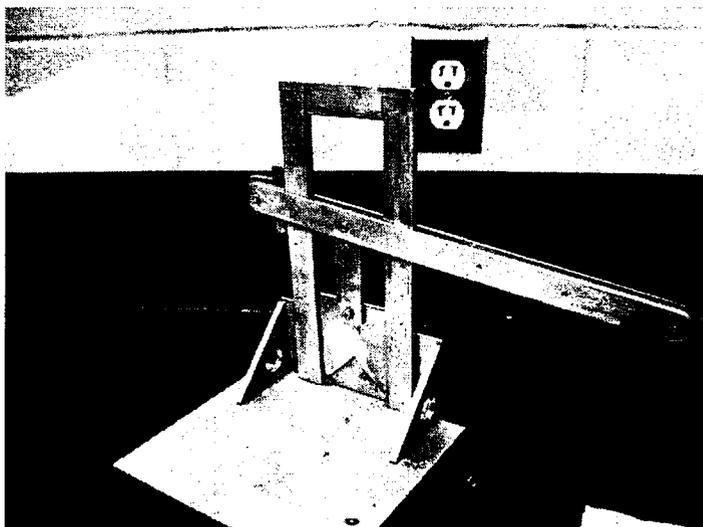
Appendix D

Paraffin Embedding and Sectioning on the Microtome

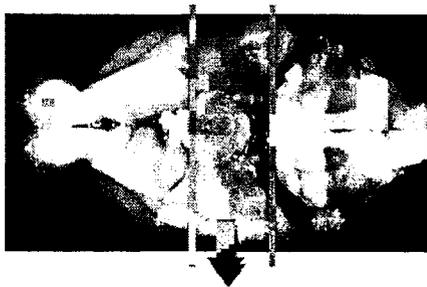Paraffin embedding and sectioning are performed in Essex 282-1.

Equipment Preparation

- Wear gloves, labcoat and protective eyewear during this procedure

- Two 50 ml tubes with melted Paraffin

- Embedding rings and steel moulds for preparing paraffin blocks

- Microtome

- Water bath at 60°C for embedding and 40°C for sectioning

- Fresh 70%, 80%, 95% and anhydrous ethanol (EtOH) and xylene

- Blades for microtome

Procedure

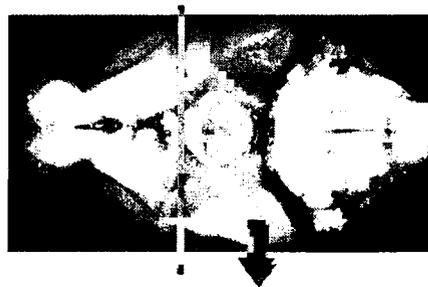- Trim the tissue. For the second phase study, we placed a coronal cut at the optic chaism and cut the brain into two pieces and saved both the pieces. (Please refer to the picture below). For the first phase of the study, a 3-4 mm piece was obtained as shown in the picture and processed.

During phase 1 study   During the phase 2 study

Tissue of interest   178   Tissue of interest

- The tissue of interest was then processed for paraffin embedding. For the 5 injection study, the tissue is incubated with following solutions:

  1. Fresh 70% EOH for 1 hour

  2. 80% EOH for 1 hour

  3. 95% EOH for 1 hour

  4. Anhydrous EOH for 3 hours

  5. Xylene 1 hour and 15 minutes

  6. Rinsed with melted paraffin and then placed in paraffin overnight in the water bath at 60°C

- Tissues were placed in a steel mould, paraffin was poured, and tissue of interest was placed in the mould, followed by the embedding ring and finally topped off with paraffin.

- The paraffin was allowed to solidify (3-4 hours at room temperature) and the mould was turned upside down and gently tapped with a pair of forceps to obtain the paraffin embedded tissue (block) was obtained.

- The block was then placed on the microtome, secured and the position was adjusted for obtaining sections.

- Initially the microtome is set at about 40 microns and the block is sectioned at this thickness till tissue starts appearing in the wax ribbon.

- The tissue is then trimmed at 20 microns and a stereotaxic rat atlas is referred to until the region of interest is reached (Figure 70- the substantia nigra pars reticulate is seen).

- Once the region of interest is reached, 6 sections are taken at 8 microns.

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- A ribbon of 6 sections is placed in the water bath at 40°C and allowed to stretch.

- A slide is used to scoop up 4 sections and then placed in a trough and dried at room temperature overnight.

- Sections are taken across the whole substantia nigra region and put onto slides.

- The slides are labelled (correctly: cage number, rat number e.g.1-1, batch slide numbers as 1,2...n) and placed in a plastic slide box that holds 100 slides until required at room temperature.

Curriculum Vitae

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Personal Profile

Languages : English, Hindi, Gujarati and Marathi

Research Experience

2004-Present	Ph.D. Thesis University of Windsor Thesis title: "Development of a paraquat induced model of sporadic Parkinson's disease in Rat: Neuroprotection by water-soluble Coenzyme Q_{10}"
2002-2004	M.Sc. Thesis University of Windsor **Thesis title**: "Role of mitochondria in neuronal cell death induced by oxidative stress: Neuroprotection by Coenzyme Q_{10}"
1998-1999	Undergraduate research project St. Xavier's College, Ahmedabad, India **Thesis title**: "Micro propagation of Mitragyna parvifolia"

Education

2004-Present	University of Windsor, Windsor, Canada Ph.D. in Biochemistry
2002-2004	University of Windsor, Canada Masters in Biochemistry
1999-2001	Gujarat University, India Masters in Biochemistry
1996-1999	St. Xavier's College, India Bachelors of Science in Biochemistry

Teaching Experience

2002-2008	Teaching Assistant, Department of Chemistry and Biochemistry

Awards

2004-Present   **Doctoral Tuition Scholarship**
University of Windsor

1999   **Dubai Friend's Circle Club Award and Scholarship**
St. Xavier's College, India

Refereed Publications

Somayajulu-Niţu, M., Parameswaran, V., Matei, A., Cohen, J., Sikorska, M., Borowy-Borowski, H., and Pandey, S. (2009). Paraquat Induced Depletion of Dopaminergic Neurons in the Substantia Nigra; Neuroprotection by Coenzyme Q_{10}. *BMC Neuroscience.* 10:88.

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Naderi, J., **Somayajulu-Niţu, M.**, Mukerji, A., Sharda, P., Sikorska, M., Borowy-Borowski H., Antonsson, B., and Pandey, S. (2006). Water-soluble formulation of Coenzyme Q_{10} inhibits Bax-induced destabilization of mitochondria in mammalian cells. *Apoptosis.* 11(8):1359-69.

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Griffin, C., Gueorguieva, D., McLachlan-Burgess, A., **Somayajulu-Niţu**, M., and Pandey, S. The Cutting Edge: Apoptosis & Therapeutic Oppourtunity. Artificial cell, Cell engineering and Therapy. Woodhead Pub. Ltd., Cambridge, UK. May, 2007.

Conference Posters

Somayajulu-Niţu, M., Schwartzenberger, E., Matei, A., Sridhar T., S., Parameswarann, T., Cohen, J., Sandhu, J., K., Borowy-Borowski, H., Sikorska, M., and Pandey, S. (2008) Induction of oxidative stress, neuronal loss and Parkinsonism in rats by pararquat: Neuroprotection by water soluble CoQ_{10}. June 3-5, 2008, Canadian Student Health Research Forum, Winnipeg, MB, Canada.

Somayajulu-Niţu, M., Sridhar, T., S., Matei, A., Parameswarann, V., Cohen, J., Sandhu, J., K., Borowy-Borowski, H., Sikorska, M., and Pandey, S. (2007) Paraquat induces oxidative stress, neuronal loss and Parkinsonism in rats: Neuroprotection by water soluble CoQ_{10}. November 11-14, 2007. XXIV International Neurotoxicology Conference San Antonio, Texas, USA. **Presentation** and Poster (Abstract # 25).

Somayajulu-Niţu, M., Vergel de Dios, J., Jasra, P., Pandey, S., Matei, A., Parameswarann, V., Cohen, J., Sandhu, J., Borowy-Borowski, H., and Sikorska, M. (2006). Paraquat Induces Oxidative Stress, Neuronal Loss in Substantia Nigra and Parkinsonism in Rats: Neuroprotection and Amelioration of Symptoms by Water-Soluble CoQ_{10}. May12, 2006. SONA Conference, Toronto, ON, Canada. Abstract # 105.

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