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Shah, Anuri Nimish

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**INVESTIGATING METABOLOMICS AND THE EFFECTS
OF OXYRESVERATROL IN EXPERIMENTAL
PARKINSON'S DISEASE**

SHAH ANURI

Ph.D. Thesis

The University of Hong Kong & King's College London

2019

Abstract of thesis titled

**Investigating metabolomics and the effects of oxyresveratrol in
experimental Parkinson's disease**

Submitted by

Shah Anuri

for the Joint degree of Doctor of Philosophy

at The University of Hong Kong & King's College London

in April 2019

Parkinson's disease (PD) is the second most common neurodegenerative disorder, with risk factors such as aging and genetic predisposition. The cardinal symptoms of PD are movement related—rigidity of the limbs, resting tremors, and bradykinesia. Brain pathologies in PD include the death of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc) resulting in diminished striatal dopamine, and the presence of intracytoplasmic spherical inclusions, known as Lewy bodies (LB). LBs comprise aggregates of the amyloid protein, α -synuclein (α -syn). Current therapeutic strategies for PD aim to replenish dopamine levels thereby providing symptomatic relief only. Compounds that can delay and slow down disease progression are an alternative strategy. Several nutraceuticals with antioxidant properties are under investigation in this regard.

Stilbenoids are a class of polyphenolics including resveratrol (RES) and its hydroxylated analogue, oxyresveratrol (OXY). Oxyresveratrol, found in the mulberry bark, is a very potent antioxidant with a wider therapeutic window than

RES. It exerts several therapeutic effects, including neuroprotection. The aim of this study was to investigate in detail the protective effects of OXY in experimental PD.

In the first part of the study, the effects of OXY on endoplasmic reticulum (ER) stress, a prime pathological pathway in PD, were studied. To induce ER stress, neurons were treated with the dopaminergic toxin 6-hydroxydopamine (6-OHDA), or transfected with familial mutants of α -syn, which form toxic oligomers. OXY mitigated ER stress in both these models, by modulating distinct pathways.

The effects of OXY exposure on rats unilaterally lesioned with 6-OHDA in the medial forebrain bundle (MFB), were then assessed. At lower doses OXY successfully rescued these rats from severe motor impairment, with a mild effect on dopaminergic loss. Subsequently, mass spectrometry-based metabolomics was employed to investigate small molecules altered in this model. The plasma was used for its translational potential, and midbrain to uncover metabolites disturbed at the site of damage.

Saturated free fatty acids were significantly upregulated in the plasma of 6-OHDA-treated rats, while monoglycerides, myo-inositol and one unidentified metabolite were decreased in their midbrain. The fatty acids showed a very high correlation with motor symptoms ($r > 0.6$) while the unidentified metabolite showed a high prediction ability, with an area under the curve (AUC) value of 100% (sensitivity = 1, specificity = 1), as seen by receiver operating curve (ROC) analysis. Moreover, phosphatidylinositol (PI 40:6 and PI 38:4) and diglycerides, lipids associated with cellular signalling, were also disrupted in the midbrain. OXY, but not RES, partially protected the metabolite imbalance in the lesioned rats.

Metabolomics also uncovered molecular targets of OXY. Levels of lactic acid, alanine and gamma-Aminobutyric acid (GABA) in the midbrain were significantly decreased in the OXY-treated rats. These metabolites are intermediates of energy metabolism in neurons, suggesting that maintaining energy homeostasis is a protective mechanism of OXY.

In summary, this study elucidated the protective effects of OXY against symptomatic and metabolic disturbances associated with experimental PD, which make it a promising prophylactic candidate for neurodegeneration.

(An abstract of exactly 486 words)

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OF OXYRESVERATROL IN EXPERIMENTAL
PARKINSON'S DISEASE**

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A thesis submitted in partial fulfilment of the requirements for
the Joint degree of Doctor of Philosophy
at The University of Hong Kong & King's College London

April 2019

Declaration

I declare that this thesis represents my own work, except where due acknowledgment is made, and that it has not been previously included in any thesis, dissertation or report submitted to this university or to any other institution for a degree, diploma or other qualifications.

A rectangular box containing a handwritten signature in black ink. The signature appears to be 'Anuri' written in a cursive style.

Signed

Anuri Shah

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List of abbreviations

(-)-epicatechin	EC
(-)-epicatechin-3-gallate	ECG
(-)-epigallocatechin	EGC
(-)-epigallocatechin-3-gallate	EGCG
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine	MPTP
1-methyl-4-phenylpyridium	MPP ⁺
3,3'-diaminobenzidine	DAB
6-hydroxydopamine	6-OHDA
78-kD glucose-regulated protein	Grp78
Activating transcription factor 4	ATF4
Activating Transcription Factor 6	ATF6
Alzheimer's disease	AD
Area under the curve	AUC
Atmospheric pressure photo ionization	APPI
Atomic pressure chemical ionization	APCI
Bicinchoninic acid	BCA
Blood-brain barrier	BBB
C/EBP-homologous protein	CHOP
Capillary electrophoresis	CE
Catechol-O-methyl transferase	COMT
Cerebrospinal fluid	CSF
c-Jun N-terminal kinase	JNK
Coenzyme Q ₁₀	CoQ ₁₀
Computed tomography	CT
Corticobasal degeneration	CBD
Cyclooxygenase	COX
Deep brain stimulation	DBS
Dementia with Lewy bodies	DLB
Desorption electrospray ionization	DESI
Diglyceride	DG/DAG
Direct infusion mass spectrometry	DIMS
Dopa decarboxylase	DDC
Dopamine transporter	DAT
Dopamine transporter scan	DaTscan
Electron impact ionization	EI
Electrospray ionization	ESI
Endoplasmic reticulum	ER
ER-associated protein degradation	ERAD
Ethylenediaminetetraacetic acid	EDTA
Eukaryotic translation initiator factor 2 α	eIF2 α
Extracellular signal-regulated kinases	ERK
Foetal bovine serum	FBS
Fourier transform ion cyclotron resonance mass spectrometers	FT-ICR-MS
gamma-Aminobutyric acid	GABA
Gas chromatography	GC

Hierarchical cluster analysis	HCA
High performance liquid chromatography	HPLC
Human Metabolome Database	HMDB
Hydrogen peroxide	H ₂ O ₂
Hydrophilic interaction liquid chromatography	HILIC
Immunoglobulin binding protein	Bip
Induced pluripotent stem cells	iPSC
Inositol Requiring 1	IRE1
Internal standard	IS
In-vial dual extraction	IVDE
Kyoto Encyclopedia of Genes and Genomes	KEGG
L-3,4-dihydroxyphenylalanine	L-dopa
Lactate dehydrogenase	LDH
Lewy bodies	LB
Lewy neurites	LN
Lipopolysaccharide	LPS
Liquid chromatography	LC
Low density lipoprotein	LDL
Lund human mesencephalic	LUHMES
Magnetic resonance imaging	MRI
Mass spectrometry	MS
Mass to charge ratio	m/z
Matrix assisted laser desorption ionization	MALDI
Median forebrain bundle	MFB
Methyl tertiary butyl ether	MTBE
Mild cognitive impairment	MCI
Monoamine – oxidase B	MAO-B
Movement Disorder Society	MDS
Multiple system atrophy	MSA
Multivariate analysis	MVA
N, O-Bis(trimethylsilyl)trifluoroacetamide	BSTFA
N-acetylcysteine	NAC
National Institute of Neurological Disorders and Stroke	NINDS
National Institute of Standards and Technology	NIST
Neuroblastoma-glioma cells	N18TG2
Non-motor symptoms	NMS
Non-steroidal anti-inflammatory drugs	NSAIDS
Nuclear Magnetic Resonance	NMR
O-methoxyamine-hydrochloride	MOX
Orthogonal Partial Least Squares-Discriminant Analysis	OPLS-DA
Oxyresveratrol	OXY
Paraformaldehyde	PFA
Paraquat	PQ
Parkinson's disease	PD
Parkinson's Disease Biomarkers Program	PDBP
Partial Least Squares-Discriminant Analysis	PLS-DA
Phosphate buffered saline	PBS
Phosphatidylinositol	PI

Phosphatidylinositol 3-kinase	PI3K
Pinostilbene	PINO
PKR-like ER kinase	PERK
Polymerase chain reaction	PCR
Positron-emission tomography	PET
Principle component analysis	PCA
Principle components	PC
Progressive supranuclear palsy	PSP
Protein disulphide-isomerase	PDI
PTEN-induced putative kinase 1	PINK1
Quality control	QC
Reactive oxygen species	ROS
Receiver operating curve	ROC
REM sleep behaviour disorder	RBD
Resveratrol	RES
Reverse transcription-polymerase chain reaction	RT-PCR
Reversed phase	RP
Single photon emission computed tomography	SPECT
Sirtuin1	SIRT1
Sodium dodecyl sulphate polyacrylamide gel electrophoresis	SDS-PAGE
Substantia nigra <i>pars compacta</i>	SNpc
Substantia nigra <i>pars reticulata</i>	SNpr
Time-of-flight	TOF
Tricarboxylic acid cycle acid	TCA
Trimethylchlorosilane	TMCS
Tris buffered saline	TBS
Tyrosine hydroxylase	TH
Ubiquitin carboxyterminal hydrolase 1	UCHL-1
Ubiquitin proteasome system	UPS
Ultra-high-performance liquid chromatography	UPLC/UHPLC
Unfolded protein response	UPR
Unified Parkinson's disease rating scale	UPDRS
Unit variance	UV
Variable importance on projection	VIP
Ventral tegmental area	VTA
Vesicular monoamine transporter-2	VMAT-2
Wild-type	WT
α -synuclein	α -syn

CHAPTER 1

Introduction

1.1 Overview of Parkinson's disease

Sir James Parkinson, the eponymous discoverer of Parkinson's disease (PD), first reported the incidence of a "shaking palsy", as he then called it, in 1817 (Hurwitz, 2016). The most ground-breaking discoveries on PD, were subsequently made in the 19th and 20th centuries. In 1872, Jean-Martin Charcot proceeded to identify "bradykinesia" as a cardinal symptom of PD, distinguishing it from other disorders. He was also responsible for renaming it "*Maladie de Parkinson*" (Parkinson's disease) (Kumar et al., 2011). The discovery of dopamine in 1910, by George Barger and James Ewens was the next breakthrough; until then the agent responsible for changes in the PD brain was unknown. In the following years, the two most important discoveries of PD were made; Friedrich Lewy discovered the presence of "spherical inclusions" in the brains of people who died of PD (Holdorff, 2002), and Konstantin Tretiakoff proceeded to demonstrate the role of the substantia nigra in PD (Lees et al., 2008). He also renamed the "spherical inclusions" as Lewy bodies (LB), after their discoverer. These are still known as the two primary pathological hallmarks of PD. PD affects almost 1% of the global population, affecting approximately seven to ten million people worldwide (Parkinson's Disease Foundation, 2016). It is estimated that the incidence of PD worldwide will double within the next decade (Dorsey et al., 2007). While it is primarily an aging-associated disease, genetic predisposition may implicate patients under fifty years of age as well, responsible for up to 4% of all cases.

However, given the rise of aging populations in countries such as Japan, Italy, Greece, China and Hong Kong, the disease is an impending burden. As the second most common neurodegenerative disease, PD results in healthcare costs that amount to \$25 billion in the United States alone (Parkinson's Disease Foundation, 2016).

Although research on pathology, etiology, therapeutic interventions and diagnosis has contributed immensely to our current knowledge of PD, we are still a long way from understanding the exact cause and finding a curative treatment to reverse PD. Elucidating the underlying mechanism of PD and emphasis on curative therapy thus seems imperative.

1.1.1 Clinical symptoms of PD

Parkinsonism refers to “a syndrome characterized by tremor at rest, rigidity, slowness or absence of voluntary movement, postural instability, and freezing” (Dauer & Przedborski, 2003). While this is an umbrella term for all conditions arising out of a depletion of striatal dopamine, PD is the most common of them (Dauer et al., 2003). PD is broadly classified as tremor dominant and non-tremor dominant. Tremor dominant PD presents resting tremors as the primary symptom and usually progresses slowly. Non-tremor dominant PD presents rigidity, gait instability and akinesia as the primary symptoms and tends to show accelerated decline (Jankovic et al., 1990). A brief timeline of all the symptoms of PD and when they manifest is illustrated in Figure 1.1.

As a movement-associated disorder, PD includes a host of motor symptoms that severely affect quality of life. While such symptoms occur starting on one side of the body first, they eventually affect the entire body. Rigidity of the muscles

results in hypokinesia (decreased amplitude of movement), bradykinesia (slowness of movement) and akinesia (complete loss of voluntary movement) (Berardelli et al., 2001). Subsequently, an interference with daily activities, such as decreased amplitude and speed of writing or difficulty with being able to dress oneself is observed (Dauer et al., 2003). Motor symptoms in late stages of PD are characterized by “freezing” episodes, wherein the patient cannot move at all, along with severe postural instability, often leading to excessive falls (Hely et al., 2005). Motor dysfunction now affects all aspects of life including but not limited to dysphagia (inability to swallow), lack of facial expressions, and hypophonia (decreased volume of speech) (Dauer et al., 2003).

Non-motor symptoms (NMS) of PD are diverse in nature (Khoo et al., 2013). Certain NMS have been shown to precede motor symptoms by years, representing the prodromal stage of the disease (Duncan et al., 2014; Khoo et al., 2013). At this stage constipation, hyposmia (reduced ability to smell) (Jellinger, 2015), REM sleep behaviour disorder (RBD) leading to sleep disturbances, depression and mood swings occur prior to motor symptoms. In some cases, these can manifest almost 12-14 years before motor symptoms start to show. Mild cognitive impairment (MCI) can also be observed at the time of diagnosis (Chaudhuri et al., 2006). In later stages of the disease, further NMS such as urinary incontinence, dementia and postural hypotension develop (Hely et al., 2005). It has also been demonstrated that the risk for developing dementia is 40% higher in PD patients (Emre, 2003). Cognitive impairment in PD includes fluctuations in attention, visuospatial memory deficits, and executive dysfunction affecting working memory (Jellinger, 2012). By now, patients become excessively solitary and passive (Dauer et al., 2003).

1.1.2 Pathology of PD

Parkinsonism results primarily from a depletion of dopamine levels in the striatum, specifically in the dorsolateral putamen (Dickson et al., 2009). This is accompanied by a loss of dopaminergic neurons in the ventrolateral substantia nigra *pars compacta* (SNpc); neurons from this region project to the dorsolateral putamen, and this nigrostriatal pathway controls voluntary movement (Figure 1.2). Neuromelanin, a dark pigment, is formed as a by-product of dopamine metabolism, giving these dopaminergic neurons a dark appearance. Therefore, a stark depigmentation of these neurons is a feature of post-mortem PD brains, indicative of cell loss in the SNpc (Zecca et al., 2003). It is purported that when symptoms start to appear, there is up to 60% cell loss in the SNpc, resulting in almost 80% of the dopamine being depleted (Bernheimer et al., 1973). However, at early stages of the disease, there is only mild loss of neurons, suggesting that early intervention could potentially salvage these neurons (Kordower et al., 2013). Interestingly, other dopaminergic neurons, such as those in the ventral tegmental area (VTA) which are part of the mesolimbic pathway, are not initially affected as much in PD (Uhl et al., 1985). It has been proposed that damage is initiated first in the putamen, which results in a “dying back” mechanism of neurodegeneration towards cell bodies of dopaminergic neurons in the SNpc (Dauer et al., 2003). However, besides damage to the nigrostriatal system, there is ample evidence for pathology observed in other regions of the brain in PD. Noradrenergic neurons of the locus coeruleus, serotonergic neurons in the raphe nuclei, and cholinergic loss in the nucleus basalis of Meynert are also observed. Pathology is also observed in the cingulate and entorhinal cortices and the hippocampus, which might be partly responsible for the cognitive decline associated with PD (Dickson, 2012).

As mentioned above in the discovery of LBs, the major pathological hallmark of PD is the presence of intracytoplasmic inclusions known as LBs and Lewy neurites (LN) in the neuronal cell body and processes, respectively (Spillantini et al., 1997). These are however, also present in other parkinsonian symptoms such as dementia with Lewy bodies (DLB) (Gibb & Lees, 1988). LBs comprise insoluble aggregates or fibrils of α -synuclein (α -syn), a pre-synaptic amyloid protein. LBs appear as 15 μ M wide eosinophilic circles, with a dense core, surrounded by a “halo” like appearance (Pappolla, 1986), as shown in Figure 1.3. While the exact initiator of fibril formation is not known, oxidative damage and certain mutations are putative inducers. These have been described in detail in the next section. Aggregates of α -syn within the inclusions are usually co-localized with residual lipids and other proteins such as ubiquitin, parkin and neurofilament (Jellinger, 2014; Spillantini et al., 1997; Spillantini et al., 1998). The process by which monomeric α -syn forms fibrils, involves an initial, slow nucleation phase, during which protofibrils or oligomers of α -syn are formed. When these grow further in size to about 10 nm in diameter, they are referred to as fibrils (Conway et al., 2000).

LBs are widespread in nature, present not just in the brain, but also the spinal cord, peripheral nervous system, enteric nervous system, salivary glands, adrenal medulla, cutaneous nerves and sciatic nerve. Braak *et al.* have proposed the prevailing theory that in early stages Lewy pathology can be found in regions of the brain stem, ultimately spreading to the midbrain and cortex (Braak et al., 2004). The “dual-hit hypothesis” suggests the involvement of the gastric system in the pathology of PD. This hypothesis proposes the initiation of PD by a pathogen, possibly entering the body via nasal and gastric routes (Hawkes et al., 2007). This theory was further strengthened by Braak and colleagues who successfully

demonstrated that inclusions of α -syn are present in innervations to the gastrointestinal (GI) tract (Braak et al., 2006). Recent evidence has further demonstrated that the gut microbiome perhaps plays an important role in the formation of PD pathology (Sampson et al., 2016). Therefore, there is an active involvement of the GI tract in Lewy body pathology. Furthermore, animal models have been used to demonstrate the prion-like mechanism of α -syn resulting in the spread of LBs (Luk & Lee, 2014).

1.1.3 Risk factors and genetic implications for PD

PD is still primarily an idiopathic disease; the exact etiology of which remains ambiguous. However, multiple risk factors have been identified. Age is the biggest risk factor for PD. An increase in age is directly proportional to the risk for developing PD, but this does reach a plateau after the age of 80 (Driver et al., 2009; Pringsheim et al., 2014). It is also known that men are at a 1.5 times higher risk for developing PD than women (Tysnes & Storstein, 2017). However, there is mild evidence to show that post-menopausal women who have undergone hysterectomies or are not on hormone replacement therapy, are at increased risk for developing PD (Dye et al., 2012). Environmental risk factors include exposure to pesticides such as rotenone, herbicides and toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Logroscino, 2005; Noyce et al., 2012). Furthermore, susceptibility to infectious microorganisms during early life also seem to play a role in the predisposition for PD (Logroscino, 2005). Consumption of well water in rural areas, head traumas and regular β -blocker use have been shown to exhibit a positive association with the risk for developing PD (Noyce et al., 2012). There are, however, some factors which have shown a tendency to reduce the risk

of PD. Cigarette smoking and caffeine are well known in this regard (Hernán et al., 2002). Consumption of non-steroidal anti-inflammatory drugs (NSAIDs) and alcohol consumption have shown a mild tendency to reduce the risk of PD (Noyce et al., 2012).

Genetic mutations account for 15-30% of total PD cases (Parkinson's Disease Foundation, 2016). A family of 18 genetic loci expressing mutations, have been grouped under the umbrella term *PARK*. These are termed as *PARK1-PARK18*. While the genes at some of these loci have been identified, and investigated for their link to PD, there are a few which remain unidentified till date. A summary of the well classified genes and their functions is shown in Table 1.1. Six genes that have a direct link to familial forms of PD have been conclusively identified so far. Two of these are genes responsible for mitochondrial function, namely, *DJ-1* (*PARK7*) and *PINK1* (*PARK6*). *PARK1/PARK4* indicates a range of mutations on the *SNCA* gene which encodes for α -syn. The two most common familial forms of mutant α -syn are Ala⁵³ $\rightarrow$ Thr (A53T) and Ala³⁰ $\rightarrow$ Pro (A30P) (Polymeropoulos et al., 1997). *SNCA* is also the most widely studied gene, due to the close association of α -syn and its mutants with LB pathology. Furthermore, there is ample evidence for the ability of familial mutants of α -syn (A30P and A53T) to accelerate oligomer formation and aggregation (Li et al., 2001). Moreover, genes implicated in protein degradation pathways such as *UCHL-1* (*PARK5*) and *Parkin* (*PARK2*) also play a role in PD. *LRRK2* (*PARK8*), encoding the signalling protein dardarin, is another common *PARK* gene associated with PD. Additionally, *ATP134A* (*PARK9*) encoding ATPases, has been identified. PD cases with this mutation show a distinct phenotype. Besides genes associated with the *PARK* family, the most common risk factor for PD, is a mutation in the *GBA* gene, encoding for β -glucocerebrosidase.

1.1.4 Etiology and pathogenesis of PD

Although the primary cause of PD remains unknown, several propositions have been made to understand the underlying mechanisms behind PD progression. The roles and relationship of all these mechanisms are highlighted in Figure 1.4.

1.1.4.1 Oxidative stress and mitochondrial dysfunction

Dopaminergic neurons are more susceptible to oxidative stress, as the metabolism of dopamine results in the formation of certain reactive oxygen species (ROS) as by-products (Graham, 1979; Spina & Cohen, 1989). Auto-oxidation of dopamine and L-3,4-dihydroxyphenylalanine (L-dopa), its precursor, generate quinones (Meiser et al., 2013), whereas enzymatic oxidation leads to formation of hydrogen peroxide (H_2O_2) and superoxide radicals (Graham, 1978). However, this theory cannot explain why dopaminergic neurons in some parts of the brain are spared of such damage.

Another theory also proposes the role of increased iron levels, which were found associated with the ROS (Dexter et al., 1991). Generally, ROS also accumulate in the cell with age (Balaban et al., 2005; Beckman & Ames, 1998). Decreased levels of ROS quenchers such as glutathione, glutathione peroxidase and superoxide dismutase have also been found in the SNpc of PD patients (Napolitano et al., 2011; Saggu et al., 1989; Sian et al., 1994), whereas indicators of oxidative damage such as malondialdehyde, a marker of lipid peroxidation, are found to be increased (Dexter et al., 1989). Besides oxidation of lipids (Dexter et al., 1994), there is also clinical evidence for oxidative damage to DNA in the PD brain (Alam et al., 2002). Although, it must be noted that most the post-mortem tissues used for these studies, comprised only microglia and non-dopaminergic neurons. Therefore, directly linking the evidence of oxidative damage to dopaminergic neurons alone,

is not wholly accurate. Interestingly, oxidative stress is also related to protein aggregation. Not only do the levels of oxidised proteins in the brain increase with age (Beckman et al., 1998), but LBs are also known to contain oxidized α -syn in part (Giasson et al., 2000).

Mitochondria are heavily implicated in PD as these organelles are directly linked with oxidative stress. In addition, there are also several mutations in genes encoding mitochondrial proteins such as DJ-1, parkin and PTEN-induced putative kinase 1 (PINK1), resulting in familial forms of the disease. These are reviewed in section 1.1.3 above. Several studies have revealed a deficiency in oxidative phosphorylation (Schapira et al., 1989) and mitochondrial complex I (Greenamyre et al., 2002; Schapira et al., 1990) in the SNpc and platelets (Parker et al., 1989) of PD patients. It must be noted that some reports found mitochondrial dysfunction and oxidative damage occurring simultaneously in nigral neurons (Dexter et al., 1991; Gu et al., 1998; Mann et al., 1994).

While oxidative stress, the accumulation of protein aggregates and mitochondrial dysfunction are part of the disease mechanisms reported from different research groups, experimental models have elucidated the role for alternative mitochondrial pathways in PD progression. Impaired calcium homeostasis has been reported to affect the mitochondrial permeability transition pore (Frei & Richter, 1986). Parkinsonian toxins have been reported to be associated with increased mitochondrial fragmentation, an induction of slower mitochondrial transport (Kim-Han et al., 2011) and the presence of fewer mitochondria in the processes of dopaminergic neurons (Liang et al., 2007).

1.1.4.2 Protein aggregation, the ubiquitin proteasome system and autophagy

LBs consist of amyloid α -syn fibrils. The relationship between amyloid proteins and neurodegeneration has been studied in detail, over the years. Currently, there are two schools of thought about the exact role of α -syn inclusions. Some studies suggest that inclusion formation is a protective mechanism potentially initiated to scavenge toxic soluble, misfolded proteins (Cummings et al., 2001; Kopito, 2000). The other prevailing theory stems from the ability of chaperones to alleviate neurodegeneration associated with protein aggregates, in experimental models of PD (Auluck et al., 2002; Cummings et al., 2001; Muchowski, 2002). These findings suggest the potential neurotoxicity of amyloid proteins found in inclusions. Dopamine and its metabolites tend to stabilize oligomers and protofibrils of α -syn, which can cause further damage to the implicated neuron (Perez et al., 2002). It is also interesting to note that the ability of cells to sequester misfolded proteins declines with age (Sherman & Goldberg, 2001).

While oxidative stress is one of the mechanisms initiating protein misfolding (Schapira et al., 1990), impairment of the ubiquitin proteasome system (UPS) and autophagy, are others. The UPS and autophagy are processes responsible for the degradation of misfolded, aged proteins and organelles in proteasomes and lysosomes, respectively. The role of UPS proteins such as parkin and ubiquitin carboxyterminal hydrolase 1 (UCHL-1) in the context of PD has been discussed previously. Moreover, reduced proteasomal function (Leroy et al., 1998; McNaught et al., 2003) and structural changes of proteasomal subunits (McNaught et al., 2002, 2001) have been observed in the SNpc of PD patients. An impairment of the proteasome associated with aging *C. Elegans* further justifies the role of UPS in

aging associated neurodegeneration (Ben-Zvi et al., 2009). While macroautophagy is not responsible for disposing α -syn in the normal state, there is evidence for its involvement in purging neurons of A53T- α -syn (Alvarez-Erviti et al., 2010). Alternatively, autophagosomes are increased in the SNpc of PD patients, whereas LBs also contain increased concentrations of the autophagic protein, LC3II (Chu et al., 2009; Dehay et al., 2010).

While the UPS and autophagy play a role in degradation of aggregated proteins, there is a cellular phenomenon that can upregulate protein misfolding: endoplasmic reticulum stress.

1.1.4.3 Endoplasmic reticulum stress and unfolded protein response

A build-up of misfolded proteins in the cell results in endoplasmic reticulum (ER) stress. Activation of ER stress leads to a series of downstream pathways that can either protect the cells from protein damage or lead to programmed cell death. Post-mortem studies have shown dopaminergic neurons containing α -syn inclusions to be positive for ER stress markers (Hetz et al., 2013). An upregulation of ER stress-associated protein chaperones has also been elucidated in nigral neurons (Conn et al., 2004; Slodzinski et al., 2009), while α -syn has been found to be accumulated at the ER in PD patient brains (Colla et al., 2012).

Cellular models have been successful in elucidating upregulation of ER stress by over-expressing α -syn (Colla et al., 2012), using mutants (Bellucci et al., 2011) or post-translational modifications (Sugeno et al., 2008). Other familial PD associated proteins such as parkin (Tsai et al., 2003) and DJ-1 (Yokota et al., 2003) are also linked to upregulation of ER stress in cell culture experiments. Furthermore, parkinsonian mimetics and environmental toxins can also upregulate

ER stress (Holtz & O'Malley, 2003; Ryu et al., 2002). More detailed clinical studies are however warranted to better understand the relationship of ER stress with idiopathic and familial PD.

1.1.4.4 Inflammation, microgliosis and immune response

The role of inflammation and microgliosis in PD has received increasing attention (Hirsch et al., 2003). Glial cells in PD post-mortem brains have been shown to exhibit an upregulation of pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α) (Boka et al., 1994) and its associated signalling factor, nuclear factor kappa B (NF- κ B) (Wilms et al., 2003). Altered cytokines have been found in the cerebrospinal fluid (CSF) of PD patients (Hirsch et al., 2005). Furthermore, activation of microglia after treatment with lipopolysaccharide (LPS), a bacterial endotoxin, has been shown to elicit selective toxicity on dopaminergic neurons in both cell culture and animal experiments (McNaught et al., 1999). Pharmacological inhibition of inflammation with the use of NSAIDS, has been shown to successfully delay degeneration in animal toxin models of PD, further strengthening the idea that inflammation in the brain is important for dopaminergic neurons and even development of parkinsonism (Aubin et al., 1998).

Markers of phagocytic microglia, such as CD8 are also upregulated in the SNpc of patients, indicating the activation of an immune response (Croisier et al., 2005). In experimental models, the presence of CD8⁺ and CD4⁺ T cells in the brain were observed (Brochard et al., 2009). A recent study also showed the role of human helper T-cells in recognizing specific epitopes of α -syn, indicating their role in possibly attacking α -syn inclusion positive neurons (Sulzer et al., 2017). Such T-cell responses were also responsible for the death of neurons overexpressing α -syn

in mice (Harms et al., 2013). These findings are however, contradictory, and whether the role of microglia in PD is causative or protective is still debatable.

1.1.5 Experimental models of PD

The first line of studies for understanding PD pathogenesis or developing treatments requires robust cellular models. Cell culture models are generally easier to replicate. There are several cell lines used for this purpose, which come from varying sources and can be used to represent a vast array of PD models. To mimic the loss of dopaminergic neurons as observed in PD, dopaminergic cell lines are usually exposed to toxins such as MPP⁺, 6-hydroxydopamine (6-OHDA) or other external agents. Alternatively, siRNA/shRNA knockdown studies are often used to understand the effects of over-expression, familial mutants or functions of specific genes in the context of PD. Protein aggregation, especially of α -syn can also be induced by ER stress inducers, intracellular calcium (Follett et al., 2013), inhibitors of the proteasome or oxidative stress inducers. The most common cell line used is the SH-SY5Y, human neuroblastoma cell line. SH-SY5Y cells were sub-cloned from the parent SK-N-SH, neuroblastoma cells, derived from a human bone marrow biopsy (Biedler et al., 1978). SH-SY5Y cells express the dopamine transporter (DAT), thereby facilitating uptake of dopamine and parkinsonian mimetic toxins that rely on DAT (Alberio et al., 2010). Moreover, SH-SY5Y cells express neuron like properties, after differentiation with different methods (Encinas et al., 2000; Schneider et al., 2017; Teppola et al., 2016). SH-SY5Y cells are known to be catecholaminergic, based on their expression of tyrosine hydroxylase (TH) (Ross & Biedler, 1985), an enzyme responsible for dopamine and noradrenaline synthesis. These cells have been used in various models, successfully representing PD pathology that have led to advancements in the field. From oxidative stress inducers

like H_2O_2 , dopaminergic toxins like 6-OHDA and 1-methyl-4-phenylpyridium (MPP^+), the active form of MPTP, to environmental toxins like rotenone, and proteasome inhibitors like lactacystin, have been used successfully on SH-SY5Y cells to understand their mechanism of actions, and the effects of therapeutic interventions better (Xicoy et al., 2017). SH-SY5Y cells have also been successfully transfected to overexpress most familial forms of α -syn, as well as mutants of LRRK2, PINK1 and parkin (Xicoy et al., 2017).

Another common cell line employed in PD cell culture is the pheochromocytoma PC12 cell line, derived from the rat adrenal medulla (Greene & Tischler, 1976). PC12 cells can also be differentiated to induce neuron-like properties (Greene et al., 1976). PC12 cells have been successfully used to mimic PD pathology using similar toxins (Grau & Greene, 2013). Progenitor cells, such as the MN9D and Lund human mesencephalic (LUHMES), and now being widely accepted for PD related studies. (Hermanson et al., 2003), (Lotharius et al., 2002, 2005), (Scholz et al., 2011). Lastly, the Mes23.5 dopaminergic cell line, a hybrid between the murine neuroblastoma-glioma cells (N18TG2) and rat embryonic mesencephalic neurons, is a suitable model for mimicking cells of the substantia nigra, without using primary cultures (Crawford & Appell, 1992). Apart from expressing properties of nigral dopaminergic neurons (Crawford et al., 1992), these cells do not need to be differentiated before treatment in most cases; making a compelling case for their use in experimental PD models.

Animal models are generally used in the next stage to validate findings from cell culture studies. While cellular models can represent PD pathology in most cases, animal models are used not only to closely mimic pathology, but also symptomatic changes. Moreover, animal models are especially useful when

studying pathologies affecting different brain cells, such as neuroinflammation. Animal models can also be used to investigate the effects of parkinsonism mimetics and toxins. Commonly used toxins are 6-OHDA and MPTP, while environmental toxins such as rotenone also represent compelling PD models. A summary of the different animal models in use, is described in Table 1.2. MPTP was discovered serendipitously when a batch of heroin that was contaminated by it, resulted in parkinsonian symptoms developed by its users (Langston et al., 1983). MPTP is converted into its toxic form, MPP^+ in microglia, astrocytes and other non-dopaminergic neurons, which is subsequently released (Nicklas et al., 1985). MPP^+ is then taken up actively by the dopaminergic neurons via DAT (Javitch & Snyder, 1984). Uptake of MPP^+ by the serotonin and noradrenergic neurons has also been demonstrated (Javitch et al., 1985; Mayer et al., 1986). The actions of MPP^+ in the cell are diverse in nature. MPP^+ can accumulate in the mitochondria to inhibit regular functioning of complex I; an integral part of the electron transport chain driving mitochondrial respiration. There is also evidence showing the ability of MPP^+ to bind the vesicular monoamine transporter-2 (VMAT-2), which in turn transports MPP^+ into synaptosomal vesicles containing dopamine (Del Zompo et al., 1993). Alternatively, if MPP^+ accumulates in the cytoplasm, it can also inhibit various cytosolic enzymes to perturb cellular functions (Klaidman et al., 1993; Ramsay & Singer, 1986). MPTP readily crosses the blood-brain barrier (BBB) (Markey et al., 1984) and works best on non-human primate models. MPTP is usually injected systemically. There is evidence to show that MPTP-treated primates produce symptoms and pathology that very closely resemble idiopathic PD, such as resting tremors, akinesia as well as rigidity (Jenner, 2003). The α -syn inclusions seen with MPTP, however, do not resemble classical LBs very closely

(Forno et al., 1992). On the other hand, rodents are less sensitive to MPTP, with the exception of the C57BL/6 mouse strain, although classical symptoms and α -syn aggregates do not appear in this model (Sedelis et al., 2001).

In contrast to the high risk associated with handling MPTP, 6-OHDA is relatively easier to use. 6-OHDA has been first used as a model of PD (Ungerstedt, 1971). 6-OHDA cannot cross the BBB, and thus needs to be injected stereotactically into the brain (Sotelo et al., 1973). The most common sites for injection are the striatum (Przedbroski et al., 1995), the medial forebrain bundle (MFB) and the SNpc (Deumens et al., 2002; Perese et al., 1989). The MFB is a bundle of nerve fibres arising from regions of the mid-brain, projecting to the forebrain. While injection of 6-OHDA into the MFB or SNpc leads to rapid degeneration of dopaminergic neurons (within 24-72 hours) (B. Jeon et al., 1995), striatal lesions are a better representation of slowly progressing, idiopathic PD (Przedbroski et al., 1995; Sauer & Oertel, 1994). 6-OHDA is taken up by monoaminergic i.e., dopaminergic and noradrenergic neurons (Luthman et al., 1989). Desipramine, a noradrenergic blocker, is therefore usually administered beforehand to ensure specific targeting of dopaminergic neurons only (Breese & Traylor, 1971).

6-OHDA works well with rodent and primate models, and is usually injected unilaterally (Ungerstedt & Arbuthnott, 1970).. Owing to the ease of injection into rodents, these are more frequently employed with 6-OHDA, with rats being the most commonly used. A good degree of nigral TH loss, and striatal dopamine loss are observed with 6-OHDA (Lee et al., 1996), making it a useful model for preclinical studies. On the other hand, LB like inclusions are rarely observed with 6-OHDA toxicity. In addition, unilateral models have the added advantage of being

less detrimental to the animals while making it easier to monitor the effects of treatment. Unilateral 6-OHDA models can show typical PD like symptoms such as rigidity and akinesia (Olsson et al., 1995). Furthermore, when 6-OHDA-treated rats are administered with dopamine agonists like apomorphine, or stimulators of dopamine release, such as amphetamines, the animals display rotational behaviour, which is often directly proportional to the extent of the lesion (Hudson et al., 1993; Ungerstedt et al., 1970). The mechanism of this behaviour, although still ambiguous, can be explained by striatal dopamine receptor sensitivity (Simola et al., 2007). This property of 6-OHDA however, is very useful in monitoring the intensity of the lesion, as well as easily quantifying the protective effects of experimental therapies.

The mechanism of 6-OHDA is attributed to a range of properties, with a primary focus on oxidative stress. 6-OHDA, upon entering dopaminergic neurons, is subjected to auto-oxidation which can lead to accumulation of free radicals such as quinones, peroxides and superoxides (Padiglia et al., 1997; Palumbo et al., 1999). Studies have also shown this property of 6-OHDA to be enhanced in the presence of ions such as ferrous and manganese (Simola et al., 2007). In addition to forming ROS, 6-OHDA is also known to inhibit endogenous free radical scavengers (Perumal et al., 1992) and complex I activity (Glinka & Youdim, 1995), thereby exacerbating oxidative stress. 6-OHDA is also one of the oxidative metabolites of dopamine found in small quantities endogenously (Linert et al., 1996; A. Napolitano et al., 1995), making a further case for its relevance to PD.

Environmental toxins such as the herbicide paraquat (PQ) and rotenone, a common insecticide, have several implications in the context of PD (Kiebertz & Wunderle, 2013). PQ has structural similarities to MPP⁺, and it also enters

dopaminergic neurons via DAT (Shimizu et al., 2001). PQ is normally administered systemically as it shows successful BBB penetration. PQ is commonly used on mice and rats, and leads to successful dopaminergic loss and LB formation, upon exposure (Bastías-Candia et al., 2015; Bastías-candia et al., 2018; Brooks et al., 1999; McCormack et al., 2002). Its mechanism of action is attributed mainly to accumulation of ROS and apoptosis. PQ also inhibits free radical scavengers (Somayajulu-Nițu et al., 2009) such as glutathione (Kang et al., 2009), and alters the redox cycle within the mitochondria (Cristóvão et al., 2009). PQ accumulation can lead to c-Jun N-terminal kinase (JNK) signalling induced apoptosis in dopaminergic neurons (Peng et al., 2004).

Rotenone is a member of a class of naturally occurring compounds known as rotenoids. Rotenone is also permeable to the BBB, facilitating systemic administration of the toxin (Talpade et al., 2000; Uversky, 2004). The mechanism of rotenone toxicity is mainly via inhibition of mitochondrial complex I (Betarbet et al., 2000). Animal experiments using rotenone have successfully resulted in loss of dopaminergic neurons, but some studies have shown its selective affinity for striatal damage only (Ferrante et al., 1997). Therefore, stereotactic use of rotenone is also employed, for selective degeneration (Heikkila et al., 1985). Interestingly, LBs containing ubiquitin, resembling idiopathic PD pathology have been observed in rotenone models (Betarbet et al., 2000), further establishing its relevance as an experimental PD model. Rotenone however, does not enter cells via DAT, and thus has greater chances of damaging other neurons as well, which makes this model debatable (Di Monte et al., 2002).

As seen in section 1.1.3 above, there are a host of genetic risk factors for PD. Therefore, transgenic models not only further the understanding between the

genetic function and relation to PD pathogenesis but are also useful in understanding differences between idiopathic and familial PD. Currently, α -syn transgenic models are the primary focus of such studies. Mice and fruit flies are the most commonly used species for genetic studies. There are several discrepancies in mouse models overexpressing α -syn. While most of these models consistently showed the presence of LB-like cytoplasmic inclusions containing ubiquitin, degeneration of dopaminergic neurons and depletion of striatal dopamine levels were not successfully achieved (Giasson et al., 2002; Lee et al., 2002; Masliah et al., 2000; Matsuoka et al., 2001; Van der Putten et al., 2000).

1.1.6 Diagnosis of PD

Current diagnosis for PD relies on examination of clinical symptoms. There are two main challenges underlying setbacks associated with accurate and timely diagnosis of the disease. The first challenge is an overlap of symptoms of PD with similarly manifesting conditions such as DLB, progressive supranuclear palsy (PSP), multiple system atrophy (MSA), essential tremors and corticobasal degeneration (CBD) (Hughes et al., 2002; Meara et al., 1999). The second factor driving misdiagnosis is a late manifestation of discernible symptoms, which do not always fall under the criteria currently in place (Schrag, 2002). Current strategies for diagnosing PD include a broad assessment of symptoms, which is eventually narrowed down by eliminating any symptoms overlapping with the above-mentioned disorders. The presence of at least two of the four cardinal symptoms of PD must be present. Additional features are, an asymmetry of symptoms at time of onset, and alleviation of symptoms after common PD medication, such as L-dopa (Hughes, 1992).

The Hoehn and Yahr scale helps neurologists monitor symptomatic decline and score patients, in turn aiding with course of treatment (Hoehn & Yahr, 1967). This system categorizes the progression of motor symptoms, as a function of time. The Unified Parkinson's disease rating scale (UPDRS) is a more definitive method of symptomatic classification (Ramaker et al., 2002). The Movement Disorder Society (MDS), has further refined the UPDRS incorporating more non-motor symptoms in the scale (Goetz et al., 2007; Goetz et al., 2003). This makes a more comprehensive scale for monitoring disease progression, after initial diagnosis. However, a definitive conclusion can only be made on histological samples from post-mortem brains, to confirm the presence of PD pathology.

More recently, some neurological tests that have been adopted to aid with diagnosis. The dopamine transporter scan (DaTscan), a recently approved technique, is used to quantify DAT in the brain, based on single photon emission computed tomography (SPECT). This aids diagnosis of PD by differentiating it from similar diseases that do not affect DAT, such as drug induced parkinsonism, or tremors (Walker et al., 2015). In some cases, magnetic resonance imaging (MRI), computed tomography (CT) or positron-emission tomography (PET) scans might aid in eliminating the presence of related disorders.

Owing to the challenges associated with accurate diagnosis of PD, a large body of research is now committed to investigating biomarkers for this purpose. Biomarkers will not only pave the way for a more empirical diagnosis, but will be effective in better distinction between similarly presenting conditions, and precision medicine, for varying cases of PD. The World Health Organization has defined a biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease”. Biomarkers

can be biochemical, genetic, or imaging biomarkers. Proteomics based techniques are the most commonly used when investigating biochemical biomarkers for PD. The current proteomics-based biomarker candidates have been summarised in Figure 1.5. Besides proteomics, metabolomics and genomics are gaining attraction in the context of biomarker development. Genetic loci implicated in PD have been explained in section above. Additionally, polymorphisms in the monoamine oxidase B (*MAO-B*), N-acetyltransferase 2 (*NAT2*) and glutathione S-transferase theta 1 (*GSTT1*) genes have also been associated with an increased risk for PD (Tan et al., 2000). Metabolomics is useful in discovering small molecules associated with PD in clinical and experimental studies. These studies have been described later, in section 1.3.4.

1.1.7 Treatment strategies for PD

Current treatment options for PD are not curative in nature. These strategies provide symptomatic relief only. Based on motor and non-motor symptoms, therapeutic intervention is categorized, giving respite from both. However, since PD is a progressive disease, medication can provide relief to an extent. Early diagnosis is therefore very important. Furthermore, current treatments can also have disagreeable side effects.

The first, and still the rifest therapeutic intervention is L-dopa or levodopa, a precursor of dopamine (Guggenheim, 1913). Its therapeutic effects lie in its ability to be converted into dopamine, by the enzyme dopa decarboxylase (DDC) after crossing the BBB (Blaschko, 1942). This acts as a counteractive measure against depleting dopamine levels in the basal ganglia of PD patients (Birkmayer & Hornykiewicz, 1961). Newly diagnosed patients are usually first put on L-dopa

treatment. L-dopa is often administrated with carbidopa, an inhibitor of DDC, which is a strategy to prevent peripheral metabolism of L-dopa, and increase the probability of cerebral dopamine synthesis (Markham et al., 1974).

A primary challenge associated with long-term L-dopa therapy is the prevalence of “on-off” effects manifesting after long term L-dopa treatment (Marsden & Parkes, 1976). The “on” phase that usually begins upon administration of a dose of L-dopa, wherein the patient exhibits symptomatic relief, usually lasts for shorter periods of time towards the administration of treatment (Marsden et al., 1976). As L-dopa levels start peaking, there is a quicker onset of the “off” phase resulting in motor impairment. Although this phenomenon is not experienced all by patients taking long term L-dopa, it poses quite a challenge and has also led to stigma against L-dopa therapy among patients in recent times.

Several other drug candidates with varying mechanisms of action have been developed for symptomatic relief of PD. These are usually prescribed along with L-dopa therapy. These are classified as dopamine agonists, catechol-O-methyl transferase (COMT) inhibitors, and monoamine – oxidase B (MAO-B) inhibitors. Dopamine agonists are chemicals which are structurally similar and can activate post-synaptic dopaminergic terminals (Schapira, 2002). Their advantage lies in the ability of these compounds to cross the BBB, as opposed to dopamine. Current available agonists are pramiprexole, ropinirole, bromocriptine, rotigotine, pergolide and apomorphine.

COMT is an enzyme responsible for the methylation of catecholaminergic neurotransmitters i.e., dopamine, epinephrine and norepinephrine (Mackinnon, 1957). This enzyme also works on catecholaminergic structures such as L-dopa,

thus deactivating it (Kurth, 1998). Use of COMT inhibitors aims at reducing the metabolism of L-dopa, thereby extending its half-life (Tai & Wu, 2002). They are thus used in conjunction with L-dopa treatment, especially at later stages. Entacapone and tolcapone are the most common COMT inhibitors used (Rinne et al., 1998). Monoamine oxidase is another class of enzymes, with different subtypes. Interestingly, only selective inhibitors of MAO-B can increase dopamine levels, while selective inhibitors of MAO-A such as clorgyline cannot (Riederer, 1993). MAO-B inhibitors are therefore used to prolong dopamine levels in the brain of PD patients (Riederer & Laux, 2011; Youdim & Bakhle, 2006). Rasagiline and selegiline are the two selective MAO-B inhibitors currently available. Current therapeutic strategies aimed at supplanting lost dopamine levels are depicted in Figure 1.6. Besides strategies aimed at the nigrostriatal pathway, anticholinergics can be used as adjuvant therapy used in PD. Anticholinergics such as oxybutynin can manage non-motor symptoms of PD such as frequent urination (Bennett et al., 2004). Amantadine, a glutamate antagonist, can be prescribed as an adjuvant in some cases. There is evidence to show it helps control PD related tremors, but the mechanism is unknown (Schwab et al., 1969).

For patients where a satisfactory level of symptomatic relief is not seen with therapeutic intervention, or patients experiencing severe side effects, deep brain stimulation (DBS) may be recommended. This procedure involves surgically introducing electrodes into parts of the basal ganglia such as the subthalamic nucleus or pars interna of the globus pallidus (Follett et al., 2010). These electrodes when activated, can then stimulate neuronal activity temporarily. The electrode activity is usually controlled by a distant pulse stimulator that the surgeon implants in the abdominal wall or near the shoulders.

1.2 Alternative therapeutic strategies for PD

One alternative strategy to try and contain PD progression includes mild physical exercise (Kolk & King, 2013). While this has shown some benefits in managing cognitive decline (Duchesne et al., 2015), it may not be the most effective or sustainable as the disease progresses. Alternative therapies such as herbal treatments and nutritional interventions have also gained traction in the context of PD. Their roles in the prevention and delay of onset of PD have been described in detail here.

1.2.1 Overview of nutraceuticals currently under research for PD

Nutraceuticals, including dietary supplements in some form or the other, are now much more commonly consumed. These include herbal medicines and plant extracts such as ginseng, vitamins, enzymes, minerals and some animal extracts such as fish oils. Such products are available in a wide range of formulations, ranging from gelatin capsules to chewable and non-chewable tablets, powders and even drinks. Nutraceuticals are regulated differently than food products. Dietary supplements can be used for a range of purposes, such as vitamin and mineral supplements to boost overall health, or herbal extracts for specific conditions.

The main drawback of current therapeutic intervention for PD, is its inability to slow down or prevent progression of the disease. Several dietary supplements are currently under study for their protective effects against slowing or preventing neurodegeneration in PD. Owing to the abundant role of oxidative stress in driving neurodegeneration in PD, many antioxidants are under review in this context. Besides the role of anti-inflammatory compounds (Pan et al., 2000), anti-apoptotic (Mercer et al., 2005) agents are also under review.

1.2.1.1 Vitamins

Vitamin C (ascorbic acid) and vitamin E (tocopherol) are the two most reviewed vitamins, for their antioxidant actions in PD. A meta-analysis of eight studies, by Etminan and co-workers, found that among vitamin C, vitamin E and β -carotene, a moderate intake of vitamin E is the most effective in decreasing the risk of developing PD (Etminan et al., 2005). However, another retrospective study found that food rich in vitamin E and carotenoids could increase the risk for developing PD (Scheider et al., 1997). On the other hand, a study by Anderson and co-workers on the effects of vitamin rich foods, found no association between foods rich in vitamin C, E or A and the risk for PD (Anderson et al., 1999). However, this study reported a significant risk for developing PD, associated with the intake of vitamin A-rich foods. Two clinical trials showed contradictory results on the effects of vitamins in PD patients. While one showed that administration of vitamin C and E to early stage PD patients, delayed the use of L-dopa treatment (Fahn, 1991), the clinical trial on effects of Vitamin E showed no such effects in delaying L-dopa therapy. This trial, famously known as the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP), compared the potential of deprenyl (selegiline) and tocopherol, in delaying the need for initiation of L-dopa therapy. They found that that deprenyl showed significant effects, but tocopherol did not (Shoulsen et al., 1989).

A comprehensive analysis by Newmark *et al.* found a link between Vitamin D deficiency, especially in vulnerable populations, and pathogenesis of PD (Newmark & Newmark, 2007). While the authors make a case for dietary intervention to delay the progression of PD, it must be noted that this study was based on the US

population only. Lastly, a study on MPTP-treated mice, found that folate deficiency increased the vulnerability of neurons to MPTP induced insult (Duan et al., 2002).

1.2.1.2 Coenzymes

Coenzyme Q₁₀ (CoQ₁₀), also known as ubiquinone or commonly known as coenzyme Q, is a ubiquitous coenzyme found in most species. This fat soluble molecule is a vital component of the electron transport chain (Ernster & Dallner, 1995). Owing to its close relevance to mitochondrial function, and promising antioxidant activity, CoQ₁₀ has been under review for its therapeutic potential in PD for a while.

One study found a high correlation between CoQ₁₀ intake and increased mitochondrial complex I levels in PD patient platelet mitochondria (Shults et al., 1997; 1999). This study also demonstrated the protective effects of CoQ₁₀ in preserving the nigrostriatal pathway in MPTP treated mice. Clinical trials have also demonstrated the protective effects of CoQ₁₀ in PD, using different assessment parameters. Shults and co-workers used periodic UPDRS scores to monitor patient decline after administration of different doses of CoQ₁₀ (Shults et al., 2002). Their findings suggest that high doses of CoQ₁₀ (1200 mg/day) showed the least change in UPDRS scores, compared to the placebo group. On the other hand, a trial conducted by Müller *et al.*, showed that CoQ₁₀ was mildly successful in providing symptomatic relief, compared to placebo, when administered for just four weeks (Müller et al., 2003).

Interestingly, another trial on 131 PD patients did not find any significant reduction in UPDRS scores between a three-month CoQ₁₀ regimen and placebo (Storch et al., 2007). It must be noted that all these trials report stable CoQ₁₀ kinetics

and a lack of adverse effects, making a case for its high tolerance in patients. Beal *et al.* also showed a lack of evidence for protective effects of CoQ₁₀ in PD, based on UPDRS and Mini Mental State Examination (MMSE) scores, which helps in assessing cognitive decline (Beal *et al.*, 2014).

1.2.1.3 Creatine

Creatine is an organic acid, whose function in the body is to maintain adenosine triphosphate (ATP) levels, by recycling and phosphorylating adenosine diphosphate (ADP) (Barcelos *et al.*, 2016). When the effects of creatine were tested on an MPTP induced PD model, a reduction of neurodegeneration in the SNpc was observed (Matthews *et al.*, 1999). Furthermore, a combination treatment of creatine and CoQ₁₀ proved to be effect against neurodegeneration in PD and Huntington's disease (Yang *et al.*, 2009). Additive effects of creatine and a cyclooxygenase (COX) inhibitor were also seen in MPTP-treated mice (Klivenyi *et al.*, 2003). Lastly, protective functions of creatine were also shown in a two year long clinical trial wherein creatine was successful in reduction of dopamine enhancing therapy, but had no significant effect in changing UPDRS scores (Bender *et al.*, 2006).

1.2.1.4 Saponins

Saponins are glycosides found in abundance in several plant species. The most common saponins used for therapeutic purposes are ginsenosides, found primarily in the ginseng root (*P. ginseng*) (Chang-Xiao & Pei-Gen, 1992). The two major types of ginsenosides are protopanaxatriol (Rg1, Rg2, Re, Rf, and Rh1) and protopanaxadiol (Rb1, Rb2, Rc, Rd, Rg3, Rh2, Rh3). Rg1 has been shown to exert protective effects in MPTP-treated mice, by suppressing iron levels (Wang *et al.*, 2009). One mechanism of action of ginsenoside Rg1, has been attributed to modulation of the Wnt/ β -catenin pathway as seen in *in vivo* and *in vitro* models of

PD (Zhou et al., 2016). Rg1 can also modulate other signalling pathways such as extracellular signal-regulated kinases (ERK), JNK and phosphatidylinositol 3-kinase (PI3K/Akt) (Gao et al., 2009). Ginseng extract has been shown to be neuroprotective in MPTP-treated SH-SY5Y cells (Hu et al., 2011), mice and MPP⁺-treated rats, as seen by improved motor function and reduced dopaminergic loss (Van Kampen et al., 2003). This extract was also useful in preventing microgliosis and α -syn aggregation (Van Kampen et al., 2014). Another saponin, astragaloside IV (AS-IV) also exhibited protective effects against 6-OHDA-induced toxicity, in nigral cultures (Chan et al., 2009). An in-depth analysis of the exact mechanism of ginseng extract and its constituents is warranted before further research can be done.

1.2.1.5 Natural L-dopa

The extensive role of L-dopa in providing symptomatic relief in PD, has already been discussed before. Research has also been directed at finding natural sources of L-dopa to meet its increased demand. L-dopa can be found and isolated from legumes such as velvet bean (*Mucuna pruriens*, *Stizolobium deeringianum*) and broad bean (*Vicia fava*). *M. pruriens* is commonly used in Ayurvedic practice to treat PD and related disorders (Manyam & Sánchez-Ramos, 1999). In recent times, clinical trials have also been conducted to assess the effects of *M. pruriens* seed powder. Vaidya *et al.* found that the extract was tolerated better than traditional L-dopa by PD patients (Vaidya & Mankodi et al., 1978), while Katzenschlager *et al.* showed the superior effects of seed powder with respect to duration of action and adverse effects such as dyskinesia (Katzenschlager et al., 2004).

1.2.1.6 Other anti-oxidants

There are several other experimental antioxidant therapies under review for PD. Several sulphur-containing compounds are being tested for this purpose, of

which the first was N-acetylcysteine (NAC). Such compounds can be found in the *Allium* genus of plants like onions and garlic. The antioxidant ability of such compounds has been attributed to glutathione synthesis, thereby reducing ROS and mitigating programmed cell death (Banaclocha, 2001). In a MPTP-treated model, the effects of four cysteine containing compounds including NAC were studied (Chen et al., 2007). NAC showed the most promise with regards to its anti-inflammatory and antioxidant abilities. Furthermore, the amide of NAC, N-acetylcysteine amide (NACA), showed protective effects in PC12 cells by increasing glutathione levels (Penugonda et al., 2005). In a separate study, the additive effects of L-dopa with sulphur containing antioxidants such as cysteine, methionine and bucillamine was demonstrated (Pinnen et al., 2009). However, while these antioxidants seemed to increase glutathione levels, they decreased the bioavailability of L-dopa.

1.2.1.7 Polyphenols

Polyphenols comprise a wide range of compounds sharing a similar chemical structure. Structurally, they contain an aromatic/phenyl ring, with one or more hydroxyl groups. An official definition proposed for polyphenols, is a class of compounds that are “generally moderately water-soluble compounds, with molecular weight of 500–4000 Da, with >12 phenolic hydroxyl groups and with 5–7 aromatic rings per 1000 Da” (Quideau et al., 2011). Polyphenols comprise a variety of different structures, such as flavonoids, tannins, quinones, coumarins, catechins, theaflavins, curcuminoids and even caffeine and its derivatives. Most of these compounds have been studied in the context of PD.

1.2.1.7.1 *Flavonoids*

Flavonoids comprise six major types of molecules, namely, anthocyanidins, flavan-3-ols, flavonols, flavanones, flavones, and isoflavones. These compounds, some of which also classify as pigments, are found abundantly in the diet, mainly fruits and vegetables. Most of the evidence for the role of flavonoids in mediating neuroprotection against PD, is still in the experimental stages. One study tested the effects of 48 flavonoids on α -syn aggregation and found that most of these were effective in delaying or preventing the formation of fibrils (Uversky et al., 2010). The authors also suggested a correlation between the antioxidant activities of flavonoids and their α -syn aggregation preventing ability. The anti-inflammatory activity of flavonoids in reducing microglial activation and neuroinflammation in toxin models of PD has also been shown (Lee et al., 2014; Leem et al., 2014; Patil et al., 2014). Besides their antioxidant activity, an antihypoxic activity of some flavonoids has also been shown, which resulted in enhanced neuroprotection in MPTP-treated mice (Smirnova et al., 2016). Lastly, the effects of flavonoids from fruit peels, clover and cocoa seeds were compared with catechins, found in grape seeds, for their effects on 6-OHDA lesioned rats. The former showed enhanced neuroprotection (Datla et al., 2007).

Catechins are a sub-type of flavonoids known as flavol-3-ol. Besides fruit seeds, these are widely present in green tea. Catechins from green tea have shown protective effects against neurodegeneration in a wide range of studies. Major catechins found in green tea include (-)-epicatechin (EC) (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin-3-gallate (EGCG), of which EGCG is the most abundant. Catechins resemble catechols in structure, which are known as potent antioxidants and iron chelators (Guo et al., 1996). The

protective actions of catechins in PD, have been attributed to this property, and other mechanisms as well. In MPTP-treated models, it has been shown that catechins can inhibit depletion of striatal dopamine (Levites et al., 2001), as well as block the DAT, thereby preventing MPP⁺ from entering neurons (Pan et al., 2003). In 6-OHDA treated rats, green tea polyphenols led to neuroprotection by containing ROS and nitric oxide build-up and lipid peroxidation in the striatum and midbrain (Guo et al., 2007). Furthermore, the involvement of EGCG in promoting cell survival through activation of protein kinase C (PKC), has also been elucidated (Levites et al., 2002). Additionally, Lu *et al.* demonstrated the ability of catechins in inhibiting COMT, the enzyme responsible for metabolism of L-dopa, making a case for its use as adjuvant therapy (Lu et al., 2003). Lastly, a cohort study that compared the effects of green versus black tea on the risk for PD, found that while green tea demonstrated no correlation with the incidence of PD, dietary components of black tea other than caffeine, showed a negative correlation with the risk for developing PD (Tan et al., 2008).

1.2.1.7.2 *Black tea and its components*

Primary components of black tea, besides caffeine, include theaflavins and thearubigins. These components are synthesized by the condensation of flavon-3-ols during an enzymatic process which is used in the production of black tea. Besides the promising results of black tea components in reducing the risk for PD, as seen above, its protective actions have been attributed to its anti-inflammatory, antioxidant and anti-apoptotic activities. It has been shown that black tea polyphenols are as potent antioxidants as green tea phenols (Dreosti, 1996). Anandhan and co-workers have shown via MPTP-treated rats that theaflavin exerts neuroprotection in these mice by reducing apoptotic proteins (Anandhan et al.,

2012) and mitigating neuroinflammatory markers (Anandhan et al., 2013). Moreover, the strong antioxidant effect of black tea extract resulted in improvement of locomotor function and dopamine metabolism in 6-OHDA treated rats (Chaturvedi et al., 2006).

1.2.1.7.3 Curcuminoids

Curcuminoids are compounds which are a major constituent of turmeric, a spice heavily used in Asian food. Primary curcuminoids are diferuloylmethane (curcumin), demethoxycurcumin and bisdemethoxycurcumin, of which curcumin is being investigated extensively for its protective roles in PD. Curcuminoids are also known to have anti-inflammatory and antioxidant properties (Yang et al., 2015). Curcumin can protect dopaminergic neurons against LPS induced neuroinflammation (Yang et al., 2008). In line with its antioxidant function, studies have reported the effects of curcumin in restoring depleted glutathione levels in damaged neurons and mouse models (Jagatha et al., 2008). When 6-OHDA-treated rats were first exposed to curcumin and other flavonoids, it was observed that curcumin was the most effective in reducing neurodegeneration (Zbarsky et al., 2005). Its antioxidant property was also helpful in protecting mitochondrial function in the brain, by regulating glutathione levels (Mythri et al., 2006). The protective effects of curcumin against MPTP-induced toxicity in SH-SY5Y cells was shown in two different studies, wherein mitochondria driven apoptosis (Chen et al., 2006) and oxidative stress were alleviated (Yu et al., 2010).

Curcumin is not limited to protect against α -syn overexpression and aggregation in cellular models (Pandey et al., 2008; Wang et al., 2010), but also A53T- α -syn induced macroautophagic dysfunction (Jiang et al., 2013). Moreover, neuroprotection mediated by curcumin has also been attribute to its iron chelating

(Du et al., 2012) and MAO-B inhibiting (Rajeswari & Sabesan, 2008) properties. Conversely, Ortiz-Ortiz and co-workers showed the apoptosis-enhancing ability of curcumin when mesencephalic cells were co-treated with paraquat (Ortiz-Ortiz et al., 2009).

1.2.1.7.4 Caffeine and its derivatives

While caffeine is the most popular constituent of coffee, other active ingredients are caffeoyl quinic acid derivatives, tannic acid and nicotinic acid (Dórea & Da Costa, 2005). A series of studies have aimed to assess the relationship between caffeine consumption and the risk of PD. The general consensus about this relationship hints at the ability of caffeine to reduce the risk of developing PD (Ascherio et al., 2003; Costa et al., 2010; Hernán et al., 2002; Ross et al., 2000; Tan et al., 2003). Interestingly, Ascherio and colleagues also observed that men required greater coffee consumption compared to women, to exhibit an inverse relationship with the risk of developing PD (Ascherio et al., 2001). Decaffeinated coffee was not effective in either case.

The neuroprotective mechanism of caffeine is still under way, but several theories have been proposed. Nakaso *et al.*, found in SH-SY5Y cells that caffeine shows anti-apoptotic effects by activating the PI3K/Akt pathway (Nakaso et al., 2008). Alternatively, a host of studies have also proposed that the mechanism of action of caffeine in facilitating neuroprotection is due to its A2A receptor antagonist activity (Chen et al., 2001; Kalda et al., 2006; Schwarzschild et al., 2003). This was supported by the finding that other A2A receptor antagonists also exerted similar neuroprotection.

1.2.1.7.5 *Stilbenoids*

Stilbenoids are a class of polyphenols which have a C₆-C₂-C₆ skeleton in their structure. They are the hydroxylated derivatives of stilbene (1,2-diphenylethene) and can be classified as glycosides, which are stilbenoids linked to sugars, and aglycones. Stilbenoids are found primarily in plant sources and some bacteria (Xiao et al., 2008).

Stilbenoids have been studied extensively in PD research for their potential neuroprotective properties. The most popular stilbenoid is resveratrol (RES), owing to its wide range of properties and uses. Other common stilbenoids include oxyresveratrol (OXY), a hydroxylated derivative of resveratrol, pinostilbene (PINO), a methylated derivative, and pterostilbene (Figure 1.7). A detailed overview of resveratrol, oxyresveratrol and their neuroprotective effects has been described next.

1.2.2 *Resveratrol*

Resveratrol or 3, 4', 5- trihydroxystilbene is a phytoalexin found in several plant species. Phytoalexins are chemical compounds produced by plants as defence against parasites, injuries and infection. Resveratrol is found in grape skins (Romero-Pérez et al., 2001), grape juice (Romero-Pérez et al., 1999), red and white wine (Király-Veghely et al., 1998; Ribeiro De Lima et al., 1999; Soleas et al., 1997), with lesser quantities present in cranberry juice (Wang et al., 2002), blueberries (Lyons et al., 2003), peanuts (Sanders et al., 2000; Tokuşoğlu et al., 2005) and pistachios (Tokuşoğlu et al., 2005). The putative beneficial effects of RES were observed early on, in a phenomenon known as the “French paradox” (Kopp, 1998; Renaud & de Lorgeril, 1992). It was observed that although people in Southern

France consumed a diet rich in saturated fats, ones who regularly consumed red wine, had a very low incidence of heart disease. It is now known, after several preclinical and clinical studies, that RES does have beneficial effects not only to the heart, but in cancer, metabolic syndrome and neurological conditions as well.

RES is an antioxidant and a sirtuin1 (SIRT1) activator (Chung et al., 2010). SIRT1 is a mitochondrial protein that drives cellular stress, survival, senescence, endothelial function and even protein folding. The antioxidant effects of RES have been shown to be beneficial for several conditions. RES has been shown to decrease lipid peroxidation, malondialdehyde content, nitric oxide levels, scavenge free radicals and increase intracellular glutathione levels, *in vivo* (Frankel et al., 1993; Han et al., 2004; Wenzel et al., 2005). RES is also known to be an inhibitor of COX (Kundu et al., 2006), the enzyme responsible for driving inflammation and modulation of the NF- κ B, Akt/Wnt and PI3K driven signalling pathways (Vanamala et al., 2010).

In the context of cardio-protection, clinical trials have demonstrated the ability of RES to lower low density lipoprotein (LDL)-cholesterol levels in patients with coronary heart disease (Magyar et al., 2012). Reduced diastolic pressure was observed in a one-month long trial on subjects with hypertension (Biesinger et al., 2016), whereas inhibition of platelet aggregation *in vitro* (Bertelli et al., 1995), and the vasodilatory activity of RES, by regulating K⁺ channels has also been reported (Nicholson et al., 2008). The greatest body of research on RES has been done to elucidate its anti-tumorigenic effects. It began with the study by Jang and co-workers on mouse models, which showed for the first time that RES when used topically, could reduce the size of skin tumours (Jang et al., 1997). RES also reduced angiogenesis promoted by tumours (Tseng et al., 2004), as well as

promoted the survival of mice with neuroblastomas (Chen et al., 2004). Human trials have shown evidence for apoptotic effects of RES in colorectal cancer patients (Howells et al., 2011), along with impeding tumour cell proliferation (Patel et al., 2010). Certain beneficial effects were also seen in prostate cancer patients (Paller et al., 2015). Additionally, RES showed promising results in diabetes trials wherein administration of RES for three months resulted in improved hypoglycaemia, cholesterol levels and blood pressure (Bhatt et al., 2012). Decreased insulin resistance, plasma glucose levels and slower glucose peaks were also observed in another study (Brasnyó et al., 2011).

With regards to neurological conditions and aging, while most studies are still in the preclinical phase, some interesting observations have been made. Rats with cerebral artery occlusion, representing a stroke model, when treated with RES, showed less impaired motor function (Sinha et al., 2002). Additionally, RES also delayed neuronal death and glial activation in a model of ischaemic stroke (Wang et al., 2002). SIRT1, one of the targets of RES, is known to play a role in extending life span. Studies on SIRT1 knockout mice have shown developmental defects and a shorter life span, in these animals (McBurney et al., 2003). A study on aged fish brains showed that consumption of RES led not only to a 60% extension in life span, but also sharpened motor performance with age, and reduced levels of aggregated proteins (Valenzano et al., 2006). RES showed protective effects in several models of PD. The study by Jin *et al.* demonstrated the potential of RES in rescuing rats against 6-OHDA-induced lesion by inhibiting neuroinflammation (Jin et al., 2008). Moreover, another study showed that a two-week pre-treatment of RES followed by injection of 6-OHDA in rat striata, resulted in improved motor function and inhibition of free radical accumulation (Khan et al., 2010). Lastly,

clinical trials in AD patients have shown the ability of RES in reducing neuro-inflammation and preserving cleaved A β i.e., A β 42 and A β 40 levels in the CSF (Moussa et al., 2017; Turner et al., 2015).

It must be noted that despite its therapeutic potential as a nutraceutical, a major drawback of RES has been its limited bioavailability (Walle, 2011). Owing to its quick metabolism and excretion from the body, RES administered via the oral route has a very limited half-life. Additionally, the strong apoptotic properties of RES, which are exploited in cancer research, also make it a delicate dietary candidate to work with.

1.2.3 Oxyresveratrol

Oxyresveratrol or 2, 3', 4, 5'-*trans*-trihydroxystilbene is the hydroxylated form of RES. It can be isolated by the hydrolytic activation of Mulberroside A, a compound found primarily in the root of *Morus alba* (White mulberry). However, OXY was first isolated from the heartwood (wood chips) of *Artocarpus lakoocha* (monkey fruit). OXY is also found in the roots, stems and leaves of other plants such as *Smilax china* (china root), *Veratrum nigrum*, and *Maclura pomifera* (osage orange) (Xu et al., 2014).

To study the cellular permeability of OXY, Chao et al treated SH-SY5Y cells with 10 μ M OXY for 6 hours or 24 hours (Chao et al, 2008). Intracellular extracts were then subjected to chromatographic separation. It was found that a peak of OXY could be observed in the extract even after 6 hours, which was reduced to half by 24 hours. These results suggested the gradual but steady uptake and metabolism of OXY within cells. Although OXY is more hydrophilic than RES there is evidence to support its adequate BBB penetration (Breuer et al., 2006). In this

study, Male Wister rats were subjected to middle cerebral artery occlusion. OXY penetration to the infarct region was then estimated. Using *in vivo* microdialysis of the striatum it was found that while OXY had relatively low BBB penetration in control animals, the infarct region had up to 660% better OXY penetration. Another independent study attempted to compare the pharmacokinetics of OXY and RES in rats (Huang et al, 2007). Rats were administered with 1g/kg *S. China* (equivalent to 180 mg/kg OXY and 80 mg/kg RES). Plasma concentrations of the two drugs were then determined. While both OXY and RES reached maximal plasma concentrations within 15 minutes, they were both detected in the plasma up to 6 hours after. While most pharmacokinetic studies on OXY have been performed in experimental models, kinetics from clinical studies will give the most accurate depiction of its absorption, distribution, metabolism and excretion.

OXY has also been demonstrated to have a host of therapeutic effects which are both, similar and ancillary to RES. Several reports have demonstrated the greater antioxidant effects of OXY over RES (Lorenz et al., 2003; Povichit et al., 2010). While the former showed the enhanced antiglycation effects of OXY, the latter showed the effects of OXY in combatting nitrosative and oxidative stress, with a reduced cytotoxicity than RES. Interestingly, the synergistic activities of OXY and RES against oxidative stress have also been demonstrated, making a case for the use of both stilbenoids together (Aftab et al., 2010). Lastly, out of the various stilbene constituents of *Cortex mori*, it was reported that OXY had the highest free radical scavenging capacity (Zhang et al., 2008).

Besides anti-oxidative abilities, OXY is also purported to have anti-inflammatory, antibacterial, antiviral (Likhitwitayawuid et al., 2006) and tyrosinase inhibitory effects. Chung and colleagues showed the anti-inflammatory effects of

OXY *in vivo* and *in vitro*. It was seen that OXY successfully inhibited prostaglandin E2 synthesis, nitric oxide production and NF- κ B activation (Chung et al., 2003). Moreover, in an LPS-treated model, OXY could modulate interleukin 6 (IL-6) dependant signalling (Mouihate et al., 2006). The anti-inflammatory effects of OXY with regards to neuroinflammation, however, remain to be discovered fully. The anti-tyrosinase activity of OXY has implications in pigmentation disorders; tyrosinase being a key enzyme in melanin formation. In fact, the anti-tyrosinase activity of OXY is shown to be not only more potent than RES, but also already established skin whiteners such as kojic acid and azelaic acid (Kim et al., 2010; Zheng et al., 2012). OXY is also known to protect against ultraviolet B (UVB) radiation induced melanogenesis in guinea pigs (Park et al., 2011). Additionally, OXY has also been under study for its anti-tumorigenic (Li et al., 2010; Wu et al., 2010) and hepato-protective effects (Zhang et al., 2008). However, only the neuroprotective effects of OXY have been highlighted in detail here.

In a model of ischaemic stroke, OXY exerted neuroprotective effects against oxidative and nitrosative stress (Breuer et al., 2006). In cerebral artery occluded rats, OXY not only reduced infarct volume, but also prevented DNA fragmentation, induced by apoptosis (Andrabi et al., 2004). This was further corroborated by reduced cytochrome *c* and release and caspase activity. In a cell culture model of trauma, wherein neuronal cultures were exposed to extensive glutamate levels, OXY significantly delayed neuronal death (Weber et al., 2012). With respect to neurodegeneration, Jagtap *et al.* observed that OXY treatment resulted in reduced microglial loss (Jagtap & Bapat, 2010).

OXY also exerted protective effects against A β , in experimental AD models. In a study by Ban *et al.*, OXY alleviated A β (25-35) (another toxic species

generated from β -amyloid) induced damage by interfering with Ca^{2+} mediated glutamate release (Ban et al., 2006). Interestingly, OXY has also been shown to inhibit β -secretase activity, which is the enzyme responsible for cleaving $\text{A}\beta$ (Jeon et al., 2007). Apposite to PD, the neuroprotective effects of OXY on reducing apoptosis and ROS formation in the SH-SY5Y cell line have been shown (Chao et al., 2008). OXY was demonstrated to also activate SIRT1 in the model and modulate JNK and Akt-mediated signalling. This study also revealed that higher doses of OXY were less toxic than higher doses of RES in the SH-SY5Y neuroblastoma cells. Furthermore, exposure to OXY prior to 6-OHDA, showed the most neuroprotective effects, compared to a post-treatment or washout. The effects of OXY on toxin-based animal models will give a better understanding of its effects in PD.

1.3 Metabolomics

1.3.1 General overview

Dating back to 1500-2000 B.C., doctors in ancient China first proposed the idea that diabetes mellitus could be diagnosed based on the sweet taste of the patient's urine (Van Der Greef & Smilde, 2005). This historical finding was used as a standard for years to come and underlies the basic principle of metabolomics; changes of metabolites in the body can be used to predict and reflect disease status. Metabolomics or metabonomics is defined as “quantitative measurement of the dynamic multiparametric metabolic response of living systems to physiopathological stimuli or genetic modifications” (Nicholson et al., 1999). It is therefore the study of “the complete set of metabolites/ low-molecular-weight intermediates (metabolome)” which vary according to the developmental,

physiologic or pathologic state of the cell, tissue or organism (Raamsdonk et al., 2001).

“Omics” studies traditionally comprised only genomics (functional and structural study of the genome), transcriptomics (mapping mRNA and the transcriptome) and proteomics (large scale study of proteins). Together, these studies can give information about the etiology and pathogenesis of disease. The pathway from genes to transcription to protein translation is clearly understood and well-defined. However, relying on only traditional omics techniques is not a feasible approach anymore. This is because, factors that influence disease status do not directly affect the genome, transcriptome or proteome, and in cases where they do, these changes are not reflected promptly enough to detect them (Klupczyńska et al., 2015; Nicholson & Lindon, 2008). Furthermore, with a shift in focus toward precision medicine and our increased understanding of the multitude of endogenous and exogenous factors capable of modifying disease, this information is not enough (Holmes et al., 2008; Nicholson, 2006). These drawbacks lead to the advent of a fourth branch, metabolomics, which has revolutionized our understanding of systems biology.

Metabolites are small molecules (< 1 kD) found at cellular and tissue levels in the body (Fiehn, 2002). A complete set of metabolites found in any given tissue is known as its “metabolome” (Nicholson et al., 2008). Metabolites are very sensitive to internal and external stimuli, ranging from genetic variations and stress, to environmental factors, diet, gut microflora and therapeutic intervention (Holmes et al., 2008). Metabolite changes are also seen in “real time” i.e., they are an immediate function of a stimulus, as opposed to gene and protein expression changes which can take much longer (Nicholson et al., 2002). Owing to this

sensitivity and promptness, metabolic changes accurately reflect the health status of an individual. Furthermore, metabolic pathways are interrelated with enzyme function, either as end products, co-factors or intermediates. Synthesis of metabolites is often part of the final step of biochemical reactions, representing the end product of RNA and protein expression (Fiehn, 2002). This makes metabolites the outcome of any biological change, linking them closely to phenotype (Figure 1.8).

Biochemically, metabolites comprise lipids, fatty acids, amino acids, carbohydrates, nucleotides, neurotransmitters, pigments and vitamins, among others (Klupczyńska et al., 2015). Metabolic pathways have indispensable functions in the body. They are responsible for energy production, energy storage, and are the building blocks of genetic material and proteins (Schuster et al., 2000). Due to their universal presence and function, metabolites can be measured in tissues or biofluids, depending on the purpose. This approach has been used in human as well as *in vivo* and *in vitro* experimental studies. Metabolites are conserved well between species, making a strong case for the use of *in vivo* models in metabolomics studies (Holmes & Nicholson, 2007). Metabolomics was adopted quickly after its introduction, because it is a relatively easier and cheaper technique; the metabolome comprises much fewer molecules than the transcriptome, genome or proteome (Patti et al., 2013; Wishart et al., 2009). Despite this, metabolomics has a wider set of applications, and is employed in medicine for biomarker discovery, drug development and monitoring disease progression. Metabolomics also finds applications in plant biology, food and environmental science and microbiology (Alonso et al., 2015). It must be noted however, that the most comprehensive information can only be obtained when a combined “omics” approach is used.

Metabolomics is classified as “targeted” or “untargeted”, based on the approach used (Patti et al., 2013). Untargeted metabolomics, which uses a “top down” strategy measures changes in the total set of metabolites in any given tissue of an organism (Vinayavekhin & Saghatelian, 2010). Based on changes observed, a hypothesis can then be made about the causal relationship between the stimuli in question and metabolites altered (Alonso et al., 2015). While this approach is often more time consuming, and requires an in-depth understanding of data treatment and metabolite identification strategies, vast amounts of information can be generated (Dunn et al., 2013) . On the other hand, targeted metabolomics measures changes in a pre-determined set of metabolites, based on a hypothesis (Dudley et al., 2010; Schuster et al., 2011). Because of the specific nature of this approach, analytical methods can be varied to as to highlight the metabolites in question without generating a large amount of data. It is therefore a faster and easier set of analysis to perform, with no high skillset required for metabolite identification (Dudley et al., 2010; Griffiths et al., 2010; Roberts et al., 2012; Schuster et al., 2011).

In recent years, traditional analytical techniques have been refined extensively, which has made the acquisition of vast amounts of metabolite information possible. The two most commonly used techniques are Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) based metabolomics. The tool employed usually depends on cost and time considerations, the depth of information required, sensitivity, and the sample source used for analysis. Both these techniques are described in detail in the next section.

1.3.2 *Analytical techniques in metabolomics*

1.3.2.1 *Nuclear magnetic resonance*

Nuclear magnetic resonance spectroscopy works on the principle of the difference in energy absorbed and emitted by certain atomic nuclei, when exposed to an external magnetic field. The most common nuclei are ^1H , followed by ^{13}C due to their abundance in biological compounds (Ross et al., 2007). NMR spectra are easily quantifiable; the peak area generated corresponds directly to the molar concentration of the specific nuclei present (Kim et al., 2010). Concentrations of different metabolites can therefore be compared in a straightforward manner, without the use of calibration curves. NMR spectra can be generated from pure compounds, or mixtures, without any prior separation (Klupczyńska et al., 2015). Therefore, analysis of complex biological samples using NMR is relatively easy. Furthermore, the NMR spectral pattern is indicative of the chemical structure of each metabolite (Alonso et al., 2015).

Other advantages of NMR are the ease of sample preparation and its less destructive nature (Bothwell & Griffin, 2011; Lindon & Nicholson, 2008). Unlike mass spectrometry, which is explained next, NMR analysis does not result in chemical destruction of the sample used. This can be particularly useful for samples that are not easily procured, such as CSF. NMR analysis is also less affected by matrix additives.

Although NMR is the more traditional metabolomics approach, its primary disadvantage over MS is a lack of sensitivity (Putri et al., 2013). It has been shown that NMR studies can only detect up to 10% of the total metabolites present in any biological sample (Hall, 2006). This is a problem, especially when using the

untargeted metabolomics approach. Another issue with NMR analysis is the presence of overlapping peaks leading to structural ambiguity, which sometimes cannot be overcome even with 2D-NMR, especially in the case of unknown metabolites (Ward et al., 2007).

1.3.2.2 Mass spectrometry

Mass spectrometry (MS) is a relatively newer technique applied to metabolomics. Traditionally, it was used for identification of chemicals, drugs and impurities in the pharmaceutical industry. However, when coupled with separation techniques such as chromatography, it gives a great deal of information about metabolites in any biological matrix. Mass spectrometry generates a spectrum of the mass to charge ratios (m/z) of the ions derived from each analyte, based on their relative intensity. Consequently, for MS analysis the samples first need to be ionized. The ions generated from each metabolite are in a gaseous form. The ions are then separated according to their m/z , and subsequently detected. Each compound thus has its own spectral fingerprint, based on the ions fragmented formed and detected, which aids in its identification (Dunn & Ellis, 2005).

Mass spectrometry is a highly reproducible and sensitive technique, although it does have certain drawbacks. Extensive sample preparation is required prior to analysis, especially when coupled with gas chromatography (GC). Moreover, during ionization, the samples are destroyed. On the other hand, due to its sensitive nature, small quantities of samples are required for analysis ($< 10\mu\text{l}$) and trace amounts (up to femtomoles) of metabolites can be detected (Dunn et al., 2005; Sumner et al., 2003). Another drawback of MS includes a loss of sample during preparation, which can make quantification less accurate. To account for this loss, a known concentration of radio-labelled internal standard (IS) is often added to the

sample for the purpose of semi-quantification (Wu et al., 2005). Moreover, a variety of ionization techniques are available, which are not compatible with all classes of metabolites (Dettmer et al., 2007). Selection of the appropriate ionization technique and separation method beforehand is therefore of utmost importance.

Ionization methods commonly used with MS, include electrospray ionization (ESI), electron impact ionization (EI), atomic pressure chemical ionization (APCI) and matrix assisted laser desorption ionization (MALDI) (El-Aneed et al., 2009; Zhou et al., 2012). ESI is the most common technique, which when coupled with liquid chromatography (LC), makes the most commonly employed metabolomics tool. The main advantage for using ESI is its ability to minimize fragmentation of the analyte, making identification less complicated. It is therefore known as a “soft” technique (El-Aneed et al., 2009). However, ion suppression is a common problem observed with ESI, wherein several components entering the ionizer cannot be ionized successfully at the same time. The use of hyphenated techniques such as Liquid chromatography-Mass spectrometry (LC-MS) and Gas chromatography-Mass spectrometry (GC-MS) that use chromatography facilitating separation and resolution of analytes, have reduced this problem (Lei et al., 2011). A more traditional method is EI, now seldom used due to its “hard” nature, causing the ions to fragment extensively. It is however, still a popular choice when using GC (Hall, 2006; Scalbert et al., 2009). When a laser is employed for the purpose of ionization, such as in MALDI, this technique can be applied in *in situ* imaging studies and for compounds with very large molecular weights (Greving et al., 2011; Miura et al., 2012; Yanes, 2013).

High resolution mass spectrometry (HRMS) has made it possible to effectively separate and analyse compounds with the same nominal mass, but different exact

masses (Junot et al., 2014). Examples of MS incorporating high resolution analysers, which are currently in use, are Fourier transform ion cyclotron resonance mass spectrometers (FT-ICR-MS), time-of-flight mass spectrometers (TOF-MS) and Orbitraps (Ghaste et al., 2016). Traditional low-resolution MS uses a quadrupole or ion trap analyser. The function of the analyser is to separate the ions based on m/z and send them to the detector. Furthermore, tandem MS, MS/MS or MS² is another increasingly popular approach, which involves two steps. The precursor ions are first separated, followed by fragmentation to give fragment ions which are subsequently separated and detected. Fragmentation is usually achieved using collision-induced dissociation (CID) or photodissociation (Taylor, 2005).

The three most commonly used chromatographic techniques which aid in the separation of metabolites for MS, are GC, LC and capillary electrophoresis (CE). The differences between each, lie in the way analytes, which are carried by a mobile phase, interact with the stationary phase.

GC-MS is the more traditional metabolomics technique. However, this technique is still popular, especially when analysing plant components such as terpenes and essential oils. GC-MS is also particularly efficient for analysing certain hydrophilic metabolites such as fatty acids and sugars, as well as glycerides (Kopka, 2006; Tsugawa et al., 2011). The mobile phase in GC is a carrier gas (such as nitrogen or helium), which carries volatile samples through a capillary column. The separation achieved by the GC capillary column is far superior compared to packed columns used in high performance liquid chromatography (HPLC) (Bartle & Myers, 2002). However, the analytes need to be volatile and thermostable. Non-volatile compounds are usually rendered volatile in a step called as derivatization (Tsugawa et al., 2011). The derivatization step however, can often lead to loss of

sample (Lenz & Wilson, 2007). GC-MS gives stable and reproducible analysis using EI ionization. GC is usually coupled with single quadrupole MS, or TOF, which is a more expensive set-up. Overall GC-MS is a sensitive and high resolution metabolomics technique (Kind et al., 2010; Simón-Manso et al., 2013; Sumner et al., 2003).

The breadth of compounds that can be separated by LC, however, is not restricted to volatile compounds and LC can separate metabolites having diverse polarity. Based on the polarity of metabolites in question, either reversed-phase (RP) or hydrophilic interaction liquid chromatography (HILIC) chromatography is used (Zhou et al., 2012). RP chromatography uses C₈ or C₁₈ columns and HILIC is used to separate primarily ionic and highly polar metabolites (Zhou et al., 2012). Furthermore, MS analysis in the positive and negative mode, enables ionization of most metabolites effectively. Despite its sensitivity and precision, LC-MS comes with a drawback of more analytical variation and therefore less reproducibility than GC-MS. To overcome this setback, quality control (QC) samples are commonly used between samples. QC samples are generally pooled analyte samples and are used to determine inter-run variability.

The advent of ultra-high performance liquid chromatography (UPLC/UHPLC) has made chromatographic resolution even more sophisticated (Plumb et al., 2004). A smaller particle size (< 2 μ) in the columns, and high-pressure pumps, leads to better and faster separation. Gradient elution i.e., using a mixture of mobile phases, further enables separation of a variety of metabolites effectively (Theodoridis et al., 2008). LC is usually coupled with ESI, in both positive and negative modes. LC-MS is more suitable when analysing unknown metabolites. While ESI leads to formation of fewer fragment ions, this does make identification of metabolites more

difficult than GC-MS. Regardless of its drawbacks, LC-MS is still the go-to analytical technique in most metabolomics studies.

A summary of the most common analytical tools used in metabolomics is shown in Table 1.3.

1.3.3 Data treatment strategies in metabolomics

Data treatment strategies in metabolomics differ based on whether a targeted or non-targeted approach is used. Since the goal of untargeted metabolomics is to identify metabolites which are altered between groups, the main challenge associated with this technique is identifying the correct metabolites from the large quantity of data produced. This involves additional steps such as data pre-processing and pattern recognition, which are not required for targeted metabolomics.

In the first stage, certain peak picking methods such as deconvolution and baseline correction first help select peaks with a suitable signal to noise ratio. This process helps eliminate any low frequency artefacts that would obscure peaks of interest, and help filter out any noise that may arise from instrumental interference (Katajamaa & Orešič, 2007; Yi et al., 2016). Deconvolution helps match metabolites with their corresponding peaks, within samples (Castillo et al., 2011). Subsequently normalization and scaling help make data between samples more comparable. Normalizing individual peak intensities to total peak intensity, logarithmic normalization, quantile normalization are different approaches that help make different group data more comparable (Roy & Becker, 2007). On the other hand scaling methods such as Pareto scaling, unit variance (UV) scaling or centroid scaling, help make data features more comparable (Yi et al., 2016). The

scaling method used is based on the nature of data generated. For example, Pareto scaling is usually used to highlight metabolites of low abundance with a smaller risk of amplifying false positive noise data (Yi et al., 2016).

The next step in data treatment involves pattern recognition, to identify any trends in metabolites. Due to the large number of metabolites generated, multivariate analysis (MVA) is used. As the name suggests, MVA is a statistical tool applied when more than two variables are involved. The primary advantage of multivariate analysis is dimension reduction, wherein the data is reduced into a matrix of N observations (each sample spectrum represents an observation) and K variables (features in each spectrum). MVA is thus a simplified projection of data in a 2D matrix. Variables with similarities are clustered together, and those with large differences can be enhanced (Hair et al., 1998).

Multivariate methods are unsupervised or supervised. Unsupervised methods include principle component analysis (PCA) or hierarchical cluster analysis (HCA). PCA analysis is a projection method that helps visualize trends between groups, without any information on sample identity (Gaude et al., 2013). PCA, thus, does not give as a model any information about factors driving the separation between groups. In PCA analysis, the data is reduced into a 2D space comprising principle components (PC) (Jackson, 1991). HCA enables clustering of samples with similar trends, with samples groups that most different, furthest away from each other (Webb & Copsey, 2011).

Supervised methods use the previously known class (group) information for prediction of new data. Class information refers to the basis of classification, such as diet, treatment, gender, etc. The most common supervised methods include

Partial Least Squares-Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA). PLS-DA combines the principles of PCA and multiple regression, between the classes and variables. PLS-DA can thus highlight variables which give the maximum class separating power. Furthermore, PLS-DA can account for collinear data (with highly correlated predictor variables), which is a challenge in regression analysis (Barker & Rayens, 2003; Worley & Powers, 2012; Yi et al., 2016). OPLS-DA is a further refined method, giving better visualization of data and is the most reliable for biomarker discovery (Westerhuis et al., 2010; Ai-hua Zhang et al., 2013). OPLS-DA analysis narrows down the data into predictive components in a scores plot, with the x-axis component giving inter-group separation and orthogonal (y-axis) component giving intra-group separation. While a scores plot gives patterns between classes, its corresponding loadings plots can give information about the variables contributing to the separation.

Another advantage of using supervised models, is the availability of various variable ranking and selection methods associated with them. Variable importance on projection (VIP) scores is the most common variable selection method, which ranks the different variables based on their importance on the projection of data. Variables with scores of ≥ 1 are usually considered the most important (Favilla et al., 2013). Another method of variable selection is using the cross validation, which uses a different number of variables each time, to calculate the prediction accuracy of the model. The aim is to use the subset of variables with the least prediction error (Yi et al., 2016).

Other common statistical data treatment tool used in prediction analysis and thus biomarker discovery is the receiver operating curve (ROC) analysis. ROC

curves are generally used to assess the prediction accuracy of variables selected from multivariate analysis. Sensitivity refers to the ability of the diagnostic test to correctly identify diseased individuals. Specificity refers to the ability of the diagnostic test to correctly identify non-diseased individuals. This analysis is based on plotting a true positive rate (sensitivity) against the false positive rate (1-specificity), essentially giving a trade-off between the two, at different thresholds or cut-off points (Greiner et al., 2000). The area under the curve (AUC) represents the prediction accuracy, with an accuracy closer to 1 giving better prediction (Hanley & McNeil, 1982). ROC curve analysis is a way to differentiate variables with the best class separation and least error.

The last step of data treatment involves feature identification and pathway analysis. Tandem MS, which gives a complete metabolic fingerprint of parent and fragment ions, has made identification easier. Spectra are usually compared to databases such as the Human Metabolome Database (HMDB) (Wishart et al., 2009) and Metlin (Smith, 2005).

1.3.4 Metabolomics in PD

As seen above, one of the prime applications of metabolomics studies is biomarker discovery. As with many neurodegenerative conditions, in recent years biomarker discovery for PD has greatly escalated. While most traditional efforts focused on proteomics and genomics, metabolomics studies have contributed a great deal to the field. A summary of all the human metabolomics studies for PD can be found in Table 1.4. An additional application of biomarker discovery for PD, besides predicting the onset of the disease, is monitoring disease progression and the incidence of cognitive decline.

A recent study by Lewitt *et al.* used LC-MS based metabolomics to analyse the plasma and CSF of patients with mild PD (Lewitt et al., 2017). A baseline and 24-month post follow-up was done, to find biomarkers associated with PD progression. Their results revealed 15 plasma metabolites that were highly correlated with UPDRS scores between this period. These metabolites included medium and long chain unsaturated fatty acids. In another study, Han *et al.* used serum samples of PD patients and controls to conduct targeted metabolomics to assess the amine/phenol “sub-metabolome”. Samples were collected over 3 years at 18-month intervals (Han et al., 2017). They discovered two distinct panels of metabolites, one that could differentiate the PD group from healthy controls and another interesting panel, which could predict PD patients who would develop dementia by year 3. In continuing the theme of PD and cognitive decline, Burté *et al.* attempted to correlate serum metabolites from early stage PD patients, with clinical measures for cognitive impairment. Their findings suggest a high predictive accuracy between the fatty acid oxidation pathway in the serum and the onset for PD along with MCI (Burté et al., 2017).

Metabolomics also has some unique applications in the context of PD biomarker discovery. Untargeted metabolomics on the plasma samples of age matched PD and control subjects using LC coupled with Fourier transform MS, revealed the confounding effects of L-dopa therapy in correctly identifying metabolic features changed between the two groups. The most significantly changed metabolites between PD patients and controls were metabolites of L-dopa therapy. The authors concluded that these confounding effects of L-dopa should be taken into consideration for any similar future studies. This study also elucidated

the role of altered glycosphingolipid metabolism in PD patients (Branco et al., 2018).

Several studies found the role of altered energy metabolism in PD. Ahmed *et al.* conducted an NMR based metabolomics study on the plasma of PD patients and controls, and found increased pyruvic acid levels, but decreased tricarboxylic acid cycle acid (TCA) levels in PD patients (Ahmed et al., 2009). Trupp *et al.* found reduced creatinine levels in the CSF of PD patients (Trupp et al., 2014), which was supported by another small-scale NMR study that discovered the role of decreased sugars and creatinine leading to altered energy metabolism in the CSF of PD patients (Öhman & Forsgren, 2015). They also found reduced alanine levels in the CSF of PD patients. There is further evidence for the role of abnormal amino acid metabolism in PD. While Trupp *et al.* found increased alanine, serine, threonine and methionine levels in the plasma (Trupp et al., 2014), Luan *et al.* showed increased histidine, tryptophan and phenylalanine in the urine of PD patients (Luan et al., 2015a; Luan et al., 2015b). Abnormal purine metabolism in the plasma (Bogdanov et al., 2008; Johansen et al., 2009; Lewitt et al., 2017) and glutathione metabolism in the plasma (Bogdanov et al., 2008) and CSF (Lewitt et al., 2013) has also been shown.

The breadth of metabolic pathways implicated in PD, as seen above gives several insights into the extent of pathways that are altered in PD. Most biomarkers studied for PD are either prognostic, diagnostic, predictive or used for monitoring disease progression. A combination of such biomarkers is required to determine the ideal course and duration of treatment. The number of subjects in the human studies discussed above, is quite small, in turn warranting larger studies for validation. Moreover, since PD is primarily a brain-based disorder, the most effective insights

can only come from neurological studies. Relying on post-mortem samples for metabolomics studies is often less practical and may not always give a representation of the disease in real time. This challenge in PD metabolomics has led to the increasing use of experimental models for this purpose.

1.4 Aims of study

The increasing financial burden and lack of curative therapy for PD, will pose a severe threat in the future. Several advances have been made in PD research which give us a lot more information about the disease today. However, there are still several unanswered questions about the etiology and pathology of PD, which makes developing therapeutic strategies difficult. To add to the conundrum, it is difficult to monitor changes in the brain, which makes specialists rely on clinical diagnosis only. These pathological changes in the brain, however, start years before symptoms manifest, making neurodegeneration at the time of diagnosis, irreversible. Additionally, several risk factors have been identified for PD. This finding should keep vulnerable groups such as aging populations, on alert. Given all these challenges, finding a therapeutic candidate that shows prophylactic effects or at least slows down disease progression, seems like a promising strategy to reduce the burden of PD.

Currently no such candidates have been approved. Given the promise antioxidants have shown in experimental models of PD, the overall aim of this study is to investigate whether OXY shows potential as a nutraceutical, the regular administration of which, will help slow down neurodegeneration associated with PD. Parkinson's disease is a multifactorial disease that is the result of an interplay of many pathological pathways. Oxidative stress is heavily implicated in PD

pathogenesis and is interrelated to most other disease mechanisms, as discussed earlier. Therefore, I chose OXY, because of its enhanced antioxidant effects which have been discussed in detail in section 1.2.3. Moreover, OXY shows a wider range of beneficial effects and is less cytotoxic than RES, its famous counterpart. Based on these properties, I hypothesized that OXY can slow down neurodegeneration, by modulating pathological and metabolic pathways that are altered in PD.

The first aim of this study is to assess whether OXY shows neuroprotection against other mechanistic pathways in PD in cell culture models, specifically ER stress, which is driven by oxidative stress as well as protein misfolding. The second aim is to see if OXY shows protection against motor decline and dopaminergic loss in an animal model of PD. In the third part, I aim to assess metabolic alterations in this animal model which are closely associated with the disease. In the fourth and final part of the study, I aim to use a detailed approach to investigate in detail, the metabolic targets of OXY, suggestive of its mechanism of action and specific roles in PD. Finally, I also attempt to compare all these effects of OXY with RES, to determine if it has enhanced effects in the context of PD as well. A schematic describes the aims of this study in Figure 1.9.

Figure 1.1. Motor and non-motor symptoms at different stages of PD. Adapted from (Kalia & Lang, 2015).

Figure 1.2. Pathological hallmarks of PD (dopaminergic loss). The nigrostriatal pathway, transmitting dopamine for normal voluntary movement (top figure, adapted from <https://www.neuroscientificallychallenged.com>). In PD, there is a loss of dopaminergic neurons in the substantia nigra *pars compacta* (transmitting neurons) which leads to depletion of dopamine in the receiving neurons in the striatum (bottom figure, obtained from <https://www.clinicaladvisor.com>).

Figure 1.3. Pathological hallmarks of PD (Lewy bodies). Intracytoplasmic inclusions known as Lewy bodies, with a dense eosinophilic core are observed in the brain (top figure, obtained from PubMed Health). Spread of Lewy body pathology, as proposed by Braak et al. (Braak et al., 2003) (bottom figure, obtained from (Hansen & Li, 2012)).

Symbol	Gene/protein	Inheritance	Function	Phenotype
<i>PARK1/</i> <i>PARK4</i>	<i>SNCA/</i> α -syn	Autosomal dominant	Vesicular trafficking (Kahle et al., 2002), membrane remodelling (Eliezer et al., 2001)	Early onset PD with rapid decline, amyloid fibrils (Conway et al., 1998), cognitive dysfunction.
<i>PARK2</i>	<i>Parkin</i> /Parkin	Autosomal recessive	Ubiquitin ligase (Shimura et al., 2000) (carries misfolded proteins and damaged mitochondria to the proteasome for degradation (Narendra et al., 2008))	Typically early onset (before 30 years) with some cases of juvenile PD. LB pathology is rare (Mizuno et al., 2001).
<i>PARK5</i>	<i>UCHL1/</i> Ubiquitin Carboxyterminal hydrolase 1	Autosomal dominant	Recycling of ubiquitin (Wilkinson, 2000)	Axopathies (Saigoh et al., 1999)
<i>PARK6</i>	<i>PINK1/</i> PTEN-induced putative kinase 1	Autosomal recessive	Mitochondrial degradation (Youle & Narendra, 2011)	Early onset PD
<i>PARK7</i>	<i>DJ-1/DJ-1</i>	Autosomal recessive	Mitochondrial protein overseeing oxidative stress (Canet-Aviles et al., 2004; Junn et al., 2005), protein chaperone (Shendelman et al., 2004)	Early onset PD (Abou-Sleiman et al., 2003)
<i>PARK8</i>	<i>LRRK2/</i> Leucine-rich repeat kinase 2 (dardarin)	Autosomal dominant	Protein signalling (MacLeod et al., 2006)	Later onset, slow progression, dementia is rare (Giasson et al., 2006)
<i>PARK9</i>	<i>ATP13A2/</i> Lysosomal ATPase	Autosomal recessive	ATPase function (Ramirez et al., 2006)	Juvenile PD (Di Fonzo et al., 2007), very rapid progression, dementia, supranuclear gaze palsy, pyramidal symptoms (Ramirez et al., 2006)
-	<i>GBA/</i> B-glucocerebrosidase	Autosomal recessive	Glycolipid metabolism (Klein & Schlossmacher, 2006)	Classical PD (Sidransky et al., 2009)

Table 1.1. Summary of known genetic factors for PD.

Figure 1.4. Pathogenesis of PD.

Model	Mechanisms	Advantages	Drawbacks
MPTP	Inhibition of mitochondrial complex I and cytoplasmic enzymes.	Results in tremors, rigidity, cytoplasmic inclusions of α -syn. Readily crosses blood brain barrier.	Toxic to handle. Inclusions do not resemble classical Lewy bodies. Rodents are less sensitive to MPTP.
6-OHDA	Oxidative stress Inhibition of mitochondrial complex I	Results in a good degree of nigral loss. Typical parkinsonian symptoms seen. Works well with rodent models.	Does not cross blood brain barrier; must be injected stereotactically. Does not result in α -syn inclusions.
Paraquat	Oxidative stress	Mimics classical PD pathology. Results in locomotor dysfunction. Crosses blood brain barrier.	Toxic to handle.
Rotenone	Inhibition of mitochondrial complex I	Typical parkinsonian symptoms seen. Can induce Lewy body pathology.	Selective cell loss; must be injected for site specific damage.
Transgenic α -syn	Classical Lewy body pathology	Can be used in rodent/fruit fly models. Locomotor dysfunction seen. Used for familial genetic variants of α -syn.	Nigral/striatal cell loss not always induced.

Table 1.2. Summary of experimental animal models of PD.

Figure 1.5. Summary of PD biomarker candidates from different sites (proteomics-based studies).

Figure 1.6. Synthesis, metabolism of dopamine and treatment strategies. Treatment strategies at several stages are shown in the blue boxes. (AAAH = aromatic amino acid hydroxylase, AADC = aromatic L- amino acid decarboxylase, MAO = monoamine oxidase, AD = aldehyde dehydrogenase, COMT = catechol-o-methyltransferase, DBH = dopamine beta-hydroxylase).

Resveratrol (5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol)

Oxyresveratrol (4-[(E)-2-(3,5-dihydroxyphenyl)ethenyl] benzene-1,3-diol)

Pinostilbene (3-[(E)-2-(4-hydroxyphenyl) ethenyl]-5-methoxyphenol)

Pterostilbene (4-[(E)-2-(3,5-Dimethoxyphenyl)ethenyl] phenol)

Figure 1.7. Chemical structures and IUPAC names of the common stilbenoids.

Figure 1.8. Summary of the "omics" techniques. Adapted from (Ussher et al., 2016).

Technique	Principle	Advantages	Drawbacks
Nuclear magnetic resonance (NMR)	Difference in energy absorbed and emitted by certain atomic nuclei, when exposed to an external magnetic field	<p>No prior separation of analytes required.</p> <p>Does not damage the sample; can be reused.</p> <p>Identification and semi-quantification is easy.</p>	<p>Sensitivity is low.</p> <p>Identification of less than 100 metabolites with low specificity.</p>
Mass spectroscopy	The analytes in sample are ionized, separated according to their m/z and subsequently detected.	<p>Very high sensitivity (can detect up to femtomolar concentrations of analyte).</p> <p>Hyphenated techniques (GC-MS and LC-MS) result in analysis of high resolution and well separated analytes. (usually 700 metabolites or more can be identified).</p> <p>Metabolites with a wide range of polarities can be analysed based on the separation technique adopted.</p>	<p>Destruction of samples.</p> <p>Sample preparation is required with varying degrees.</p> <p>Identification of metabolites can be difficult.</p>

Table 1.3. Summary of the primary analytical techniques used in metabolomics.

Study design	Platform	Sample	Findings
PD subjects (n=49); Samples collected at baseline and final (after 24 months)	LC-MS GC-MS	Plasma CSF	15 plasma metabolites (3 xanthine structures and 4 medium or long chain fatty acids) had positive correlation with change in UPDRS scores (Lewitt et al., 2017).
PD subjects (n=43) Control subjects (n=42); Samples collected at 18-month intervals over 3 years.	LC-MS	Serum	Vanillic acid, 3-hydroxykynurenine, isoleucyl-alanine, 5-acetylamino-6-amino-3-methyluracil, and theophylline discriminated PD from controls. His-Asn-Asp-Ser, 3,4-dihydroxyphenylacetone, desaminotyrosine, hydroxy-isoleucine, alanyl-alanine, putrescine [-2H], purine [1O] and its riboside distinguished PD patients who developed dementia from those who didn't (Han et al., 2017).
PD (n=41) Control subjects (n=40)	LC-MS GC-MS	Serum	Fatty acid oxidation metabolites had good prediction accuracy for early stage PD and MCI (Burté et al., 2017).
PD (n=21) Control subjects (n=13)	LC-MS	Plasma	L-dopa metabolites changed the most between PD and control groups. Glycosphingolipid metabolism decreased in PD subjects (Branco et al., 2018).
PD (n=43) Control subjects (n=37)	NMR	Plasma	Increased pyruvic acid and decreased TCA metabolites in PD patients (Ahmed et al., 2009).
PD (n=20) Control subjects (n=20)	GC-MS	Plasma CSF	Increased pyroglutamate and 2-oxoisocaproate in PD subjects in the plasma. Decreased 3-hydroxyisovaleric acid, tryptophan and creatinine in CSF of PD subjects (Trupp et al., 2014).
PD (n=10) Control subjects (n=10)	NMR	CSF	Alanine, creatinine and mannose decreased in the PD subjects (Öhman et al., 2015).
PD (n=106) Control subjects (n=104)	LC-MS	Urine	Steroidogenesis, fatty acid beta-oxidation, histidine metabolism, phenylalanine metabolism, tryptophan metabolism, nucleotide metabolism,

			and tyrosine metabolism altered in PD subjects (Luan, et al., 2015).
PD (early, middle and advanced stages, n=91) Control subjects (n=65)	LC-MS GC-MS	Urine	Branched chain amino acid metabolism, glycine derivation, steroid hormone biosynthesis, tryptophan metabolism, and phenylalanine metabolism differentiated between PD and control subjects, and showed prediction for disease progression (Luan, et al., 2015).
Idiopathic PD (n=41) <i>LRRK2</i> PD (n=12) Control subjects (n=15)	LC-electrochemistry	Plasma	Distinct metabolome of idiopathic and <i>LRRK2</i> PD patients. Reduced uric acid metabolism in idiopathic and <i>LRRK2</i> associated PD, compared to controls. Purine metabolites differentiated <i>LRRK2</i> associated PD and controls (Johansen et al., 2009).
PD (n=66) Control subjects (n=25)	LC-electrochemistry	Plasma	Uric acid decreased, but glutathione increased in PD patients (Bogdanov et al., 2008).
PD (n=47) Control subjects (n=58)	LC-MS GC-MS	CSF	3-hydroxykynurenine, oxidised glutathione, N-acetylated amino acids differentiated PD subjects from controls (Lewitt et al., 2013).

Table 1.4. Summary of PD metabolomics studies on human samples.

Figure 1.9. Schematic of the aims of the study. (I) To investigate the protective effects of OXY against ER stress in cell culture models. (II) To investigate the protective effects of OXY against motor dysfunction and dopaminergic loss in an animal model. (III) To investigate metabolic pathways altered in an animal model of PD. (IV) To investigate metabolic pathways altered by OXY, in an animal model of PD.

CHAPTER 2

Oxyresveratrol exerts neuroprotection against endoplasmic reticulum stress in *in vitro* models of Parkinson's disease

2.1 Introduction

There are two pathological hallmarks of Parkinson's disease (PD): degeneration of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc) and the presence of Lewy Bodies (LB), which are inclusions of α -synuclein (α -syn) in the aggregated form. The formation of α -syn aggregates via oligomerization has been discussed in Chapter 1. While aggregates are the primary form seen in LBs, the toxic role of oligomers, has also been demonstrated. Winner *et al* introduced mutants of α -syn which facilitate oligomer formation, into rat SNpc, through a lentivirus injection system (Winner et al., 2011). They successfully demonstrated the enhanced ability of these oligomers to induce dopaminergic loss in the SNpc. The membrane disrupting ability of α -syn oligomers has also been shown (Conway et al., 1998, 2000), along with their propensity to induce cell death in non-mammalian PD models (Karpinar et al., 2009) and *in vitro* (Caughey & Lansbury, 2003; Danzer et al., 2007). Given their toxic nature, halting oligomer formation early on, might be a promising strategy is slowing neurodegeneration in PD.

Although the exact cause of PD is not known, these pathologies have been attributed to mitochondrial oxidative stress (Devi et al., 2008; Piccoli et al., 2008), inflammation (Hirsch et al., 2003), and endoplasmic reticulum (ER) stress (Conn et al., 2004; Hoozemans et al., 2007). The ER is the main organelle for proper folding

and post-translational modifications of newly synthesized proteins. These processes confer proteins the ability to function properly. Damage or stress to the ER can however disrupt these mechanisms, in turn increasing load on the ER. Oxidative stress, mutations and increased protein demand are some of the factors that can lead to impaired ER function, resulting in ER stress. ER stress is also activated by a build-up of misfolded proteins in the ER lumen (Kaufman, 1999). Misfolding of proteins can be induced by oxidative stress, certain mutations and in the specific case of PD, an elevation of dopamine levels (Mor et al., 2017; Yamakawa et al., 2010)

A cascade of signals attempts to restore homeostatic balance in the ER. If this fails, then the signalling shifts towards programmed cell death. This cascade is known as the unfolded protein response, or the UPR. The main effectors of the UPR are three ER transmembrane proteins known as Inositol Requiring 1 (IRE1), PKR-like ER kinase (PERK), and Activating Transcription Factor 6 (ATF6) (Ron & Walter, 2007). Under normal physiological conditions these proteins are bound to the ER chaperone immunoglobulin binding protein (Bip) also known as 78-kD glucose-regulated protein (Grp78). When the cell detects ER stress Bip detaches itself and the three signalling molecules are activated (Ron et al., 2007). A series of events then controls the cell's fate based on the intensity of stress.

PERK undergoes dimerization followed by auto-phosphorylation and subsequently phosphorylates one of its downstream effectors eukaryotic translation initiator factor 2 α (eIF2 α), which can halt total protein translation (Harding et al., 2000). However, phosphorylation of eIF2 α (p-eIF2 α) upregulates translation of activating transcription factor 4 (ATF4) (Ameri & Harris, 2008), which is responsible for regulating autophagy, protein folding, redox metabolism and pro-

survival genes at this stage (Ameri et al., 2008). ATF6 is cleaved at the Golgi apparatus, generating a 50 kD transcription factor: ATF6f, which controls ER-associated protein degradation (ERAD), along with upregulation of chaperones like Grp78 and protein disulphide-isomerase (PDI) (Yamamoto et al., 2007). IRE1 dimerizes and auto-phosphorylates itself, leading to splicing of X box-binding protein 1 (XBP1s) (Calton et al., 2002). XBP1s also controls ERAD and protein folding (Lee et al., 2003).

Under conditions of chronic or prolonged ER stress, the three pathways aim at inducing programmed cell death. ATF4 upregulates the pro-apoptotic transcription factor C/EBP-homologous protein (CHOP). CHOP drives apoptosis via the mitochondrial pathway (Fomby & Cherlin, 2011). IRE1, on the other hand, leads to activation of the c-Jun N-terminal kinase (JNK) driven pro-apoptotic signal (Urano et al., 2000), which also regulates macroautophagy (Ogata et al., 2006).

There is plenty of clinical and experimental evidence to support the role of ER stress in PD. ER-chaperones such as Grp78 and PDI were upregulated in the SNpc of PD patients (Conn et al., 2004). Phosphorylated PERK (p-PERK) and eIF2 α was also found in post-mortem brains of PD patients (Hoozemans et al., 2007). Interestingly, neurons that were immuno-positive for ER-stress markers also contained inclusions. Phosphorylation and certain post-translational modifications of α -syn upregulated the UPR (Sugeno et al., 2008); whereas an increase in the ER burden was induced by familial mutants of α -syn, in the SH-SY5Y cell line and transgenic synuclein mice (Bellucci et al., 2011). Parkinsonian toxins 6-hydroxydopamine (6-OHDA) or 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) also activated ATF4 and CHOP in experimental models of PD (Holtz et al., 2003; Ryu et al., 2002). Furthermore, *UCHL1* and *DJ-1* mutations increased ER

stress in the Neuro2a neuronal cell line. (Yokota et al., 2003). Owing to the significant role of ER stress in the pathogenesis of PD, strategies to contend it are now under scrutiny.

In a rotenone model of PD, candesartan cilexetil, an Angiotensin II receptor antagonist, was shown to reduce ER stress by downregulating the expression of ATF4 and subsequently CHOP (Wu et al., 2013). Overexpression of XBP1 exhibited protective effects against MPP⁺-treated cells having impaired proteosomal function (Sado et al., 2009). Another study showed that overexpression of the ER chaperone Bip using adeno-associated virus (AAV) attenuated α -syn induced toxicity in a transgenic PD model (Gorbatyuk et al., 2012). On the other hand knocking out CHOP was shown to promote cell survival in toxin based models of PD (Harding et al., 2000).

The effects of oxyresveratrol (OXY) as a potent antioxidant have been discussed in chapter 1. Chao and co-workers have also elucidated the role of OXY in mitigating cellular toxicity exerted by 6-OHDA in neurons (Chao et al., 2008). However, the mechanism of this neuroprotection is still unclear. Pinostilbene (PINO), the methylated derivative of resveratrol (RES) was also protective against 6-OHDA induced oxidative stress (Chao et al., 2010). Since oxidative stress is closely related with ER stress, I hypothesized that OXY is also protective against ER stress in PD. Assessing the effects of stilbenoids on ER stress mediated neurodegeneration will give better insights into their mechanism of action and extent of neuroprotection.

2.2 Aims of study

The aim of this study was to investigate the effects of OXY on ER stress in different models of PD. For this purpose, a toxin and a gene-based culture model was used. For the former, the aim was to investigate the potential of OXY in reducing 6-OHDA mediated ER stress. The Mes23.5 cell line was treated with an appropriate dose of 6-OHDA, and ER stress pathways that were subsequently upregulated were determined. The effects of OXY treatment on upregulated pathways was then assessed; RES and PINO were used to compare the effects of all three stilbenes on ER stress. The experimental design is explained in Figure 2.1.

For the genetic model, SH-SY5Y cells were transfected to express wild-type (WT) or familial (A30P, A53T) mutants of α -syn. The aim here was to investigate the effects of OXY on α -syn oligomers, α -syn expression and ER stress induced by them. RES and PINO were used to compare the effects of all three stilbenes on ER stress. The experimental design is described in Figure 2.7.

2.3 Materials and methods

2.3.1 Study design

The Mes23.5, a hybrid rat embryonic mesencephalon and murine neuroblastoma cell line, and the human neuroblastoma cell line, SH-SY5Y, were used for this part of the study. Concentration response curves were performed using the lactate dehydrogenase (LDH) assay to determine appropriate concentrations of all drugs and toxin, prior to the study.

The Mes23.5 cells were treated with the dopaminergic toxin 6-hydroxydopamine (6-OHDA) or tunicamycin (TM), for a chosen time point, after

one-hour treatment with vehicle, oxyresveratrol (OXY), resveratrol (RES) or pinostilbene (PINO). The time of pre-treatment determined by Chao *et al.* (Chao *et al.*, 2008), was slightly modified. Treatment was halted by collecting the media for LDH assay, and cells were subsequently harvested for Western-blot analysis of protein and RT-PCR for gene expression.

The SH-SY5Y cell line was used to overexpress wild-type (WT) and familial mutants of α -synuclein (α -syn), using stable transfection. Subsequently, all transfected cell lines were treated with vehicle, OXY, RES or PINO. Treatment was halted by collecting the media for LDH assay, and cells were subsequently harvested for immunoblotting and immunofluorescence.

All materials were purchased from Sigma Aldrich (United Kingdom), unless stated otherwise.

2.3.2 *Cell culture and treatment*

Both cell lines were grown in a 5% CO₂ incubator maintained at 37°C. The Mes23.5 mesencephalic cells (generously given by Professor Weidong Le from Baylor College of Medicine, U.S.A) were cultured in Dulbecco's Modified Eagle medium F12 (DMEM-F12) (Gibco, U.S.A) with Sato supplements, 5% heat-inactivated foetal bovine serum (FBS) (Gibco, U.S.A), penicillin (50 units/ml, Gibco, U.S.A) and streptomycin (50 µg/ml, Gibco, U.S.A) solution. Cells were grown at a seeding density of 3×10^5 per well onto 6-well plates (SPL Life Sciences, Korea). For SH-SY5Y (ATCC, U.S.A), the cells were grown in DMEM supplemented with 10% FBS, penicillin (50 units/ml) and streptomycin (50 µg/ml) at a seeding density of 5×10^5 per well onto 6-well plates.

All treatments were done when cells reached 80% confluence. Stock solutions of OXY (purified by Dr. Mingfu Wang at The University of Hong Kong), RES, PINO and TM (5 $\mu\text{g/ml}$) were prepared in dimethyl sulfoxide (DMSO) while the hydrobromide salt of 6-OHDA was dissolved in syringe-filtered saline. All drugs were diluted in the relevant growth medium and added one hour before 6-OHDA or TM treatment. Drug treatments lasted 48 hours for the SH-SY5Y cells.

2.3.3 *Stable transfection*

The α -SYN plasmid constructs pCEP4-WT- α -SYN, pCEP4-A53T- α -SYN and pCEP4-A30P- α -SYN were kindly given by Dr. Atsushi Takeda (Department of Neurology, Graduate School of Medicine, Tohoku University, Sendai, Japan). In these constructs, the cDNA of the WT- α -SYN, A53T- α -SYN and A30P- α -SYN gene were cloned in Xho I and Hind III sites of the MCS of the pCEP4 plasmid.

For stable transfection, a seeding density of 4×10^5 cells onto a 35-mm plate (SPL Life Sciences) was used. When cells reached 70% confluence, the culture medium was replaced with DMEM. Each plasmid at 4 μg was subsequently added to 250 μl Opti-MEM (Gibco, U.S.A) and was incubated. Lipofectamine[®] 2000 (4 $\mu\text{l}/\mu\text{g}$ of plasmid, Thermo Fischer Scientific, U.S.A) was diluted in 250 μl Opti-MEM, mixed with the plasmid and incubated for 15-20 minutes. Lipofectamine[®] 2000 – plasmid complex (500 μl) was then added to the culture medium and incubated for four hours. Thereafter, 10% DMEM containing 0.5 g/l hygromycin B (Invitrogen, U.S.A) was used as culture medium, with swaps every two days. The surviving cells were grown until 80% confluence and passaged further. Cell treatments were done after the surviving cells had been grown for 30 days.

2.3.4 *Concentration response curves and lactate dehydrogenase assay*

The LDH assay (Roche, Switzerland) can be used as a measure of cytotoxicity and was thereby used for all concentration response curves. To choose the appropriate dose of 6-OHDA that induces toxicity in the Mes23.5 cell line, cells were incubated with 0 μ M, 5 μ M, 10 μ M, 25 μ M and 50 μ M 6-OHDA for 24 hours. The Mes23.5 and SH-SY5Y (transfected and non-transfected cells) were then treated with a range of doses of OXY, RES and PINO for 24 hours, to select a dose for subsequent use. Finally, the protective effects of all drug being tested on 6-OHDA-induced neurotoxicity was compared. The cell incubation media were collected for each treatment group, to carry out the LDH assay.

The procedure for LDH assay was followed according to the manufacturer's protocol and previous publications in the laboratory (Chao et al., 2010, 2008; Ho et al., 2007). Briefly, culture medium was collected and spun down at 3000 rpm at 4°C, in an Eppendorf centrifuge for five minutes. The LDH assay reaction mixture was subsequently incubated with an equal volume of culture medium onto 96-well plates (SPL Life Sciences) without direct light for 30 minutes. The resulting absorbance was measured at 492 nm. All samples were run in triplicates, and the average reading was normalized to control. Results were expressed as a fold of control.

2.3.5 *Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting*

The culture medium was aspirated, and neurons were briefly washed with ice cold tris-buffered saline (TBS). Neurons were then scraped in ice-cold lysis buffer (containing 10 mM Tris at pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1

mM NaF, 20 mM Na₄P₂O₇, 1% Triton X-100, 1% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride) supplemented with protease and phosphatase inhibitor cocktail, as previously described (Chao et al., 2008; Hung et al., 2018; You et al., 2018). The lysate was homogenized by sonicating for 10 seconds on ice, spun at 13,000 rpm for 30 minutes (at 4°C) and the supernatant was collected. Total protein concentrations were determined using the bicinchoninic acid (BCA) assay (Thermo Fischer, U.S.A).

For the Mes23.5 cells, 40 µg of protein was separated on a 12% or 15% native SDS-PAGE gels. For the transfected SH-SY5Y cells, 40 µg of protein was separated on 10% native SDS-PAGE gels and subsequently transferred onto polyvinyl difluoride (BioRad, U.S.A) membranes. Membranes were then blocked with bovine serum albumin (BSA) (BioRad) or 5% skim milk (BioRad) for one hour. The membranes were then washed with TBS containing 0.1% Tween-20 (TBS-T) three times before incubating with antibodies for α -synuclein (1:500, Santa Cruz, U.S.A), Grp78 (1:500, Cell Signaling Technology, U.S.A), p-eIF2 α (1:1000, Cell Signaling Technology), eIF2 α (1:1000, Cell Signaling Technology), p-PERK (1:500, Santa Cruz Technology), PERK (1:500, Cell Signaling Technology), and CHOP (1:500, Santa Cruz) overnight at 4°C. Membranes were washed again with TBS-T, and respective secondary antibodies were incubated (1:5000, Dako) for two hours. Protein bands were developed on X-ray film (Fujifilm, Japan) using the enhanced chemiluminescent (ECL) Western Bright detection kit (Advansta, U.S.A). All membranes were then stripped with a mild stripping buffer, and re-probed for β -actin (1:5000) or α -tubulin (1:5000) as loading controls. All protein bands were analysed and quantified using ImageJ (NIH). Results were normalized to loading control and expressed as fold of control.

2.3.6 *cDNA synthesis and reverse transcription-polymerase chain reaction (RT-PCR)*

RNA was isolated from the cells by lysing with TRIzol® reagent (Thermo Fischer). Extracted RNA purity and concentrations were determined using a NanoDrop (Thermo Fischer). Only purified RNA with an absorbance ratio 260 nm: 280 nm > 1.8 was used. 500 ng of RNA was reverse transcribed into cDNA using the PrimeScript™ Master Mix Kit (Takara, Japan) according to the manufacturer's protocol. Briefly, the RNA was incubated with the reverse transcription master mix from the kit and nuclease free water at 37°C for 15 minutes and 85°C for 5 seconds in the Veriti Thermal cycler (Life Technologies). cDNA at a concentration of 25 ng/μl was subsequently obtained.

For amplification, cDNA (20 μg) was mixed with 5 μl of 2x SYBR® Green (Thermo Fischer), and 0.5 μl of forward and reverse primers (Integrated DNA Technologies, U.S.A). Amplification was done using the StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA) and the conditions used were:

95°C for one minute followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for one minute. All samples were run in triplicates and $\Delta\Delta C_T$ values obtained by normalizing to the endogenous control (GAPDH) values were expressed as fold of control. Primer sequences were as follows:

Gene	Forward primer	Reverse primer
ATF4	5'- TAT GGA TGG GTT GGT CAG TG -3'	5'- CTC ATC TGG CAT GGT TTC C -3'
CHOP	5'- CCA CAC CTG AAA GCA GAA AC -3'	5'- CAC TGT CTC AAA GGC GAA AG -3'
GAPDH	5'- TCA AGA AGG TGG TGA AGC AG -3'	5'- AGG TGG AAG AAT GGG AGT TG -3'

2.3.7 Immunofluorescence staining

The transfected SH-SY5Y cells were grown on cover slips coated overnight with 30 µg/ml laminin (Thermo Fischer). Drug treatments were done as in section 2.3.2 above, after which media was aspirated. Cells were washed mildly with TBS and fixed with 4% paraformaldehyde for 15 minutes. Permeabilization was facilitated with 0.1% Triton-X 100 for seven minutes, and 5% goat serum (Thermo Fischer) was used as a blocking agent for 1 hr before incubation with primary antibody for α -syn (1:500, BD Biosciences) or Grp78 (1:500, Santa Cruz) at 4°C overnight. Coverslips were washed again, prior to secondary Alexa-Fluor-conjugated antibody (Life technologies) incubation. After incubating with the secondary antibody for two hours at 4°C, cells were co-stained with DAPI (Thermo Fischer), coverslips were washed and mounted onto pre-coated slides (Thermo Fischer) with ProLong Gold (Thermo Fischer). Z-stack images were captured for at least three distinct fields per slide, on a Carl Zeiss LSM780 (Germany) multiphoton inverted confocal system at 40x magnification. Fluorescence intensities were measured using ImageJ (NIH) and normalized to the DAPI count for each section. Results were expressed as fold of control (WT).

2.3.8 Statistical analysis

All analysis was done using GraphPad Prism 6 (GraphPad, U.S.A). Statistical analysis was done using either Kruskal-Wallis test followed by Dunn's *post-hoc* comparison or One-way ANOVA followed by Tukey's or Dunnett's multiple comparison *post-hoc* test, wherever applicable. All results are expressed as mean $\pm$ standard error of the mean (S.E.M) of at least three independent experiments. The

p values and exact number of independent experiments are indicated in each figure legend.

2.4 Results

2.4.1 Concentration response curves for 6-OHDA and drug treatments in the Mes23.5 cell line

To identify suitable doses of 6-OHDA and the three drugs, the lactate dehydrogenase (LDH) assay was used. The LDH released in the media, due to membrane rupture, was used as a measure of cytotoxicity. Mes23.5 cells were treated with increasing concentrations of 6-OHDA (2.5 μ M, 5 μ M, 10 μ M, 25 μ M and 50 μ M) for 24 hours. 10 μ M and higher concentrations of 6-OHDA showed a significant increase in LDH levels, based on which 10 μ M was used for the study (Figure 2.2A).

Mes23.5 cells were also treated with increasing concentrations of OXY, RES and PINO (5 μ M, 10 μ M, 25 μ M and 50 μ M) for 24 hours to assess cytotoxicity of the drugs. None of the treatments of any drugs resulted in significant LDH release, indicating all drugs were well tolerated by the cells in this concentration range (Figure 2.2B). A dose of 10 μ M was selected for the drug treatments. A concentration of 5 μ M was also used for OXY treatment to assess its potency.

2.4.2 OXY mitigates cytotoxicity induced by 6-OHDA

My next aim was to determine whether OXY showed neuroprotection against 6-OHDA induced cytotoxicity in the Mes23.5 cell line. Cells were treated with selected concentrations of OXY, RES and PINO, or an equal volume of the vehicle (DMSO) for the control group, for an hour. It was confirmed previously that up to

50 μ l/ml of DMSO had no significant effect on the cells. 10 μ M 6-OHDA, or an equal volume of vehicle (saline) for the control group, was subsequently added for 24 hours. In line with the previous result, 10 μ M 6-OHDA, without any drug treatment, induced a two-fold increase in the LDH released. 10 μ M OXY treatment resulted in a significant reduction in the LDH released, compared to the 6-OHDA group (Figure 2.3). 10 μ M RES and PINO did not show any significant change compared to the 6-OHDA group.

2.4.3 6-OHDA induces ER-stress in the Mes23.5 cell line in a time-dependant manner

To determine the potential of 6-OHDA in inducing ER-stress in the Mes23.5 cell line, I chose four different time points. The cells were treated with 10 μ M 6-OHDA for 30 minutes, one, two and four hours. Tunicamycin (TM), an inhibitor of protein glycosylation, and therefore inducer of ER stress, at a concentration of 5 μ g/ml, for four hours, was used as positive control (Osłowski & Urano, 2011).

Grp78 is a UPR chaperone upregulated as a protective mechanism to restore proteostasis. However, Grp78 expression was not upregulated by 6-OHDA treatment, at any time point (Figure 2.4A, 2.4B). Furthermore, TM treatment did not increase expression of Grp78 (Figure 2.4B). Therefore, I hypothesized that this dose of 6-OHDA and TM might be too high for protective ER stress mechanisms to be effective.

Next, I investigated the branch of UPR downstream of PERK by measuring the phosphorylation of PERK, phosphorylation of eIF2 α and expression of CHOP, a pro-apoptotic transcription factor. Immunoblot results showed that the phosphorylation of PERK (Figure 2.4A), the phosphorylation of eIF2 α (Figure

2.4C) and the expression of CHOP (Figure 2.4D) were significantly elevated at the four-hour time-point. Similar results were seen after TM treatment. These results confirmed the activation of prolonged ER-stress at this time-point. A four-hour treatment of 10 μ M 6-OHDA was therefore chosen to investigate effects of OXY on ER stress in this model.

2.4.4 *OXY modulates ER stress signalling by suppressing transcription of ATF4*

My next aim was to look at changes in the elevated markers of ER-stress, when the Mes23.5 cells were treated with OXY, RES and PINO for one hour prior to the four-hour 10 μ M 6-OHDA treatment (Figure 2.5A). Immunoblot results revealed a propensity of low dose (5 μ M) OXY to inhibit the phosphorylation of eIF2 α and expression of CHOP (Figure 2.5), although these results were not significant. 10 μ M RES and PINO treatment did not seem to decrease the activation of any of these markers. Since Grp78 levels were not altered by 6-OHDA at any time point, the effects of OXY on this marker were not investigated.

I then proceeded to investigate the effects of OXY on the transcription of ATF4 and CHOP. Transcription of ATF4 is upregulated downstream of eIF2 α phosphorylation, and in turn controls the fate of CHOP transcription. Results revealed that the transcription of ATF4, which was significantly upregulated after addition of 6-OHDA, was reduced by both doses of OXY as well as 10 μ M RES treatment (Figure 2.6A). OXY and RES also showed a similar trend in downregulating the transcription of CHOP (Figure 2.6B), but without any statistical significance. However, PINO did not show any such changes.

2.4.5 Concentration response curves of the three drugs in the SH-SY5Y cell line.

The LDH assay was employed to assess the tolerance of different concentrations of all three drugs on the non-transfected and the three transfected cell lines. All the cells were treated with OXY, RES and PINO (5 μ M, 10 μ M, 25 μ M and 50 μ M) for 24 hours. While 50 μ M of all three drugs were cytotoxic in the non-transfected cells (Figure 2.8A), higher doses of OXY were tolerated well in the WT- α -syn (Figure 2.8B), A30P- α -syn (Figure 2.8C), and A53T- α -syn (Figure 2.8D), expressing cells. Based on these LDH results and the protective effects of 25 μ M OXY on SH-SY5Y cells reported previously (Chao et al., 2008), a drug concentration of 25 μ M was selected for subsequent studies.

2.4.6 OXY reduces soluble oligomeric species in the A30P mutant

Formation of soluble oligomeric species is the first step in protein misfolding (Conway et al., 2000). The familial mutants of α -syn I chose, are known to form oligomeric species, and lead to aggregates at late stages (Conway et al., 1998, 2000). Accumulation of misfolded proteins leads to perturbations in the ER. In this part of the study, I first aimed to elucidate the effects of OXY on soluble oligomers in the mutants. For this purpose, the transfected cell-lines were exposed to 25 μ M OXY, RES or PINO for 48 hours. Subsequent immunoblotting results showed that higher molecular weight, soluble oligomeric species (95 kD) did not exist in the WT (Figure 2.9A) but were present in the A30P and A53T mutants (Figure 2.9B). None of the drug treatments showed any effect on A53T oligomers (Figure 2.9C). Exposure to 25 μ M OXY significantly reduced the oligomers of A30P (Figure 2.9D) while 25 μ M RES or PINO did not show such inhibitory effects.

2.4.7 *Effects of OXY on total α -syn expression and ER stress*

Overexpression of proteins can also induce ER stress in experimental neurodegenerative models (Auluck, 2002; Terro et al., 2002). In line with this, I attempted to assess if the arsenal of OXY mechanisms extended to mitigating overexpression in the A30P mutant. As mentioned previously, Grp78 is an ER associated chaperone which is activated by the UPR to contain the build-up of misfolded proteins and maintain proteostasis. Therefore, I also anticipated the involvement of Grp78 regulated ER stress in the α -syn model. To confirm this and to assess any co-localization between Grp78 and α -syn, I co-stained the WT and A30P transfected cells for α -syn and Grp78.

Total α -syn and Grp78 expression was determined, relative to the number of cells in each field, obtained using DAPI staining. When normalized to the WT, it was observed that the A30P mutant had significantly more total α -syn (Figure 2.10A). No co-localization was observed between α -syn and Grp78 in this model (Figure 2.10B). OXY did not have any effect on α -syn expression (Figure 2.10C). Interestingly, the A30P mutant also had significantly more Grp78 expression than the WT (Figure 2.10A). These increased Grp78 levels were prevented by the 48-hour exposure to OXY (Figure 2.10D).

2.5 Discussion

In this part of my study, I used two models of PD exhibiting ER stress to assess the protective functions of OXY, in each. The findings from this study indicate that OXY is a potent ER stress preventing agent in both models, albeit with distinct mechanisms. While RES was partially protective against 6-OHDA induced ER

stress, PINO had no effects on either model. The inducers of ER stress were different in both models. 6-OHDA is an oxidative stress inducer and the role of its toxicity via mitochondrial dysfunction is well established (Cohen & Heikkila, 1974; Henze et al., 2005; Tirmenstein et al., 2005). However, in the case of mutant α -syn, the formation of oligomers and their accumulation in the ER lumen triggers ER-stress (Colla et al., 2013; Smith et al., 2005). In this study, 6-OHDA lead to exacerbated ER stress, as seen by activation of the pro-apoptotic factor CHOP. Misfolding of α -syn, on the other hand, is a slower phenomenon. Therefore, ER stress induced in this case, can be surmountable at early stages. This is in line with real life scenarios, where idiopathic PD leads to a more rapid decline than familial PD.

This is the first study to show the upregulation of ER stress by 6-OHDA in the Mes23.5 cell line. Although the three drugs at doses higher than 10 μ M did not show any significant toxicity in the Mes23.5 cells, I chose to assess the effects of 10 μ M and 5 μ M OXY, to see its efficacy and potency. Here, I showed that OXY even at 5 μ M, intervened and halted the transcription of ATF4. Transcription of ATF4 is upregulated in response to the phosphorylation of eIF2 α . Under prolonged ER-stress, it will activate the pro-apoptotic marker, CHOP. These results indicate that inhibition of signalling molecules upstream of CHOP activation, is the prime target of OXY. 10 μ M RES also showed promise in this regard. Prior studies have reported the ER stress inducing ability of RES at a higher concentration (100 μ M) (Chow et al., 2014; Vamsi & Haifan, 2009). In this study however, a low dose (10 μ M) of RES partially mediated ER stress. It is already known that higher concentrations of OXY are less cytotoxic than RES (Chao et al., 2008). Taken

together these findings further confirm that although RES is protective at lower doses, it has a narrower therapeutic window than OXY.

ER stress is a conjoint mechanism between three different branches. In this study, I only measured pathways downstream of the PERK. Besides being the primary pathway of the UPR, the implication of the PERK pathway in 6-OHDA mediated toxicity has been shown before (Holtz et al., 2003; E J Ryu et al., 2002). Moreover, results from other laboratories support the role of this branch of ER stress in post-mortem tissues (Hoozemans et al., 2007).

Overexpression of Grp78 plays a protective role (Gorbatyuk et al., 2012). This might explain why it was not upregulated in a model geared towards apoptosis. The present findings are also consistent with a previous study that suggested Grp78 remained unchanged after 6-OHDA treatment at early time points, albeit in a different cell line (Ryu et al., 2002). An increase in transcription and translation of ATF4, following by the phosphorylation of eIF2 α , is well reported (Chan et al., 2017; Holtz et al., 2003). At earlier stages, this shows protective functions such as restoring redox and amino acid metabolism (Ameri et al., 2008). When the damage is too extensive, it increases transcription and translation of the apoptotic transcription factor CHOP (Fomby et al., 2011). Apoptosis initiated by CHOP is purported to its Bcl-2 inhibitory function (McCullough et al., 2001). However, since other signalling molecules such as ATF6 can also activate CHOP (Li et al., 2000), their involvement in this experimental model, needs to be confirmed. There are studies that have reported the role of ATF6- and Xbp1-mediated signalling in 6-OHDA mediated toxicity, in neuronal cell lines (Holtz et al., 2003; Ryu et al., 2002; Yamamuro et al., 2006). The effects of OXY on the other branches of ER stress triggered by 6-OHDA will need to be studied further. It must also be noted that

although direct effects of OXY on 6-OHDA-induced apoptosis have been elucidated before (Chao et al., 2008), this was not directly measured in this study. The ability of OXY to reduce CHOP associated apoptosis will also need to be determined by assessing the transcription and expression of specific apoptotic markers such as Bcl-2.

Idiopathic PD patients are often undiagnosed until there is a significant degree of neuronal damage. Previous findings (Chao et al., 2008) coupled with the present study, show that a prior exposure to OXY treatment can alter signalling pathways and exert neuroprotection. Early treatment of OXY might therefore help slow down degeneration induced by ER stress and help preserve the remaining dopaminergic neurons.

For the second model, I transfected the SH-SY5Y neuroblastoma cell line to overexpress WT, A30P and A53T- α -syn. I chose the SH-SY5Y cell line for this part of the study, as it retains certain properties of dopaminergic neurons, but does not constitutively express α -syn. The SH-SY5Y cells were more sensitive to the highest doses of the drug treatments and showed higher LDH levels than the Mes23.5 cells. This could be due to inherent differences in the genotype of the cell lines and possibly different rates of drug uptake by both. It is worthwhile to investigate the latter further in order to make the results more comparable. Since no accelerating agents were used here, oligomerization of α -syn was catalysed by the virtue of mutations only. The A53T and A30P are familial mutants which have been demonstrated to form oligomers and fibrils faster than WT- α -syn (Conway et al., 1998, 2000; El-Agnaf et al., 1998; Uversky et al., 2002). Moreover, there is evidence to show that ER stress is triggered by mutants of α -syn in experimental models (Colla et al., 2012; 2013). In one study, ER-stress was upregulated in a

transgenic mouse model, observed by the co-localization of Grp78 with α -syn, in inclusions (Bellucci et al., 2011).

OXY significantly reduced the higher molecular weight (>90 kDa) soluble oligomeric species of A30P α -syn, but not A53T. This can be attributed to the slower aggregation rate of A30P compared to A53T (Conway et al., 1998; Narhi et al., 1999). Oligomerization is a rate-limiting step in protein misfolding (Orte et al., 2008). Therefore, halting the process at this stage is another strategy to protect neurons from long-term ER stress. Whether OXY modulates the oligomeric species via direct interactions, remains to be investigated. RES and PINO, however, did not show such effects. A significant increase of Grp78 and overexpression of A30P- α -syn compared to WT was also observed here, but with no co-localization. Since Grp78 is a chaperone, it is likely that it was upregulated to stabilize the formation of oligomers. OXY reduced expression of Grp78 in the A30P mutant, thereby mitigating ER stress, but with no effects on A30P- α -syn overexpression. It must be noted that higher molecular weight insoluble aggregates of α -syn were not observed by immunocytochemistry. These species would have to be investigated further using more specific antibodies. Overexpression of α -syn also has toxic effects; demonstrated in primate (Eslamboli et al., 2007) and mouse (Masliah, 2000) models. Interestingly, it was observed that in the 25 μ M OXY-treated A30P cells, lower molecular weight species seemed to increase, while oligomers decreased (Figure 2.9A). However, the exact relationship between these species, and the direct effects of OXY will need to be characterized further. In summary, these results show the potential of OXY in mitigating ER stress related to α -syn toxicity as well. There are reports elucidating the upregulation of CHOP by overexpression of WT

α -syn (Jiang et al., 2010). Therefore, it is also worthwhile to investigate the activation of CHOP and its subsequent effects in the A30P mutation.

This study also outlines the differential mechanisms by which OXY and RES exert protective effects in experimental PD. While OXY showed an ability to mitigate both levels of ER stress, RES worked with the oxidative stress model only, playing no role in α -syn induced ER stress. Finally, although neuroprotection exerted by PINO against 6-OHDA induced toxicity has been demonstrated before (Chao et al., 2010), it did not show protective effects in the context of ER stress.

2.6 Conclusion

Owing to the widespread manifestation of ER stress and the UPR in the pathology of PD, several drug targets are under investigation for their effects in this regard. ER stress is upregulated in familial and sporadic PD and is therefore a common cause of neuronal degeneration. This study highlights the promising roles of OXY in counteracting ER stress in both such models. These effects need to be further validated in toxin and transgenic models. The key to effective treatment in all cases of PD is accurate and timely diagnosis. Protective agents like OXY that prolong neurodegeneration and prevent pathological mechanisms, can be a promising strategy in the therapy for PD.

Figure 2.1. Experimental design for the Mes23.5 cell line.

A.

B.

Figure 2.2. Concentration response curves for 6-OHDA and the three drugs. Based on the LDH cytotoxicity assay, 10 μ M 6-OHDA (A), and 10 μ M OXY, RES and PINO (B) were selected for the study. All samples were run in triplicates, and data represents mean $\pm$ S.E.M of at least three independent experiments (* indicates $p < 0.05$ compared to control, using Kruskal-Wallis and Dunn's *post-hoc* test).

Figure 2.3. Comparison of the protective effects of the three drugs on 6-OHDA induced toxicity in the Mes23.5 cell line. 10 µM OXY significantly reduced LDH levels. RES and PINO showed no changes at this dose. All samples were run in triplicates, and data expressed as mean $\pm$ S.E.M of at least three independent experiments (* indicates $p < 0.05$, ** indicates $p < 0.01$ compared to control, and # indicates $p < 0.05$ compared to 10 µM 6-OHDA, using ordinary One-way ANOVA and Tukey's *post-hoc* test).

Figure 2.4. 6-OHDA initiates ER stress in the Mes23.5 cell line in a time dependent manner. Representative blots shown in (A). Four-hour treatment of 10 μ M 6-OHDA and 5 μ g/ml TM did not alter Grp78 expression (B) but increased phosphorylation of eIF2 α (C) and expression of CHOP (D). Data are expressed as mean $\pm$ S.E.M of at least three independent experiments (* indicates $p < 0.05$ compared to control, using Kruskal-Wallis test and Dunn's *post-hoc* test).

A. **B.** **C.**

Figure 2.5. Effects of drugs on prolonged ER stress. Representative blots shown in (A). Immunoblot results indicate a trend of low dose (5 μM) OXY in reducing phosphorylation of eIF2α (B) and CHOP expression (C). RES and PINO showed no changes. Data are expressed as mean ± S.E.M of at least three independent experiments (* indicates $p < 0.05$ compared to control, using One-way ANOVA and Tukey's *post-hoc* test).

Figure 2.6. Effects of OXY on ER stress related transcription factors. 10 µM 6-OHDA induced an 8-fold increase in the transcription of ATF4 (A). 5 µM, 10 µM OXY and 10 µM RES treatment significantly reduced ATF4 mRNA expression (A). OXY and RES showed a similar trend with CHOP mRNA expression (B). 10 µM PINO showed no effects on either. Samples were run in triplicates and data are expressed as mean $\pm$ S.E.M of at least three independent experiments (* indicates $p < 0.05$, compared to control and # indicates $p < 0.05$, compared to 10 µM 6-OHDA using ordinary one-way ANOVA and Tukey's *post-hoc* test).

Figure 2.7. Experimental design for the SH-SY5Y cell line.

Figure 2.8. Concentration response curves of all three drugs on all SH-SY5Y cell lines. Effects of increasing concentrations of OXY, RES and PINO on LDH released in the non-transfected (A), WT- α -syn (B), A30P- α -syn (C) and A53T- α -syn (D) transfected SH-SY5Y cell line. Based on these results, 25 μ M was chosen as a suitable dose of the drugs for the study. Samples were run in triplicates and data are expressed as mean $\pm$ S.E.M of at least three independent experiments (* indicates $p < 0.05$, compared to control using Kruskal-Wallis test and Dunn's *post-hoc* test).

Figure 2.9. Effects of drug treatments on oligomeric α -syn. Immunoblot results confirmed the absence of higher molecular weight soluble oligomeric species (95 kD) in the WT (A) but their presence in the A30P and A53T mutant (B). 25 μ M OXY had no effect on the A53T (C) mutant but significantly reduced the presence of higher molecular weight oligomeric species in the A30P mutant (D). 25 μ M RES and PINO showed no effects on oligomeric species. Data are expressed as mean $\pm$ S.E.M of at least three independent experiments (* indicates $p < 0.05$, compared to A30P control using Kruskal-Wallis test and Dunn's *post-hoc* test).

Figure 2.10. Effects of OXY on total α -syn and Grp78 expression. The A30P mutant had a higher expression of α -syn (green) and Grp78 (red), compared to WT (A). A higher magnification of the squared section is shown in (B). 25 μ M OXY treatment did not alter expression of A30P α -syn (C) but reduced the expression of Grp78 (D). Data are expressed as mean $\pm$ S.E.M of at least three independent experiments (scale bar: 10 micron, * indicates $p < 0.05$, compared to WT control, and # indicates $p < 0.05$ compared to A30P control, using Kruskal-Wallis test and Dunn's *post-hoc* test).

Figure 2.11. Major pathways involved in ER-stress and the effects of OXY. ATF6, PERK and IRE1 dissociate from the chaperone Grp78, and a series of downstream signals are activated. If ER stress cannot be combatted by the cell, all three pathways can ultimately facilitate apoptosis. Molecular targets of OXY identified in this study have been highlighted in red.

CHAPTER 3

Effects of oxyresveratrol on motor function and dopaminergic loss in a 6-OHDA induced *in vivo* model of Parkinson's disease

3.1 Introduction

There are several experimental models which can mimic the pathology and symptoms of Parkinson's disease (PD). While 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are toxin models used to induce the disease, paraquat (PQ) and rotenone, found in herbicides, support the role of environmental toxins that can promote the progression of PD. Furthermore, increasing lines of research on the role of genetics in PD, has led the science community to establish transgenic mice bearing expression of human wild-type (WT) or mutated α -synuclein (α -syn). Along with the traditional primate and rodent PD models which are induced by toxins, zebrafish (*Danio rerio*) and fruit fly (*Drosophila melanogaster*) models of PD, have also been established. This variety of different PD models provides us with a gamut of tools to investigate the underlying mechanisms and effects of neuroprotective drugs.

Common sites for injection of 6-OHDA are the striatum, substantia nigra *pars compacta* (SNpc), or the medial forebrain bundle (MFB): the bundle of afferent nerve fibres projecting from the SNpc to the striatum (Figure 3.1). In PD, death of dopaminergic neurons in the SNpc, leads to a loss of 70-80% of the striatal dopamine by the time symptoms are visible (Dauer et al., 2003). Striatal infusion of 6-OHDA, results in mild lesions (Przedbroski et al., 1995). Therefore, to mimic

advanced stages of the disease, either bilateral striatal lesions or multiple unilateral injections in different striatal sites are required. On the other hand, since the SNpc is a small region, a successful injection requires very high accuracy. SNpc lesions also have the risk of damage to surrounding areas such as the substantia nigra pars reticulata (SNpr). The MFB is a widely used site for injection, wherein due to anterograde and retrograde transport, the toxin triggers degeneration of both striatal and nigral dopaminergic neurons, respectively. Furthermore, there is evidence to show the effects of MFB lesions in inducing non-motor behavioural changes related to PD. Cognitive deficits such as spatial memory deficit (Mura & Feldon, 2003) and mnemonic memory impairment (Ma et al., 2014) have been reported in the MFB model. The MFB lesion model is thus a holistic representation of PD.

Rats treated with 6-OHDA have always been a traditional model used for drug testing. Owing the fact that 6-OHDA must be injected, unilateral lesions are possible which are less detrimental. Additionally, unilateral models make it easier to monitor disease progression, and in turn the effects of anti-parkinsonian treatments (Jiang et al., 1993; Schwarting & Huston, 1996). Apomorphine- or amphetamine-induced rotations are a regular test accessing the severity of unilateral lesion. Administration of these compounds leads to contralateral or ipsilateral rotations, respectively. It has been proposed that these rotations are usually observed when there is up to a 90% destruction of dopaminergic neurons (Deumens et al., 2002). However, the rotation test is a mere indication for the severity of unilateral 6-OHDA lesion. Several other tests are used to assess an asymmetry in motor function caused by akinesia of the fore-limbs. The rotarod, initially developed to assess motor coordination after cerebellectomies (Caston et al., 1995), has now been adapted to PD models as well. The rats are made to walk on a rotating

wheel with controlled acceleration - rats with impaired motor function tend to fall off the wheel faster. The asymmetric cylinder test (Schallert et al., 2000; Tillerson et al., 2001) is another test for motor function wherein the animal is placed in a transparent cylinder, and the difference between the its tendency to use the ipsilateral limb versus contralateral, or both limbs while rearing is measured. Lesioned rats tend to use their ipsilateral limb more. The footprint analysis (Cheng et al., 1997) and beam walking tests (Wallace et al., 1980) have been used to assess gait and balance, respectively, whereas simple tests such as the open field can be used to measure total locomotor activity (Michele, 1995), which is reduced after lesion. In conjunction with the rotation test, these behavioural tests for motor function are also used as screening methods to test the effects of antiparkinsonian drugs.

Nutraceuticals play a protective role against diseases, due to their antioxidant properties. Therapies for neurodegenerative disease should ideally have high blood-brain barrier (BBB) permeability for optimal effects. It has been elucidated that oxyresveratrol (OXY), the candidate under study here, adequately crosses the BBB in rats (Breuer et al., 2006). This permeability makes OXY an ideal candidate for testing in animal models of PD. I therefore hypothesized that OXY exerts neuroprotective effects in 6-OHDA-lesioned rats, preventing motor dysfunction and dopaminergic loss.

3.2 Aims of study

In Chapter 2, I have shown the protective effects of OXY pre-treatment against 6-OHDA-induced toxicity *in vitro*. However, since PD is a neurodegenerative

disorder with predominantly motor symptoms, my aim in this part of the study was to assess protective effects of OXY on motor function and dopaminergic cell loss in an animal PD model. For this purpose, Sprague-Dawley (SD) rats injected unilaterally in the MFB with 6-OHDA were employed. The MFB was chosen as a site for injection, as it results in loss of dopaminergic neurons mimicking PD (Torres et al., 2011).

The first aim of this study was to set up and validate the MFB model. A screening study was first performed to identify potentially neuroprotective doses of OXY, against 6-OHDA-induced lesions. A range of three doses of OXY was then given to the rats by oral gavage for one week, followed by 6-OHDA lesion. The drug treatment continued for two weeks post-surgery, as described by the experimental scheme in Figure 3.2. It was then determined whether OXY at any of the above doses could reduce the intensity of the damage caused by 6-OHDA.

Once the effective dose of OXY was chosen, its protective effects were compared with the same doses of resveratrol (RES), in a larger study. Since pinostilbene (PINO) did not show any protective effects in Chapter 2, it was not used in this study. The experimental scheme is described in Figure 3.2, and all treatment groups are listed in Table 3.1.

3.3 Materials and methods

3.3.1 Study design

One hundred and forty-five 4-6 weeks old, male Sprague-Dawley rats were purchased from the Laboratory Animal Unit at The University of Hong Kong. The rats weighed 200 g at the beginning of treatment. All experimental procedures were

in accordance with the Committee on the Use of Live Animal in Teaching and Research (CULATR # 3491-14) of The University of Hong Kong. Rats of same treatment groups were held in pairs in cages, in a temperature-controlled room with 12-hour dark/light cycles and access to food and water *ad libitum*. Rats randomly were divided into sham or 6-OHDA groups, and received either vehicle, 1 mg/kg OXY, 10 mg/kg OXY, 20 mg/kg OXY, 1 mg/kg RES, or 10 mg/kg RES treatment for one week prior to injection of 6-OHDA into the right medial forebrain bundle (on day 8). Rats were allowed to recover, and drug treatment continued for two weeks post-surgery, until day 21. On day 22, behavioural tests were carried out to assess intensity of lesion and motor dysfunction. The rats were then euthanized using the appropriate method and tissues extracted for immunohistochemistry or metabolomics analysis.

All materials were purchased from Sigma Aldrich (United Kingdom), unless stated otherwise.

3.3.2 *Natural products*

OXY (generously provided by Dr. Mingfu Wang, School of Biological Sciences, The University of Hong Kong) and RES were dissolved in deionized water to make stock solutions. Rats in the sham or 6-OHDA groups received either 1 mg/kg OXY, 10 mg/kg OXY, 20 mg/kg OXY, 1 mg/kg RES, 10 mg/kg RES or vehicle treatment. Fresh 1 mg/ml stocks of OXY and RES were prepared in water every day, and vortexed until dissolved. The weight of each rat was noted before drug treatment every day, and appropriate volume of drug solution was administered by oral gavage. Drug administration was done at the same time (between 12pm and 2pm) every day for the treatment period.

3.3.3 Stereotactic injection of 6-OHDA

The rats were randomly divided into sham and 6-OHDA groups. Fresh stock solution (3 µg/µl) of 6-hydroxydopamine hydrobromide was prepared in saline (0.9% w/v NaCl) containing 0.2 mg/ml ascorbic acid. Rats were anaesthetized with 60 mg/kg pentobarbital (Alfansan International, Netherlands). 6-OHDA at 12 µg (in 4µl) was introduced into the right (ipsilateral) MFB, using a Hamilton syringe connected to a 33G needle, at the rate of 1 µl/min. The coordinates of the injection site were: ML = -1.2, AP = -4 and DV = +7.5 (below dura), with the nose bar position at 4.5, based on the atlas by Paxinos and Watson. These coordinates were slightly modified from the study by Torres *et al.* (Torres et al., 2011). Rats in the sham group were injected with the same volume (4 µl) of vehicle. The needle was left in place for five minutes before retracting, and the incision was sutured. Body temperature and heart rate of the animals was measured constantly throughout the procedure. The rats were kept under warm conditions until they regained consciousness. They were left to recover in the temperature-controlled room and administered with 1.5 mg/kg meloxicam in drinking water for 48 hours to minimize any pain.

3.3.4 Behavioural assessment

On day 22 of the treatment plan, behaviour tests were carried out to assess intensity of the unilateral lesion, as well as motor dysfunction. Behavioural assessment was done in an isolated room, in the following order:

3.3.4.1 Asymmetric cylinder test

The protocol used by Schallert *et al.* was modified slightly (Schallert et al., 2000). Rats were placed in a transparent acrylic cylinder for a total of three minutes and

recorded. During every rear, the use of ipsilateral, contralateral or both forelimbs was counted, for a minimum of three and a maximum of ten rears or three minutes, whichever was first. The cylinder was cleaned with 70% ethanol between each use. Results were expressed as % of rears with ipsilateral limb use only.

3.3.4.2 Rotarod

The accelerating protocol by Monville *et al.* (Monville et al., 2006) was modified as follows:

Training: The rats were trained on the rotarod before commencing the treatment regime, to generate a stable baseline. On the first day of training, rats were first made to walk on the wheel at four rotations per minute, for five minutes to acclimatize to the instrument. Any animal that fell off, was put back onto the wheel. The rats were then made to run on an accelerating wheel, increasing from 4 - 40 rotations per minute, gradually over five minutes. The time at which each animal fell off the wheel (latency to fall), was noted. Each animal was given three consecutive trials, for three consecutive days. Since the rats had stable latencies to fall by the third day, the average of the three trials on this day was used as the baseline.

Test: On the test day (Day 22), the rats were given three trials on the wheel again, at the same accelerating speed of 4-40 rotations per minute, over five minutes. The latency to fall was noted, and the average of three trials was measured. The results were then expressed as a % change of the latency to fall on test day, compared to the baseline.

3.3.4.3 *Apomorphine-induced rotation test*

Rats were injected subcutaneously with 0.3 mg/kg of apomorphine hydrochloride dissolved in saline. Five minutes after injection, each animal was placed in a cylinder and recorded for 40 minutes. The number of contralateral rotations in 30 minutes was measured. Minimum four rotations per minute was considered as acceptable criteria for a successful model.

3.3.5 *Immunohistochemistry*

After behavioural assessment, the mesencephalon of eight rats from each group were harvested for tyrosine hydroxylase staining as follows:

3.3.5.1 *Tissue processing and frozen-sectioning*

Rats were overdosed with 150-200 mg/kg pentobarbital, followed by intra-cardiac transfusion of ice-cold saline and subsequently, freshly prepared ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The midbrain section containing the *substantia nigra pars compacta* (SNpc) was then dissected out, post-fixed and soaked in increasing concentrations of sucrose. The tissue was then embedded in Tissue-Tek® OCT, snap-frozen and stored in -80°C until use. 15 µM slices were then sectioned using on a cryostat (Leica, Germany) and mounted. Every 6th section of the mesencephalon was collected, dried and stored at 4°C until use.

3.3.5.2 *3,3'-diaminobenzidine (DAB) staining and Imaging*

Sectioned tissues were washed with 0.1 M phosphate buffered saline (PBS) thrice, followed by blocking of endogenous peroxidase activity with 30% hydrogen peroxide (H₂O₂) in methanol for 30 minutes. Tissues were then incubated with the anti-tyrosine hydroxylase (TH) antibody (1:400, Cell Signaling Technologies,

U.S.A), in a humid slide chamber at 4°C overnight. Following biotinylated secondary antibody (1:400, Dako, U.S.A) incubation, slides were stained using the ABC staining kit (Invitrogen, U.S.A), followed by DAB (ThermoFisher Scientific), according to the manufacturers' protocols. Tissues were then counter stained with haematoxylin, dehydrated with ethanol and toluene and mounted. Slides were observed at a magnification of 5x using brightfield microscopy (Zeiss Axioplasm, Germany), and stitched using the Image Composite Editor software (Microsoft, U.S.A). TH counting was then done using ImageJ (National Institute of Health, U.S.A). TH-positive puncta were measured using 16-bit converted images at a threshold of 60-150 pixels, using the "Analyze particles" function. Total TH counts on each side were then normalized to the number of tissue sections to find the TH density, which was expressed as a % of TH density of the right ipsilateral side to the contralateral side.

3.3.6 Statistical analysis

All analysis was done using GraphPad Prism 6 (GraphPad, U.S.A). Statistical analysis was done using either unpaired t test, Kruskal Wallis test followed by Dunn's *post-hoc* test, Two-way ANOVA or One-way ANOVA followed by Tukey's or Dunnett's *post-hoc* test, wherever applicable. All results are expressed as mean $\pm$ standard deviation (S.D) of at least four rats in each group. The *p* values and number of independent rats in each treatment group are indicated in each figure legend.

3.4 Results

3.4.1 *Validation of the medial forebrain bundle 6-OHDA model*

6-OHDA (12 μ g) was injected stereotactically into the right MFB. The coordinates used were: mediolateral = -1.2 (from the bregma), anteroposterior = -4 (from the bregma) and dorsoventral = +7.5 (from the dura), with the nose bar position at 4.5, based on a study by Torres and co-workers (Torres et al., 2011). Owing to the nature of the treatment plan which involved a one-week pre-treatment of the drugs, before surgery, a high (> 80%) success rate of MFB lesion was a prerequisite. This was to ensure that rats showing a low intensity of ipsilateral lesion after injection, were doing so because of drug-mediated neuroprotection only, eliminating any unsuccessful lesions of the MFB as a confounding factor. A total of 12 male SD rats were used to test the MFB injection site for this purpose. The apomorphine-induced rotation test was used as a measure of intensity of lesion, one week after injection. Rats exhibiting $\geq$ four contralateral rotations per minute after administration of apomorphine, were considered successfully lesioned. The dopaminergic neurons in the SNpc of these rats were then examined for tyrosine hydroxylase (TH) by immunohistochemical staining to measure the cell loss induced by 6-OHDA on the lesioned side. Four animals injected with saline were used as a control group.

It was observed that 10 of the 12 rats showed successful ipsilateral lesions with an average of 143 rotations in 30 minutes (Figure 3.3). Furthermore, immunostaining of the SNpc of these rats revealed that TH density on the ipsilateral side, was half compared to the contralateral side, further indicating that 6-OHDA successfully lesioned the MFB (Figure 3.4). Since the success rate of lesions was 83.33%, the MFB model at this site was successfully validated. Additionally, since

the rats were successfully lesioned within just one week after surgery, this model was adopted for the drug treatment plan (Figure 3.2), which involved behaviour testing two weeks after surgery.

3.4.2 *Effects of different doses of OXY on the intensity of ipsilateral lesions*

The optimal therapeutic window of OXY against *in vivo* parkinsonian toxicity is unknown. Therefore, a preliminary study was done by using a high dose of OXY: Three rats were fed with 50 mg/kg OXY for a week to ensure they could tolerate such a high dose, without any adverse effects, before commencing the experimental plan. Former studies have illustrated the neuroprotective effects of OXY against stroke (Andrabi et al., 2004) and neuro-inflammation (Mouihate et al., 2006), in the range of 10-20 mg/kg. Therefore, at the first stage of the study, three doses of OXY were chosen for initial screening: 1 mg/kg, 10 mg/kg and 20 mg/kg.

After subcutaneous apomorphine injection (0.3 mg/kg) on day 22, rats in the 6-OHDA + vehicle group had a significantly higher number of contralateral rotations than the sham + vehicle group (Figure 3.5). It was observed that 10 mg/kg OXY treatment significantly reduced the number of rotations, while 1 mg/kg OXY showed a similar trend (Figure 3.5). Interestingly, 20 mg/kg OXY was not effective in decreasing the number of rotations (Figure 3.5). The intensity of ipsilateral lesion was alleviated by lower doses of OXY. The sham + 20 mg/kg OXY group was used to ensure that OXY treatment alone had no significant effect on lesions (Figure 3.5). Since this group did not show any significant contralateral rotations, it was concluded that OXY alone had no role in this regard.

3.4.3 *Effects of lesions and OXY treatment on body weight*

The mean weight of rats in each group was calculated over each week of the entire treatment course. During week 1 prior to 6-OHDA surgery, rats in each treatment group showed a similar average weight. At week one and two post-surgery, however, rats in the 6-OHDA + vehicle group weighed the least, indicating that the 6-OHDA unilateral lesion affected body weight (Figure 3.6). OXY treatment had no significant impact on body weight (Figure 3.6).

3.4.4 *Comparison of the effects of OXY and RES on motor function and lesions*

After narrowing down on the suitable dose of OXY, my next aim was to investigate the effects of OXY on motor dysfunction in the 6-OHDA model. Based on the results from 4.3.2 above 10 mg/kg OXY showed the most protective effects. 1 mg/kg OXY was also selected as it appeared to have a partial effect. The same two doses of RES were used for comparison.

Rigidity of the contralateral fore-limb, that is induced by unilateral lesion of 6-OHDA represents one of the hallmark symptoms of PD. An inability to use the contralateral limb and a decreased latency to fall off the rotating wheel were assessed using the asymmetric cylinder and rotarod tests, respectively. The 6-OHDA lesioned group showed a higher tendency to use the ipsilateral limb only while rearing (Figure 3.7A), as well as a significantly lower latency to fall off the rotating wheel (Figure 3.7B), due to motor imbalance. However, lesioned rats fed with 1 mg/kg OXY showed a significantly improved ability to use their contralateral limbs compared, to the 6-OHDA group (Figure 3.7A). The 10 mg/kg OXY treated rats also had a higher latency to fall off the rotarod, compared to the 6-OHDA group, indicating an alleviation of motor imbalance (Figure 3.6B). Sham

rats, administered with OXY or RES showed no effect on motor function, indicating that these compounds *per se* did not have any undesirable effects on motor function.

Although 1 mg/kg RES did significantly reduce the percentage of ipsilateral fore-limb use (Figure 3.7A), none of the doses of RES were effective in reducing the latency to fall off the rotarod (Figure 3.7B), or the intensity of the lesion, as seen by the apomorphine induced rotation test (Figure 3.7C).

3.4.5 *Comparison of the effects of OXY and RES on dopaminergic loss in the SNpc*

After conducting behavioural assessment, immuno-staining was done for TH, a marker of dopaminergic neurons. Frozen sections of the SNpc region (every 6th section) were stained for TH using 3,3'-Diaminobenzidine (DAB). TH count on the ipsilateral side, was expressed as a percentage of the contralateral side count.

It was observed that in the 6-OHDA + vehicle group, there was significantly greater cell loss on the ipsilateral side, when compared to the sham + vehicle group (Figure 3.8A), corroborating the efficiency of this model, in lesioning the SNpc. Rats lesioned with 6-OHDA exhibited just 25% TH count on the lesioned side (Figure 3.8B). While both doses of OXY and RES showed a trend in increasing the TH count on the ipsilateral, none of the treatments significantly reversed the cell loss of dopaminergic neurons (Figure 3.8B).

3.5 Discussion

The findings from this study indicate that OXY can successfully protect 6-OHDA-lesioned rats from severe motor dysfunction. This study was carried out after validating the efficacy of 6-OHDA in inducing MFB lesions. It was also

determined that OXY, even at a high dose of 50 mg/kg did not induce any adverse effects in the rats. After establishing the safe dosage range, and eliminating any errors due to unsuccessful lesions, the treatment plan was commenced. It was observed that lower doses of OXY (10 mg/kg or less) were more effective in reducing the extent of 6-OHDA lesion and motor dysfunction, with 20 mg/kg OXY showing no such effect. On the other hand, RES, which has previously shown a reduction in motor dysfunction at higher doses (Jin et al., 2008; Khan et al., 2010), it was not as effective at 1 mg/kg or 10 mg/kg. Based on these results, OXY is more potent than RES in reducing 6-OHDA induced damage. This also corroborates findings from Chapter 2, which elucidated the effective of low concentrations of OXY in combatting ER stress. However, the dosage range needs to be determined carefully, by validation in a larger preclinical study. There could be several explanations for the discrepancies in the effects of OXY at the different doses. As seen from Chapter 2, OXY has enhanced effects compared to RES, on pathological pathways involved in PD. Since OXY shows modulation of several cellular pathways, it is more than just an antioxidant or anti-inflammatory compound. It is possible, that at 20 mg/kg even more signalling pathways are being affected, leading to overstimulation in cells. On the other hand, high dose OXY might also be inducing noxious effects in the cells, that were not investigated in this study.

In the two previous studies investigating RES, a striatal model of 6-OHDA was used. Its beneficial effects against 6-OHDA neurotoxicity on MFB lesions have not been elucidated. Since injection of 6-OHDA into the striatum is less detrimental than that the MFB, (Yuan et al., 2005) RES might be protective against cell damage and motor dysfunction induced in the striatum, but not as effective in the MFB. This might also explain why although in the study by Jin *et al.* there was no pre-treatment

of RES before surgery, all three doses of RES (10 mg/kg, 20 mg/kg and 40 mg/kg) were still effective in mitigating motor dysfunction (Jin et al., 2008).

Although both 1 mg/kg and 10 mg/kg OXY showed improvement in motor function, it was interesting to note that 1 mg/kg OXY did not significantly increase the latency to fall off the rotarod. This dose was, however, beneficial in alleviating the intensity of the lesion as well as dependence on ipsilateral fore-limb use. A reason for this discordance in the effects of 1 mg/kg OXY could lie in the fact that rats in the 6-OHDA + 1 mg/kg OXY group had the highest average body weight, amongst all the lesioned groups. This might interfere with their ability to run on the rotarod for too long. On the other hand, a mild drop in average body weight was seen in the 6-OHDA group, which could stem from a host of reasons. In one study, considerable weight loss of rats bilaterally lesioned with 6-OHDA was attributed to reduced appetite and motivation (Ferro et al., 2005). It is possible that an extent of these effects might also be seen in a unilateral model, affecting their appetite. In a recent study, anhedonia and neurotransmitter changes leading to depression, induced by unilateral 6-OHDA lesion of the MFB were shown (Kamińska et al., 2017). Such conditions also have an impact on appetite.

Results from this chapter suggest that regular, long term OXY consumption may reduce the rate of decline in aging-associated, gradually progressing neurodegeneration. A one-week pre-treatment of OXY was chosen here before inducing parkinsonian mimetic motor deficits. Enhanced effects of OXY pre-treatment as opposed to co-treatment or post-treatment have been elucidated previously in cell culture (Chao et al., 2008). Furthermore, natural extracts tend to be more prophylactic in nature. The alleviating effects of OXY demonstrated here, imply that it can rescue motor decline, if consumed early on. Furthermore, OXY

showed promising effects at lower doses; making a case for safety even over longer periods of consumption. It must be noted however, that because this is not curative therapy, a slower progression of disease may also mean further prolonged diagnosis.

A limitation of this study is that a significant reduction in the loss of dopaminergic neurons in SNpc was not observed in OXY-treated rats. In studies investigating beneficial effects of RES on striatal neurons in rats, such effects were observed (Jin et al., 2008; Khan et al., 2010). Cell loss in PD is a gradual but irreversible process. When introduced into the MFB, 6-OHDA induces dopaminergic cell loss in both anterograde and retrograde manner, affecting both the striatum and SNpc, making this a very acute model. Additionally, dopamine levels were not measured, in the striatum or SNpc here. In PD, there is up to 80% loss of dopamine in the striatum. Therefore, measuring striatal dopamine levels is an additional means to assess protective effects of OXY. It is possible that although cell loss was not significantly improved, mitigation of motor decline was observed due to partial replenishment of depleted dopamine levels in the striatum. The striatal model also represents earlier stages of PD (Yuan et al., 2005). Therefore, investigating the prophylactic effects of OXY in a striatal model will give further insights about its protective effects in a real-life scenario. In the RES study, there was a pre-treatment of 15 days (Khan et al., 2010), while the duration of pre-treatment here, was just one week. Since the cell loss by 6-OHDA in the MFB model is acute, one week might not be enough time for OXY to slow down pathological damage. A future study with a longer pre-treatment is also warranted, to explore the full potential of OXY as prophylactic treatment.

In summary, this is the first study of its kind to demonstrate the effects of OXY, a potent extract of *Morus Alba*, on pathology and behaviour in experimental PD. Protective effects of RES on motor function in genetic and toxin models of PD have emphasized on its role as an antioxidant, anti-inflammatory and activator of SIRT1 (Albani et al., 2009; Jin et al., 2008; Khan et al., 2010; J. Zhang et al., 2015). However, OXY has been shown to have more pronounced antioxidant activity than RES (Chao et al., 2008; Lorenz et al., 2003). Moreover, OXY, but not RES, had protective effects on neurodegenerative pathways such as ER stress, as shown in Chapter 2. Therefore, the enhanced protection of OXY treatment compared to similar doses of RES observed *in vivo*, can also be attributed to the more widespread mechanisms of OXY. Neurodegenerative diseases have very complex disease pathologies. A protective agent like OXY, therefore, seems like a suitable agent to combat neurodegeneration. The mechanism and role of OXY in slowing down decline in PD should be explored further in other larger preclinical and clinical studies.

3.6 Conclusion

In this chapter, I successfully established the OXY-mediated neuroprotection against 6-OHDA related toxicity in an animal PD model. These effects, however, need to be further validated with another toxin model and a different site of lesion. OXY was also more potent compared to RES, in facilitating improvement of motor dysfunction. These protective effects of OXY against motor decline, coupled with its effects on ER stress (as shown in the last chapter), further lead to the hypothesis that OXY can modulate specific pathways associated with PD, which are not limited to just oxidative stress. Given the multifactorial nature of PD, a

comprehensive drug profile is warranted, investigating effects of lower doses of OXY on all possible disease mechanisms. To analyse this further, systems metabolomics is used in Chapter 4 and 5. Metabolomics gives detailed insights about pathways altered in disease, as well as effects of drugs in restoring such pathways.

Figure 3.1. Summary of injection sites. (Adapted from http://www.ablongman.com/html/psychplace_acts/synapse/dopamine.html)

Figure 3.2. Schematic of experimental design.

Sham (saline)	6-OHDA
Vehicle	Vehicle
1 mg/kg OXY	1 mg/kg OXY
10 mg/kg OXY	10 mg/kg OXY
20 mg/kg OXY	20 mg/kg OXY
1 mg/kg RES	1 mg/kg RES
10 mg/kg RES	10 mg/kg RES

Table 3.1. Summary of treatment groups. The rats were randomly divided into sham or 6-OHDA groups and received either vehicle or drug treatment. There were 12 different treatment groups in total over the course of the entire study.

Figure 3.3. Success rate of MFB injections. Ten of the twelve rats showed more than four contralateral rotations per minute after subcutaneous injection of 0.3 mg/kg apomorphine hydrochloride, indicating that the success rate of MFB injections was 83.33%.

Figure 3.4. 6-OHDA-treated rats show cell loss on the ipsilateral side. Representative images shown in (A). Higher magnification TH puncta shown in (B). Rats in the sham group showed similar TH staining on both sides, while the 6-OHDA treated rats had significantly less TH positive cells on the ipsilateral side compared to the contralateral (B). Data represents mean TH density on each side, of at least four rats in each group (* indicates $p < 0.05$, ipsilateral side compared to contralateral side, using unpaired t test).

Figure 3.5. Effect of three doses of OXY on number of contralateral rotations. An increase in the number of contralateral rotations observed in the 6-OHDA group, was decreased significantly by 10 mg/kg OXY. The 20 mg/kg OXY group showed no effect on the number of rotations. Data represents mean $\pm$ S.D of at least four different rats for each group (**** indicates $p < 0.0001$, compared to sham + vehicle, and ##### indicates $p < 0.0001$, compared to 6-OHDA + vehicle, using Kruskal-Wallis test followed by Dunnett's *post-hoc* test).

Figure 3.6. Average body weight of rats over the three-week treatment regime. Average weight of the rats in each group was similar over week 1 before surgery. After 6-OHDA injection, the 6-OHDA + vehicle group weighed the least Data expressed as mean $\pm$ SD of at least four different rats for each group

Figure 3.7. Comparison of low dose OXY and RES treatments on intensity of lesions and motor function. While 1 mg/kg OXY and RES significantly improved contralateral limb function as seen by the asymmetric cylinder test (A), only 10 mg/kg OXY significantly improved the latency to fall off the rotarod (B). 1 mg/kg and 10 mg/kg OXY, but not RES significantly reduced the intensity of lesions as seen by the reduction in number of rotations (C). Data expressed as mean $\pm$ SD of at least seven rats for each group (* indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, compared to sham + vehicle, # indicates $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$, compared to 6-OHDA + vehicle, ¹ $p < 0.05$, compared to 6-OHDA + 1 mg/kg OXY, using One-way ANOVA and Tukey's *post-hoc* test).

A.

B.

Figure 3.8. Effects of OXY and RES on tyrosine hydroxylase (TH) staining. Representative images shown in (A). 6-OHDA significantly reduced the TH density in the ipsilateral SNpc. Drug treatments did not significantly change the TH counts (B). Data expressed as mean $\pm$ SD of at least five rats for each group (** indicates $p < 0.01$, compared to sham + vehicle, using Kruskal-Wallis test and Dunn's *post-hoc* test).

CHAPTER 4

Systems metabolomics reveals the association of plasma and midbrain metabolites with 6-OHDA associated neurodegeneration

4.1 Introduction

Diagnosis of Parkinson's disease (PD) is primarily based on clinical symptoms and the error rate can be quite high (Bogdanov et al., 2008). Of these, the main features used for diagnosis include bradykinesia, rigidity, tremors, postural instability and freezing (Jankovic, 2008). Significant challenges include a late diagnosis due to the symptoms manifesting years after pathology develops, or misdiagnosis due to similarity of symptoms with conditions such as multiple system atrophy (MSA). In recent years, there have been plenty of efforts in advancing biomarker discovery for PD. Metabolomics is a well-defined approach used for biomarker discovery and investigating disease mechanisms.

In one longitudinal study, it was found that plasma xanthine and fatty acid metabolites showed a high correlation with progression of the disease (Lewitt et al., 2017). Several studies on urine samples of PD patients showed an increase in amino acid metabolism, including phenylalanine (Hatano et al., 2016), histidine, glycine and tryptophan/kynurenine (Luan et al., 2015). Metabolic profiling of cerebrospinal fluid (CSF) has also been particularly useful. Decreased levels of creatinine (Öhman et al., 2015; Trupp et al., 2014), amino acids and fatty acids (Trupp et al., 2014) were found in the CSF of PD patients, using Gas chromatography-Mass spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR) based metabolomics. Based on these studies, it is evident that examination of

metabolomics in biofluids such as plasma and CSF can help identify dynamic changes associated with disease progression. Given that PD is a neurological disorder, detailed brain related studies are necessary to give a more accurate depiction of mechanisms of the disease. However, post-mortem brains, which are currently the only option, cannot accurately depict dynamic changes in metabolites.

A wide range of animal models of PD are available, which have been employed to monitor metabolite changes in real-time. Studies on the mesencephalon of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD in mice have revealed the role of altered energy (Poliquin et al., 2013), ceramide and sphingolipid metabolism (Li et al., 2013). A range of lipid species including lysophosphatidylcholines and phosphatidylcholines are shown to be involved in 6-hydroxydopamine (6-OHDA) toxicity in rat midbrains (Farmer et al., 2015). Liquid chromatography-Mass spectrometry (LC-MS) based metabolomics suggests an involvement of different cardiolipins; mitochondria-associated phospholipids; in mesencephalon and plasma samples of rotenone treated rats (Lu et al., 2014; Tyurina et al., 2015). Transgenic- and toxin-treated *Drosophila* models have supported the role of kynurenine metabolism (Luan et al., 2015) along with amino acid, fatty acid and myo-inositol metabolism, (Shukla et al., 2016) respectively.

Changes in metabolites are sensitive and dependant on numerous factors. Metabolic changes are therefore an accurate depiction of disease mechanisms, disease status and effects of therapeutic intervention. More in-depth metabolomics analysis in experimental PD will aid in depicting alterations occurring on a cellular level. This understanding of the basic pathological changes is warranted for accurate diagnosis and precision medicine. Therefore, I hypothesized that metabolic pathways will be severely altered in the plasma and midbrain of 6-OHDA lesioned

rats. These metabolic changes will give insights into biochemical pathways involved in PD disease pathogenesis.

4.2 Aims of study

In this chapter, the 6-OHDA-induced rat model was employed to investigate a system wide change in metabolites induced by the toxin, using GC-MS. This model was used as it is the most common choice for preclinical studies investigating therapeutic candidates for PD. Although 6-OHDA is used for primarily unilateral lesions, the pathology and symptoms induced are a very accurate depiction of clinical manifestations. The aim of this study was, therefore, to discover the perturbed metabolic pathways in different tissues with this traditional unilateral model. Changes in the plasma can reflect the severity of any condition. Metabolites in the plasma are often sensitive to disease status and can be measured easily. The midbrain is the primary site of damage in this model and changes in midbrain metabolites represent primary pathological pathways. Lastly, the liver is the site of metabolism of all macronutrients, and because of this important role, is linked with normal functioning of all other tissues in the body. Therefore, I chose to first analyse plasma and midbrain tissues and then the liver, to follow system relevance to the disease. My next aim was to investigate the prediction ability of the metabolites selected and their correlation with motor decline. The metabolomics study design is described in Figure 4.1.

Finally, the effects of oxyresveratrol (OXY) and resveratrol (RES) treatment on any altered metabolites were also investigated. Results from Chapter 3 suggested that 1 mg/kg and 10 mg/kg OXY show neuroprotective effects against 6-OHDA

induced toxicity. Therefore, the final aim of this study was to assess if these doses of OXY can prevent disruption of metabolic pathways in any of the tissues. Only 1 mg/kg RES was used as a comparison, as 10 mg/kg RES showed no protective effects against motor dysfunction (Section 3.4.4).

4.3 Materials and methods

4.3.1 Study design

Gas chromatography - Mass spectrometry (GC-MS) based metabolomics was used for this part of the study. Plasma, midbrain, cerebellum and liver tissues from all experimental groups of the animal study were collected. All the tissue samples were homogenized, and the aqueous and non-aqueous phases were separated by in vial dual extraction (IVDE). For the first part of the metabolomics study, plasma, midbrain and liver tissues were analysed by GC-MS analysis. The cerebellum was used as a control region. After raw data pre-processing, multivariate analysis was used to pick out features driving separations between the sham and 6-OHDA treated groups, in the plasma and midbrain. These features were subsequently semi-quantified and subjected to univariate analysis to identify significant metabolic features. The correlation of these features with behaviour test scores, and their prediction abilities were also determined. Finally, the levels of significantly changed features were also measured in the drug treated groups.

All materials were purchased from Sigma Aldrich (United Kingdom), unless stated otherwise.

4.3.2 *Tissue harvest*

After behavioural assessment, eight rats from each group were asphyxiated using CO₂ and tissues were harvested for metabolomics analysis as follows:

4.3.2.1 *Plasma*

About 1 ml of blood was drawn by intra-cardiac transfusion, using an ethylenediaminetetraacetic acid (EDTA) buffer-coated syringe. The needle was removed, and blood transferred to Eppendorf tubes, and shaken. Samples were kept on ice, and then centrifuged at 4500 rpm in a 4°C Eppendorf centrifuge for 15 minutes. The supernatants were collected, and samples were stored at -80°C until use.

4.3.2.2 *Brain*

The brain was extracted and briefly rinsed in ice cold 0.1 M PBS to remove any excess blood. It was then slit down the middle to divide the right and left sides and the cerebellum and mesencephalon tissues were separated according to a previous protocol (Chiu et al., 2007), snap frozen in liquid N₂ and stored in -80°C until use.

4.3.2.3 *Liver*

A section of the liver was dissected and briefly rinsed in ice cold 0.1 M PBS to remove any excess blood. It was then snap frozen in liquid N₂ and stored in -80°C until use.

4.3.3 *Sample extraction*

4.3.3.1 *Plasma sample extraction*

In-vial dual extraction (IVDE) was slightly modified based on a previous publication in the laboratory (Whiley et al., 2012). Briefly, 20 µL of LC-MS grade water was added to 40 µL plasma, followed by 80 µL of LC-MS grade methanol

containing 10 µg/mL of succinic-d4 acid as internal standard (IS) for GC-MS and L-serine $^{13}\text{C}_3^{15}\text{N}$ (95% purity) for LC-MS. After vortexing, 400 µL of LC-MS grade methyl tertiary butyl ether (MTBE) with 10 µg/mL of tripentadecanoin as IS was added and then the samples were mixed thoroughly. Following a final addition of 100 µL LC-MS grade water, samples were centrifuged at 3000 g for 10 min at 4°C to give a clear separation of MTBE (upper) and aqueous (lower) phases with protein aggregated at the bottom. The aqueous layer (about 150 µL) and MTBE (about 200 µL) layers were collected and stored at -20°C and -80°C, respectively. Pooled samples were used as quality control (QC) samples.

4.3.3.2 *Brain and liver sample extraction*

IVDE was slightly modified based on a previous protocol (Ebshiana et al., 2015). Prior to homogenisation, 5 µL of methanol and 5 µL of IS (50µg/mL succinic-d4 acid in 80% methanol) was added per milligram of tissue. The tissue was then homogenised using a Tissuelyzer (Qiagen, Germany) for 10 cycles of 30 seconds at 25 Hz. Subsequently, 80 µL of homogenate was diluted with 120 µL of methanol. The remaining extraction procedure was similar to plasma, with addition of 40 µL of water, 1 ml of MTBE containing tripentadecanoin (10 µg/ml) followed by thorough vortexing. After addition of a further 160 µL of water, samples were then centrifuged at 3000g for 10 min at 4°C. The aqueous and MTBE layers were separated and stored, as before.

4.3.4 *Derivatization of tissues for GC-MS*

Twenty µL of plasma/brain homogenate samples were dried under a stream of N_2 . For plasma, 50 µL of O-methoxyamine-hydrochloride (MOX) in pyridine (20 mg/mL, Sigma Aldrich) was added to the residue and maintained at 70°C for

30 min. Samples were then dried again and reconstituted in a 1:1 (v/v) solution of acetonitrile and the derivatizing agent N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 1% trimethylchlorosilane (TMCS). The derivatization process was operated at 70°C for one hour. For brain tissues, the dried residues were reconstituted in a 1:1 (v/v) solution of acetonitrile and BSTFA (1% TMCS) directly and incubated at 37°C for an hour. During this process, samples first underwent oximation, followed by silylation (Fiehn et al., 2000). Oximation is often done prior to silylation, in order to protect the carbonyl group in organic molecules such as ketones and sugars, from cyclization (Koek et al., 2006; Scalbert et al., 2009). Silylation, or attaching a trimethylsilyl group to already present functional groups, is the step that renders volatility to most non-volatiles analytes, making them amenable to GC separation (Scalbert et al., 2009). All the resulting derivatized samples were transferred to amber vials with inserts for GC-MS analysis.

4.3.5 GC-MS analysis

GC-MS analysis was carried out on a Shimadzu GC-2010 Plus gas chromatograph equipped with a GCMS-QP2010 SE single quadrupole mass spectrometer (Shimadzu, Japan). Sample (0.5 μ L) was injected on a BP5MS (5% phenyl polysilphenylene-siloxane) capillary column (length 30 m, thickness 0.25 mm, diameter 0.25 mm) in the split mode with a split ratio of 1:60. The gradient temperature started from 60°C and was held for one minute, followed by a linear increase of 10°C/min to 320°C. Then, it was kept at 320°C for 4 minutes. The carrier gas (helium) flow rate was set at 40 cm/sec. Mass spectra analysis was performed using electron impact ionization (EI) at 70eV with an ion-source temperature of 200°C, an interface temperature of 320°C and an injection temperature of 280°C. The ionization voltage was kept constant at 70 eV, which

results in reproducible fragment ions, further aiding in smooth identification of metabolites, after comparing to reference libraries available (Babushok et al., 2007). Data were collected between mass to charge (m/z) 50-600 Da in SCAN mode. QC samples made from pooled corresponding samples were injected periodically.

4.3.6 *Data processing and metabolite identification*

The raw GC-MS data generated was converted to mzXML using the GC-MS Postrun Analysis software (Shimadzu, Japan). The converted files were further processed for peak picking (signal to noise threshold = 5) and retention time correction using the XCMS package in R. All semi-quantification was done using raw peak areas of selected features normalized to that of IS.

Metabolite identification was done by comparing the GC-MS fragmentation mass spectra to those found in the National Institute of Standards and Technology (NIST) database. Identification of metabolites was confirmed by comparing to pure standards, when required. Pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999).

4.3.7 *Statistical analysis*

SIMCA (Umetrics, Sweden) was used for multivariate analysis (MVA). Principle component analysis (PCA) was done on combined data from both phases for analysis of QC clustering, followed by orthogonal partial least squares-discriminant analysis (OPLS-DA), after Pareto scaling and normalization, when required, for the plasma and mesencephalon separately. The resulting S-plots of each were used for feature selection with appropriate thresholds.

Following feature selection, univariate analysis was performed on semi-quantified data using GraphPad Prism 6 (GraphPad, U.S.A). The p values obtained from Mann-Whitney tests underwent Benjamini and Hochberg correction, using q value < 0.05 as significant. Univariate analysis on all treatment groups was done using Kruskal-Wallis test, followed by Dunn's multiple comparison *post-hoc* test.

Receiver operating curve analysis and Spearman's correlation tests were performed using GraphPad Prism 6. *Post-hoc* power analysis was performed in R with package "pwr". Heat map cluster analysis was performed in R with package "gplots".

All data is expressed at mean $\pm$ S.D, of at least four rats in each group. The q values and number of independent rats in each treatment group are indicated in each figure legend.

4.4 Results

4.4.1 Metabolomic method validation and feature selection

The metabolomics workflow is described in Figure 4.1. To assess the reproducibility of the analytical method, PCA plots were used to illustrate clustering of quality control (QC) samples in both the plasma, (Figure 4.2A) and midbrain (Figure 4.2B). QC samples clustered together, showing good repeatability of the analytical method used. Up to 1,500 metabolic features in the plasma and 2,500 metabolic features in the mesencephalon regions were obtained by using this method, respectively. OPLS-DA multivariate analysis (MVA) was then applied to determine which metabolic features were the strongest discriminators between the 6-OHDA and sham groups. Since the lesion was unilateral, for mesencephalon

tissues, the metabolite levels on the lesioned side were normalized to the intact side and these ratios were subsequently used for OPLS-DA plots. This was done to compare metabolite imbalance in the midbrain caused by 6-OHDA.

OPLS-DA plots were built for both the plasma (Figure 4.3A) and mesencephalon (Figure 4.3B) tissues after excluding features with variable importance on projection (VIP) values < 1 and showed a significant separation between the 6-OHDA and sham groups. S-plots based on the OPLS-DA model were then used to select features that could contribute to this separation. Thresholds of $p[\text{corr}]$ as > 0.4 , < -0.6 and covariance $(p[1])$ as > 0.2 , < -0.2 , for the plasma, and $p[\text{corr}]$ as > 0.5 , < -0.6 and covariance $(p[1])$ as > 0.06 , < -0.06 for the midbrain were applied subsequent to which 16 metabolic features were selected (4 from plasma tissues, and 12 from midbrain). While higher $p[\text{corr}]$ values indicate a high reliability for prediction, higher $(p[1])$ values represent features with a higher influence on the model. After comparing to National Institute of Standards and Technology (NIST) library, 13 features were successfully identified with a similarity index $> 85\%$, two were identified as sugars and one remained unknown.

4.4.2 *Univariate analysis of selected features from plasma and brain*

Univariate analysis based on semi-quantified data was then performed for these 16 features using the Mann-Whitney test. Six features (two from the plasma, and four from the brain) showed a significant difference between the groups after Benjamini-Hochberg correction (Table 4.1). The two plasma metabolite features, which were identified as palmitic acid and stearic acid (similarity index $> 90\%$), were significantly upregulated in 6-OHDA group, compared to the sham ($q = 3.72 \times 10^{-2}$ for palmitate and $q = 3.84 \times 10^{-2}$ for stearate) (Figure 4.4). *Post-hoc* power

analysis yielded a statistical power of 93.2% for palmitic acid and 86.5% for stearic acid.

From the mesencephalon, all four metabolite features presented lower levels in the 6-OHDA group compared to the sham (Figure 4.5). Three of the four features were identified as mono-palmitin ($q = 4.8 \times 10^{-2}$), mono-stearin ($q = 3.72 \times 10^{-2}$) and myo-inositol ($q = 3.81 \times 10^{-2}$), while one (221_8.15) remained unidentified ($q = 3.66 \times 10^{-2}$). Mono-palmitin and mono-stearin had a similarity index of more than 90%, while myo-inositol was 88%. To further confirm the identification of myo-inositol, a 50 µg/ml standard was used, and the retention times and mass to charge (m/z) of fragments were compared (Figure 4.6). After confirming the identification, the corresponding levels of these four metabolites were measured in a similar manner in the cerebellum tissues, to account for region specific disturbance. Ideally, the cerebellum should not be affected by 6-OHDA lesions in the medial forebrain bundle. The MFB contains bundles of nerve fibres connecting areas of mesencephalon, to the forebrain and hypothalamus (Nieuwenhuys et al. 1982). After normalizing lesioned side metabolites to the intact side levels for the cerebellum, it was observed that none of the four metabolites were significantly changed (Figure 4.5). These results further confirmed that the changes observed were specific to the midbrain and indeed induced by 6-OHDA. *Post-hoc* power analysis revealed that the two monoglycerides showed a statistical power below 80% which is the most commonly used cut-off point, while myo-inositol had a statistical power of 97.4% and the unknown 98.2%.

4.4.3 Receiver operating characteristic (ROC) curve and correlation of plasma and midbrain features with behaviour tests

Spearman's correlation was done to gauge the relationship between the levels of all five features and the motor impairment seen with the behaviour tests (Section 3.4.4). Plasma palmitic acid showed a high positive correlation ($r = 0.552$, $p = 0.029$) with the intensity of the lesion (apomorphine test score) and was also highly correlated ($r = 0.674$, $p = 0.007$) with rigidity in the contralateral fore-limb movement, as seen by the asymmetric cylinder test. Stearic acid also had a high positive correlation with the apomorphine test ($r = 0.512$, $p = 0.044$) and asymmetric cylinder test ($r = 0.649$, $p = 0.011$). Both acids showed better correlation to the asymmetric cylinder test (Table 4.2).

To then elucidate the predictive abilities of the two fatty acids a receiver operating characteristic (ROC) curve was built for both (Figure 4.7). Both palmitic and stearic acid had an area under the curve (AUC) of > 0.8 , which indicates good predictive ability. ROC curve analysis also indicated that palmitic acid and stearic acid had a high specificity (0.88 for both).

On the other hand, amongst the three mesencephalon features, only normalized mono-palmitin showed moderate significant negative correlation with the apomorphine ($r = -0.679$, $p = 0.02$) and asymmetric cylinder score ($r = -0.578$, $p = 0.042$), suggesting it was the most associated with the intensity of the disease (Table 4.3). While all other mesencephalon metabolites were negatively correlated, they were not significant with either behavioural score.

To further assess if any of the brain metabolites showed predictive abilities, I also built a ROC curve for these features (Figure 4.8). It was observed that mono-

stearin, myo-inositol and the unknown (221_8.15) exhibited an AUC > 0.9, indicating excellent predictive ability. Mono-stearin showed sensitivity = 1 and specificity = 0.8, while myo-inositol had sensitivity = 0.75 and specificity = 0.83. Unknown (221_8.15) showed the most promise in this regard, with an AUC of 1, sensitivity = 1 and specificity = 1.

4.4.4 *Comparison of plasma and brain metabolite features*

Since the two monoglycerides changed in the midbrain had the same side chains as the fatty acids in the plasma, I next examined if these changes were associated. Correlation tests were done between palmitic acid in the plasma with normalized mono-palmitin in the midbrain and plasma stearic acid with midbrain mono-stearin. However, these metabolites were not significantly correlated, indicating the changes may not be directly linked as a one-step transformation (Table 4.4). To confirm further, I measured the significant plasma feature levels in the midbrain. It was observed that normalized palmitic acid and stearic acid levels remained unchanged in the midbrain between the two groups (Figure 4.9). Similarly, the levels of mono-palmitin, mono-stearin and myo-inositol also remained unchanged in the plasma of control versus 6-OHDA-lesioned rats (Figure 4.10).

4.4.5 *Corresponding changes of selected metabolites in the liver*

Palmitic acid and stearic acid are diet derived fatty acids, which was kept constant for all the rats. Therefore, I hypothesized that changes in the plasma levels of these fatty acids and their corresponding monoglycerides might be because of metabolic changes in the liver. To confirm this, I performed semi-targeted GC-MS

analysis on homogenized liver samples of each of the rats in the 6-OHDA and the sham groups.

The same univariate approach was applied to liver palmitic acid, stearic acid, mono-palmitin and mono-stearin levels. The levels of these four metabolites remained unchanged between the 6-OHDA and the sham groups (Figure 4.11). This indicates that the liver might not play a direct role in the upregulation of plasma fatty acids or brain monoglyceride imbalance and that changes in the plasma and the brain might be due to localized mechanisms.

4.4.6 Effects of drug treatments on plasma and midbrain metabolites

My final aim was to assess the effects of drug treatments on metabolites changed in the 6-OHDA group. The levels of fatty acids in the plasma, and the four metabolites in the midbrain were semi-quantified and measured in the 1 mg/kg OXY, 10 mg/kg OXY and 1 mg/kg RES groups. It was observed that 1 mg/kg OXY was effective in restoring both plasma fatty acid levels (Figure 4.12), while RES was partially successful. 10 mg/kg OXY did not show significant changes. On the other hand, both doses of OXY successfully maintained the balance of unknown 221_8.15 in the midbrain (Figure 4.13). Interestingly, both drugs did not seem to show any significant changes in the levels of midbrain mono-palmitin, mono-stearin or myo-inositol (Figure 4.13). These results were consistent with Chapter 2 and 3, where I showed that the range of neuroprotective effects of OXY is wider than RES.

4.5 Discussion

The aim of this study was to elucidate any changes of metabolites in the most commonly used toxin-model of PD. MVA uncovered 16 metabolic features that were most altered out of 4000 between the control and 6-OHDA groups, of which two metabolites were significantly changed in the plasma and four in the mesencephalon. Palmitic acid and stearic acid were significantly increased in the plasma, while an imbalance of their monoglyceride forms in the midbrain was observed. However, their changes were not significantly correlated, further confirmed by unchanged palmitate and stearate in the midbrain. These results also suggest that the changes of metabolites in the midbrain are not directly correlated with any change in the plasma or the liver and that they could be a primary effect of 6-OHDA-induced injury. Additionally, 1 mg/kg OXY treatment was successful in preventing the elevation of both plasma fatty acids, while 1 mg/kg RES treatment succeeded partially. This finding also supports the role of these fatty acids in monitoring therapeutic effectiveness in 6-OHDA induced parkinsonism.

The significant correlations between plasma palmitic acid and stearic acid and the apomorphine - induced rotation test and the asymmetric cylinder test are of particular interest, suggesting an association with severity of symptoms. Owing to a lack of biomarkers for PD, diagnosis currently relies heavily on symptoms. One study has shown a decrease of palmitic and linoleic acid in the plasma in human subjects (Trupp et al., 2014). On the other hand, only mono-palmitin amongst all the midbrain features, showed significant correlation with behaviour tests and can be used for better understanding of midbrain pathology.

To further understand the clinical relevance of these metabolites, ROC curve analysis was performed. ROC curve is an asset in the evaluation of diagnostic

ability of tests, with AUC being an effective measurement of the accuracy. A metabolite that shows high prediction ability, must have $AUC > 0.8$, ideally with high sensitivity (fraction of the diseased group correctly identified as positive) and specificity (fraction of healthy group correctly identified as negative). Palmitic ($AUC = 0.87$) and stearic acid ($AUC = 0.85$) both showed high promise in this regard, giving further evidence of their potential as a biomarker for early diagnosis of PD. Most of the mesencephalon metabolites also showed excellent prediction abilities, except mono-palmitin having $AUC = 0.8$. Power analysis estimates the likelihood of a statistical test to reject the null hypothesis, given the alternative hypothesis is fact true. A power closer to 100% is therefore an indicator that the chances of a type II error are less. The results here suggest that palmitate, stearate, myo-inositol and the unknown (221_8.15) are good discriminators, as they all showed a power of more than 80%. Moreover, the imbalance of the unknown metabolite, was also revived by 1 mg/kg and 10 mg/kg OXY treatment only. Taken together, this study shows that these metabolites might also play an important role in monitoring treatment effectiveness when using the 6-OHDA model. ROC curves after clinical tests in humans will give a clearer indication of the role these metabolites play as diagnostic markers.

Saturated free fatty acids are released into the blood by two major pathways. Lipolysis (Conner et al., 1996) is the breakdown of fats known as triglycerides in adipose tissue to release free fatty acids and glycerol into the blood, whereas *de novo* lipogenesis (DNL) is when saturated fatty acids are synthesized from glucose and its metabolites in the liver and subsequently released into the plasma to target tissues in need. There is also evidence to show that palmitic acid may be the major product of DNL (Hellerstein et al., 1991). However, the findings here show that

there was no increase of palmitic or stearic acid in the liver, indicating DNL may not be related to the elevated levels of these two fatty acids. In addition, oxidation of fatty acids, which takes place in the mitochondria, is an important way to provide energy (Houten & Wanders, 2010). As previously mentioned, mitochondrial dysfunction has also been implicated in PD (Schapira et al., 1989). Since no changes were observed in the liver metabolites here, the increased levels of stearic acid and palmitic acid could be a consequence of impaired mitochondria in extrahepatic tissues.

Saturated free fatty acids also have detrimental effects in the context of neurological conditions. In one study, a diet rich in palmitic acid when given to mice, resulted in reduced hippocampal neurogenesis (Park et al., 2011). Additionally, a diet supplemented with palmitate induced endoplasmic reticulum (ER) stress in murine hippocampi and cortices (Marwarha et al. 2016). This study also assessed effects of palmitate on human neuroblastoma SH-SY5Y cells, which revealed an upregulation of the ER stress associated pro-apoptotic factor C/EBP-homologous protein (CHOP). This observation is of particular interest, given the role of 6-OHDA-induced ER stress studied in Chapter 2. Palmitic and stearic acid induced hyperphosphorylation of tau in rat primary cortical neurons, which was facilitated by astrocyte-induced oxidative stress (Patil & Chan, 2005). Hyperphosphorylation of tau is a hallmark of Alzheimer's disease (AD), the most common neurodegenerative disease. An increased uptake of labelled palmitate into the brain was also observed in patients with metabolic syndrome compared to that of control subjects. Elevated levels of free fatty acids in the plasma have also been linked to metabolic syndrome. Palmitic acid has been shown to induce myocardial injury in mice (Wang et al., 2017), increased blood pressure (Grimsgaard et al.,

1999) and cholesterolemia (Nestel et al., 1994) in adult males. Palmitic and stearic acid, but not oleic acid also showed a positive association with the risk for diabetes, indicated by a high correlation with adiposity, triglyceride levels and insulin resistance, in one report (King et al., 2015; Mu et al., 2001). This is particularly important given that PD affects an aging population, many of who suffer from metabolic syndrome as well.

Fatty acids are transported through the blood-brain barrier (BBB) via two major routes, either passive diffusion (Hamilton & Kamp, 1999), or facilitated by transporters (Spector, 1988). In this study, however, fatty acids in the mesencephalon did not display any significant change. This could be because fatty acids are rarely found in their free form in the brain (Gnaedinger et al., 1988). Interestingly, there is evidence of increased levels of saturated fatty acids in cortical lipid rafts of PD patients (Fabelo & Martin, 2011). This also suggests that the elevated plasma free fatty acids might affect other regions of the brain, not analysed here. The direct effects of these two upregulated fatty acids in the context of PD need to be further studied.

On the other hand, monoglycerides of palmitic acid and stearic acid had a significant difference between the lesioned and non-lesioned sides of the mesencephalon, with no corresponding changes in the cerebellum. The cerebellum was used as a control region because this region remains unaffected by injecting 6-OHDA into the MFB. Any differences in metabolites these two brain regions could thus be a primary effect of 6-OHDA on the midbrain. Monoglycerides are an intermediate product formed during the breakdown of triglycerides by lipolysis (Frühbeck et al., 2014). This process is important to compensate for an increased demand of energy. No significant changes of these two molecules were found in

the plasma or the liver, further supporting a localized imbalance of these fatty acids in the midbrain. Whether the imbalance of these metabolic features can stem from the death of dopaminergic neurons must be further assessed. 1-monopalmitin and 1-monostearin have been shown to be altered in the CSF of patients with inflammatory demyelinating disease such as multiple sclerosis, a disorder affecting nerve fibres (Park et al., 2016).

An imbalance of myo-inositol was also observed in the mesencephalon. Myo-inositol levels did not show significant changes in the plasma; confirming that disturbances of myo-inositol levels in the mesencephalon were indeed a function of 6-OHDA lesion and not related to diet. In one report, the basal ganglia of genetically predisposed PD patients also exhibited an increase of myo-inositol, analysed by MR spectroscopy (Prestel et al., 2008). Myo-inositol is also purported to be a marker of glial death (Badar-Goffer et al., 1992) making it a potential indicator of neuroinflammation in PD. It is noteworthy that monoglycerides and myo-inositol are also downstream products of a cellular signalling (IP₃-DAG) pathway (Berridge & Taylor, 1988). This pathway plays a role in facilitating release of Ca²⁺, which is important in cellular growth and synaptic plasticity.

The present findings also validate results from other studies. Lu *et al.* studied changes in metabolites in the brains of goldfish treated with MPTP. ¹H NMR-based metabolomics revealed an increase of myo-inositol and linoleic acid; further evidence that perturbations of myo-inositol and fatty acid metabolism play a role in experimental PD (Lu et al., 2014). However, in this study the brain was not dissected into different regions, and thus there is no information about the region-specific metabolite changes. In another independent study, a paraquat (PQ)-treated *Drosophila* model was used to elucidate changes of metabolites (Shukla et al.,

2016). It was observed that myo-inositol, 1 mono-palmitin, 1-mono-stearin and the fatty acids, palmitate and oleate, were increased in the fly heads. The studies described above used a bilateral model and thus reported changes of metabolites in the whole brain, unlike the metabolic asymmetry observed here.

All the metabolites revealed in this study are derived primarily from the diet. Therefore, given that all the above metabolites show pathological effects at elevated levels, monitoring the diet of PD patients to prevent further metabolic disturbances should be an important consideration.

4.6 Conclusion

In summary, this study used GC-MS based metabolomics to investigate changes in several tissues of 6-OHDA-induced PD rats. Two saturated free fatty acids, palmitic and stearic acid were upregulated in the plasma of the rats that underwent 6-OHDA lesion, which was prevented by 1 mg/kg OXY treatment. Mono-palmitin, mono-stearin, myo-inositol and an unknown metabolite with m/z 221, showed an asymmetric distribution between the ipsilateral and contralateral mesencephalon. Both doses of OXY pre-treatment were also successful in restoring the balance of the unknown metabolite in the midbrain. Changes of these metabolites may be a direct consequence of neuronal loss elicited by 6-OHDA. Understanding the nature of the unknown metabolite is warranted in the future, given its promise in predicting 6-OHDA induced damage. In addition, these two plasma fatty acids also showed a high correlation with behaviour tests, indicating their association with severity of the disease and deserve a thorough investigation with patient samples.

Figure 4.1. Workflow of metabolomics study. Workflow represents the experimental design starting from sample preparation for GC-MS analysis, feature selection using multivariate and univariate analysis, and finally feature identification.

A. **B.**

Figure 4.2. PCA plots showing clustering of QC samples. PCA plots for plasma (A) and midbrain (B), illustrating a clear clustering of the QC samples.

A.

B.

Figure 4.3. OPLS-DA score plot and S-plot for plasma and midbrain samples. OPLS-DA plots (left) showing a separation between the sham and 6-OHDA groups in the plasma (A) and midbrain (B), along with their corresponding S-plots (right) from which features were selected based on appropriate thresholds.

Metabolite	Site	p-value	q- value	Fold change
Palmitic acid	Plasma	1.76×10^{-2}	3.72×10^{-2}	1.81
Stearic acid	Plasma	2.56×10^{-2}	3.84×10^{-2}	2.15
Mono-palmitin	Mesencephalon	2.4×10^{-2}	4.8×10^{-2}	- 11.72
Mono-stearin	Mesencephalon	3.1×10^{-2}	3.72×10^{-2}	- 15.11
Myo-inositol	Mesencephalon	3.81×10^{-2}	3.81×10^{-2}	- 3.32
Unknown (221_8.15)	Mesencephalon	6.1×10^{-3}	3.66×10^{-2}	- 2.51

Table 4.1. Summary of metabolic features from S-plots that were significantly altered after univariate analysis.

Figure 4.4. Changes in saturated free fatty acids in the plasma. Palmitic acid (A) and stearic acid (B) were upregulated in the plasma of 6-OHDA-lesioned rats. Data represent mean $\pm$ S.D of at least five rats in each group (* indicates q value < 0.05 using Mann Whitney test, followed by Benjamini Hochberg correction).

Figure 4.5. Comparison of brain metabolite changes between the mesencephalon and cerebellum. Midbrain mono-palmitin (A), mono-stearin (C) myo-inositol (E) and unknown (221_8.15) (G) were significantly altered while cerebellar mono-palmitin (B), mono-stearin (D), myo-inositol (F) and unknown (221_8.15) (H) remained unchanged. Data represents mean $\pm$ S.D of at least five rats in each group (* indicates q value < 0.05 using Mann Whitney test, followed by Benjamini Hochberg correction).

A.

B.

Figure 4.6. Chromatograms and mass spectra of myo-inositol. Myo-inositol peaks (highlighted in red, top figure) with their corresponding mass spectra (bottom figure) from a midbrain sample (A) and the myo-inositol reference standard (B).

Metabolite	Behaviour test			
	Apomorphine induced rotation		Asymmetric cylinder	
	Spearman's correlation coefficient	p-value	Spearman's correlation coefficient	p-value
Palmitic acid	0.551	0.029	0.674	0.007
Stearic acid	0.512	0.044	0.649	0.011

Table 4.2. Summary of correlation results of plasma free fatty acid levels with behaviour tests.

Metabolite	AUC	p-value	Sensitivity	Specificity
Palmitic acid	0.8704 (95% C.I = 0.65-1.08)	0.0184	0.83	0.88
Stearic acid	0.8519 (95% C.I = 0.64-1.06)	0.0251	0.66	0.88

Figure 4.7. Receiver operating characteristic (ROC) curve analysis of plasma metabolites.

Metabolite (R/L ratio)	Behaviour test			
	Apomorphine induced rotation		Asymmetric cylinder	
	Spearman's correlation coefficient	p-value	Spearman's correlation coefficient	p-value
Mono-palmitin	-0.675	0.024	-0.578	0.042
Mono-stearin	-0.553	0.097	-0.439	0.164
Myo-inositol	-0.420	0.193	-0.205	0.438
Unknown (221_8.15)	-0.567	0.088	-0.263	0.393

Table 4.3. Summary of correlation results of mesencephalon metabolites with behaviour tests.

Metabolite	AUC	p-value	Sensitivity	Specificity
Mono-palmitin	0.8 (95% C.I = 0.51-1.08)	0.088	0.8	0.85
Mono-stearin	0.95 (95% C.I = 0.80-1.09)	0.027	1	0.8
Myo-inositol	0.91 (95% C.I = 0.72-1.1)	0.033	0.75	0.83
Unknown (221_8.15)	1 (95% C.I = 1-1)	0.008	1	1

Figure 4.8. Receiver operating characteristic (ROC) curve analysis of mesencephalon metabolites.

Plasma metabolite	Mid-brain mono-palmitin		Mid-brain mono-stearin	
	Spearman's correlation coefficient	p-value	Spearman's correlation coefficient	p-value
Palmitic acid	- 0.33	4.2×10^{-1}	-	-
Stearic acid	-	-	- 0.26	4.2×10^{-1}

Table 4.4. Spearman's correlation between plasma fatty acids and their corresponding monoglycerides in the midbrain.

Figure 4.9. Saturated free fatty acid levels in the midbrain remained unchanged. Palmitic acid (A) and stearic acid (B) levels were unchanged between the 6-OHDA and sham groups in the midbrain. Data represents mean $\pm$ S.D of at least five rats in each group.

Figure 4.10. Levels of monoglycerides and myo-inositol in the plasma. Mono-palmitin (A), mono-stearin (B) and myo-inositol (C) remained unchanged in the plasma of 6-OHDA versus sham groups. Data represents mean $\pm$ S.D of at least five rats in each group.

Figure 4.11. Changes in liver metabolites. Palmitic acid (A), mono-palmitin (B), stearic acid (C) and mono-stearin (D) were all unchanged in the livers of the 6-OHDA versus sham groups. Data represent mean $\pm$ S.D of at least five rats in each group.

Figure 4.12. Effects of drug treatments on plasma metabolites. Levels of palmitic acid (A) and stearic acid (B) in the 6-OHDA injected rats, after OXY and RES treatment. Data represent mean $\pm$ S.D of at least five rats in each group (* indicates p value < 0.05, compared to sham + vehicle and # indicates p < 0.05, ## indicates p < 0.01, compared to 6-OHDA + vehicle, using Kruskal-Wallis test, followed by Dunn's *post-hoc* test).

Figure 4.13. Effects of drug treatments on midbrain metabolites. Levels of mono-palmitin (A), mono-stearin (B), myo-inositol (C) and unknown 221_8.45 (D) in the 6-OHDA injected rats, after OXY and RES treatment. Data represent mean $\pm$ S.D of at least five rats in each group (* indicates p value < 0.05 , compared to sham + vehicle and # indicates $p < 0.05$, ## indicates $p < 0.01$, compared to 6-OHDA + vehicle using Kruskal-Wallis test, followed by Dunn's *post-hoc* test).

Figure 4.14. Heat map showing the fold change of metabolites between the sham and 6-OHDA groups within the different tissues. Similarly changed metabolites are clustered together, while tissues with similar changes in metabolites are clustered. Significantly changed metabolites are marked for each tissue (* indicates $p < 0.05$ after Benjamini-Hochberg correction, compared to sham).

Figure 4.15. Summary of region specific metabolite changes. Arrows indicate significantly increased or decreased metabolites in the midbrain and plasma of the 6-OHDA treated rats, while those metabolites remained unchanged in the liver.

CHAPTER 5

Oxyresveratrol modulates metabolic pathways to exert neuroprotection against 6-OHDA induced Parkinsonism

5.1 Introduction

Of the current treatment options for Parkinson's disease (PD), none can slow down or halt progression of the disease. The primary hindrance in developing more therapeutic candidates, is attributed to the wide range of pathological pathways involved in PD progression. Additionally, damage to the substantia nigra *pars compacta* (SNpc) occurs years before symptoms manifest, in turn making diagnosis difficult. By the time therapeutic intervention put in place, this damage is too extensive to be reversed. Therefore, there is a dire need for therapeutic strategies capable of delaying the onset and/or severity of the disease; alternative dietary interventions are now under investigation for this purpose. Metabolomics has emerged as a successful tool in translational medicine, a key application of which is identifying small molecules involved in disease progression and thereby, therapeutic intervention. In the context of neurodegeneration alone, several metabolic pathways that are modulated by drug candidates have been identified, indicating their mechanism of action.

An extract of *Acanthopanax senticosus* Harms (EAS), a traditional Chinese medicine, was administered to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -treated mice for 20 days, in a study by Li *et al* (Li *et al.*, 2013). It was found that EAS showed protective effects against a host of metabolic pathways

altered by MPTP. LC-MS based metabolomics suggested the role of L-3,4-dihydroxyphenylalanine (L-dopa), methionine metabolism, fatty acid oxidation and tyrosine metabolism as protective mechanisms of EAS. Their study was then extended to a familial α -synuclein (α -syn) model of PD. When wild-type (WT) and A30P- α -syn transgenic mice were treated with EAS, it was observed that ubiquinone, taurine and prostaglandin metabolism were linked with the mechanism of EAS in the A30P mutant. However, the A30P mutant also had defects in pyruvate, amino acid and biotin metabolism (Zhang et al., 2015). Altered immune function is also associated with PD pathology (Mosley et al., 2012). Gendelman *et al* used metabolomics to understand better the effects of sargramostim, an immunomodulator, in PD versus control patients. Serum samples indicated modified levels of serotonin, L- kynurenine and quinolic acid associated with sargramostim treatment (Gendelman et al., 2017). Metabolomics can also be used to assess secondary effects of drug treatments, such as in the case of L-dopa. The most adverse effect of L-dopa treatment is the onset of dyskinesia in later stages (Bezard et al., 2001). In a clinical study applying Liquid chromatography-Mass spectrometry (LC-MS) based metabolomics, it was found that higher 3-hydroxykynurenine/kynurenic acid ratios in the plasma were highly associated with L-dopa induced dyskinesia (Havelund et al., 2017).

Therefore, we see that metabolomics is an emerging tool for more in-depth analysis of prophylactic candidates for PD. This is especially useful for nutraceuticals, which as dietary supplements can affect a variety of metabolic pathways in the body. Identifying pathways altered by such candidates in experimental PD, can pave the way for future strategies against neurodegeneration. Additionally, I hypothesized that the protective effects of OXY observed in chapter

3, can stem from a modulation of small molecules, keeping biochemical pathways in check.

5.2 Aims of study

In chapter 4, a panel of metabolites that showed an imbalance in the brain after 6-hydroxydopamine (6-OHDA) lesion were discovered. However, oxyresveratrol (OXY) only protected levels of the unknown metabolite. The primary aim of this study was to combine reversed-phase (RP) LC-MS and Gas chromatography-Mass spectrometry (GC-MS) based metabolomics to uncover a wider panel of metabolites. For the animal study, rats were given OXY exposure one week before 6-OHDA injection (Figure 3.2). Therefore, any modulation of metabolic pathways resulting in neuroprotection induced by OXY, would appear in the entire brain, not just the lesioned side. Therefore, in this chapter I looked at total brain changes. Plasma samples were used as an additional measure. Based on results from Chapter 4, the plasma is also helpful in identifying altered metabolites in PD and shows translational potential.

In the first part of the study, metabolic pathways associated with 6-OHDA mediated toxicity was identified from the plasma and total midbrain. The effects of OXY and resveratrol (RES) treatments on these metabolites was also determined, to compare their effects.

In the second part of the study, metabolites changed in the OXY group were determined in the total midbrain and plasma. These metabolites will give deeper insights into the specific neuroprotective mechanisms of OXY. Changes induced specifically by 1 mg/kg OXY were determined, as it is evident from Chapter 2, 3

and 4 that very low doses of OXY are the most effective. The effects of 10 mg/kg OXY and 1 mg/kg RES on these metabolites were also determined, to gauge specificity.

5.3 Materials and methods

5.3.1 Study design

Gas chromatography - Mass spectrometry (GC-MS) and Liquid chromatography - Mass spectrometry (LC-MS) based metabolomics was used for this part of the study. Plasma, midbrain and cerebellum tissues from all experimental groups of the animal study were collected. All the tissue samples were homogenized, and the aqueous and non-aqueous phases were separated by in vial dual extraction (IVDE). Raw data from the GC-MS and LC-MS runs was then combined for the plasma and midbrain separately and subjected to multivariate analysis. The cerebellum was used as a control region. Multivariate analysis was now used to pick out metabolic features driving not only 6-OHDA-induced toxicity, but the mechanism of OXY mediated neuroprotection as well. Semi-quantification followed by univariate analysis was then performed on any such metabolic features obtained to assess their levels in all the treatment groups. Significant features were identified. To analyse some of the pathways even further, semi-targeted hydrophilic interaction liquid chromatography (HILIC) separation was subsequently used.

All materials were purchased from Sigma Aldrich (United Kingdom), unless stated otherwise.

5.3.2 *Tissue harvest*

Plasma and brain tissues were harvested as described in section 4.3.2.

5.3.3 *Sample extraction*

Sample extraction was performed as described in section 4.3.3.

5.3.4 *GC-MS analysis*

Derivatization and GC-MS analysis was performed as described in section 4.3.4 and section 4.4.5, respectively.

5.3.5 *LC-MS analysis (RP/non-aqueous phase)*

Analysis was completed using a Waters ACQUITY UPLC® and a Waters Xevo® QTOF system (Waters, USA). About 5 µl of sample extracts were separated on an Agilent Poroshell 120 EC-C8 column (150mm × 2.1mm, 2.7 µm particle size). Separation was performed at 55 °C with a flow rate of 0.5 mL/min. A gradient was applied using 10 mM ammonium formate in water (mobile phase A) and 10 mM ammonium formate in methanol (mobile phase B). For analysis in the positive mode, the gradient started at 80% mobile phase B increasing linearly to 96% B in 23 minutes and was held until 45 minutes. Then the gradient was increased to 100% by 46 minutes until 49 minutes. Initial conditions were restored in two minutes ahead of a seven-minute column re-equilibration. For analysis in the negative ionisation mode the gradient started at 75% B increasing linearly to 96% B at 23 minutes, then increasing further to 100% B by 35 minutes, initial conditions were restored to allow seven minutes of column re-equilibration.

Mass spectral data was acquired between 50–1000 Da in both positive and negative ionisation modes. In both modes, all analyses were acquired in LockSpray mode

to ensure accuracy and reproducibility. Leucine-enkephalin was used as a lock mass (m/z 556.2771, m/z 278.1141) at a concentration of 500 ng/mL and a flow rate of 10 μ L/min. In the positive mode, a capillary voltage of 3.2 kV and a cone voltage of 45V was applied. The desolvation gas flow was set to 400 L/hour and the source temperature was 120°C. In the negative mode, the capillary voltage and the cone voltage were 2.6 kV and 45 V, respectively. Desolvation gas flow and source temperature were fixed at 800 L/h and 350°C. Data were collected in the centroid mode with an acquisition time of 0.1 second a scan.

5.3.6 LC-MS analysis (HILIC/aqueous phase)

Analysis was completed using a Waters ACQUITY UPLC® and a Waters Xevo® QTOF system (Waters, USA). About 5 μ L of sample extracts were separated on a Merck Sequant Zic-HILIC column (150mm $\times$ 4.6mm, 3 μ m particle size) coupled to a Merck Sequant guard column (20mm $\times$ 2.1mm). Separation was performed at room temperature with a flow rate of 0.3 mL/min. A 40-minute gradient was applied using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The gradient started at 80% mobile phase B, followed by a linear reduction to 20% mobile phase B after 30 minutes. Then initial conditions were restored to allow 10 minutes of column re-equilibration.

Mass spectral data was acquired between 50–1000 Da in both positive and negative ionisation modes. In both modes, all analyses were acquired in LockSpray mode to ensure accuracy and reproducibility. Leucine-enkephalin was used as a lock mass (m/z 556.2771, m/z 278.1141) at a concentration of 500 ng/mL and a flow rate of 10 μ L/min. In the positive mode, a capillary voltage of 3.2 kV and a cone voltage of 45 V was applied. The desolvation gas flow was set to 400 L/hour and the source

temperature was 120°C. In the negative mode, the capillary voltage and the cone voltage were 2.6 kV and 45 V respectively. Desolvation gas flow and source temperature were fixed at 800 L/h and 350°C. Data were collected in the centroid mode with an acquisition time of 0.1 second a scan.

5.3.7 *Data processing and metabolite identification*

The raw GC-MS data generated was converted to mzXML using the GC-MS Postrun Analysis software (Shimadzu, Japan). Raw LC-MS data was converted to NetCDF format using the MassLynx software (Waters, USA). The converted files were further processed for peak picking (signal to noise threshold = 5) and retention time correction using the XCMS package in R. Processed data were then combined for the positive and negative modes for LC-MS (non-aqueous phase). Additionally, the processed LC-MS and GC-MS data were combined for the plasma and midbrain respectively, for further statistical analysis. All semi-quantification was done using raw peak areas of selected features normalized to that of IS.

Metabolite identification was done by comparing the GC-MS fragmentation mass spectra to those found in the National Institute of Standards and Technology (NIST) database. Identification of metabolites was confirmed by comparing to pure standards, when required. LC-MS feature identification was done by comparing fragmentation data to the Human Metabolome Database (HMDB) (Wishart et al., 2009) and Metlin (The Scripps Research Institute, U.S.A) (Smith et al., 2005) databases. Pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999).

5.3.8 Statistical analysis

SIMCA (Umetrics, Sweden) was used for multivariate analysis (MVA). Principle component analysis (PCA) was done on combined data from both phases for analysis of QC clustering, followed by orthogonal partial least squares-discriminant analysis (OPLS-DA), after Pareto scaling and normalization, when required, for the plasma and mesencephalon separately. The resulting S-plots of each were used for feature selection with appropriate thresholds.

Following feature selection, univariate analysis was performed on semi-quantified data using GraphPad Prism 6 (GraphPad, U.S.A). The p values obtained from Mann-Whitney tests underwent Benjamini and Hochberg correction, using q value < 0.05 as significant. Univariate analysis on all treatment groups was done using Kruskal-Wallis test, followed by Dunn's multiple comparison *post-hoc* test.

Spearman's correlation tests were performed using GraphPad Prism 6. Correlation matrix was performed using Spearman's correlation in R using package "corrplot". Heat map cluster analysis was performed in R with package "gplots".

All data is expressed at mean $\pm$ S.D, of at least four rats in each group. The p values and number of independent rats in each treatment group are indicated in each figure legend.

5.4 Results

5.4.1 Method validation and multivariate analysis

The metabolomics workflow is shown in Figure 5.1. Reproducibility of the GC-MS technique was already assessed in Chapter 4. To further assess analytical

variation in the LC-MS analysis, PCA plots for the brain and plasma samples were built separately. The pooled quality control (QC) samples in the plasma (Figure 5.2A) and midbrain (Figure 5.2B) clustered well, indicating good repeatability of the LC-MC runs. After combining the processed data from both the analytical methods, for the midbrain and plasma separately, multivariate modelling was used for feature selection. Based on this combined approach, up to 4575 and 6912 metabolic features were separated in the brain and plasma respectively.

My first aim was to identify which features were altered in the midbrain and plasma after 6-OHDA injection, and whether the drugs had any effects on those pathways. Therefore, OPLS-DA plots were built between the sham + vehicle and 6-OHDA + vehicle groups for the plasma and midbrain. The midbrain plot showed better predictive ability (Q^2 value) than the plasma. The corresponding S-plot was then used to select features driving these separations, using features with variable importance on projection (VIP) values > 1 . Based on the thresholds of ($p[\text{corr}]$) as > 0.4 , < -0.4 and covariance ($p[1]$) as > 0.15 , < -0.15 , S-plots gave a total of 5 features in the plasma (Figure 5.3). Based on thresholds of ($p[\text{corr}]$) as > 0.6 , < -0.6 and covariance ($p[1]$) as > 0.1 , < -0.1 , S-plots gave 15 features in the midbrain (Figure 5.4).

To subsequently identify further therapeutic targets of OXY treatment, OPLS-DA plots between the 6-OHDA + vehicle and 1 mg/kg OXY + vehicle groups were built, again using VIP values > 1 . The midbrain plot here also showed better predictive ability (Q^2 value) than the plasma. Based on the thresholds of ($p[\text{corr}]$) as > 0.4 , < -0.4 and covariance ($p[1]$) as > 0.15 , < -0.15 , S-plots gave a total of 4 features in the plasma (Figure 5.5). Using thresholds of ($p[\text{corr}]$) as > 0.6 , < -0.6

and covariance ($p[1]$) as > 0.15 , < -0.15 , S-plots gave 6 features in the mesencephalon (Figure 5.6).

5.4.2 *Univariate analysis and metabolite identification (sham + vehicle vs. 6-OHDA + vehicle)*

Univariate analysis was done on the semi-quantified data, to pick out S-plot features that were significantly altered. A total of six metabolites changed between these two groups, after applying Benjamini-Hochberg correction; five from the midbrain and one from the plasma (Table 5.1).

Annotation of GC-MS feature: The mass to charge (m/z) of this midbrain feature was compared with the National Institute of Standards and Technology (NIST) library and identified as lactic acid, with a similarity index of $> 90\%$.

Annotation of LC-MS features: The m/z derived from the S-plots, were used to pull up candidate metabolites from Human Metabolome Database (HMDB) library. All the fragmentation peaks (from MS2 data), with a similar elution profile as the respective m/z of interest were first determined. Similar elution profiles would indicate these fragment peaks were daughter ions of the m/z in question. Subsequently, the obtained fragmentation patterns were compared with spectral patterns of the candidate metabolites from the database. Based on this method, two features from the midbrain were putatively annotated as phosphatidylinositols (PI), and two as diglycerides (DG) (Table 5.2). LP 928.59_15.93 was putatively PI (40:6) ($q = 3.5 \times 10^{-2}$), LP 887.56_16.05 was putatively PI (38:4) ($q = 4.5 \times 10^{-2}$), LP 612.56_19.7 was putatively DG (16:0/18:1) ($q = 3.6 \times 10^{-2}$) and LP 640.58_21.18 was putatively DG (18:0/18:1) ($q = 4.8 \times 10^{-2}$). The exact side chains for the two PIs could be determined. The spectrum for LP 819.66_17.43 showed fragments ion

with m/z 526.52, eluting from the same peak. However, after comparing with the database, plasma feature LP 819.66 could not be annotated.

5.4.3 *Effects of drugs on the altered metabolites*

To investigate if OXY or RES could keep any of the above metabolites intact, their levels were semi-quantified in the 6-OHDA +1 mg/kg OXY, 6-OHDA +10 mg/kg OXY groups and 6-OHDA +1 mg/kg RES groups.

It was observed that 1 mg/kg OXY significantly reduced the levels of PI (40:6), compared to the 6-OHDA + vehicle group (Figure 5.7), but did not have significant effects on the diglycerides (Figure 5.8). 1 mg/kg OXY also prevented lactic acid level changes in the midbrain (Figure 5.9A). RES did not have any effect on PI (40:6) or lactic acid levels. Additionally, all these metabolites remained unchanged in the cerebellum after univariate analysis, indicating that the changes were specific to 6-OHDA pathology induced in the MFB. Interestingly, only RES significantly protected alterations of the plasma feature LP819.66, with OXY having no such effect (Figure 5.9C).

I then aimed to investigate some of these pathways even further. Lactic acid is related to energy metabolism and is a by-product of pyruvate. Thus, pyruvic acid and glucose in the midbrain and 3-hydroxybutyric acid in the plasma were also measured. Pyruvic acid was analysed from the hydrophilic interaction liquid chromatography (HILIC) data, in the negative mode, and annotated based on an in-house database. Glucose and 3-hydroxybutyric acid were measured from the GC-MS data. The glucose peak was confirmed after running a 50 $\mu\text{g/ml}$ glucose reference standard. It was observed, however, that there was no change between the levels of these three metabolites between the sham + vehicle and 6-OHDA + vehicle

groups (Figure 5.10). To further investigate if the changes in energy metabolism could stem from mitochondrial dysfunction, I measured the levels of intermediates of the tricarboxylic acid (TCA) cycle. Impaired mitochondrial function is long known to be associated with PD (Schapira et al., 1989). Nevertheless, citric acid, malic acid and succinic acid, measured from the GC-MS data, showed no changes between the sham + vehicle and 6-OHDA + vehicle groups (Figure 5.11), suggesting that changes in levels of lactic acid could be linked to an alternative energy related pathway or enzyme dysfunction.

5.4.4 *Univariate analysis and metabolite identification (6-OHDA + vehicle vs. 1 mg/kg OXY + vehicle)*

Univariate analysis showed three features significantly changed between the 6-OHDA + vehicle and 1 mg/kg OXY + vehicle groups, after applying Benjamini-Hochberg correction (Table 5.3). Two features were from the midbrain, and one from the plasma. All three features were based on the GC-MS data.

Annotation of GC-MS features: m/z of the features were compared with the NIST library. The two midbrain features were identified as alanine and gamma-Aminobutyric acid (GABA), with a similarity index of > 90%. Interestingly, the plasma feature was also identified as alanine. The levels of these features were then measured in all treatment groups but 10 mg/kg OXY and 1 mg/kg RES treatment showed no effects on midbrain alanine (Figure 5.12A), plasma alanine (Figure 5.12C), or midbrain GABA (Figure 5.13A).

These three features were not significantly altered between the sham + vehicle and 6-OHDA + vehicle groups. To ensure that these metabolite changes induced by 1 mg/kg OXY treatment, were indeed protective against 6-OHDA induced damage,

their levels were also measured in the cerebellum (Figure 5.12B, 5.13B). OXY did not alter these metabolites in the cerebellum, indicating these effects were specific to the midbrain alone; the site of 6-OHDA pathology.

GABA is an inhibitory neurotransmitter that also plays a role in basal ganglia circuitry (Fisone et al., 2007). I then proceeded to investigate whether the effects of 1 mg/kg OXY treatment seen on GABA, could be extended to related neurotransmitters. The levels of glutamate and glycine were therefore measured subsequently. However, no changes were observed in glutamic acid (Figure 5.14A) or glycine levels (Figure 5.14B) between any of the groups.

5.4.5 *Correlation of metabolites and behaviour test scores after OXY treatment*

My next aim was to elucidate the relationship between the metabolite changes induced by 1 mg/kg OXY and its effects on motor function (Section 3.4.4). This would give insights into whether the metabolite changes observed after OXY treatment are related to its protective effects on locomotor impairment. A correlation matrix between the levels of metabolites and behaviour test scores after OXY treatment was built for this purpose (Figure 5.15). The levels of plasma and midbrain alanine, lactic acid or GABA, did not seem to correlate significantly with any of the behaviour test scores. However, it was interesting to note that levels of midbrain lactic acid, showed significant positive correlation with midbrain alanine ($r = 0.9$, $p = 8 \times 10^{-4}$) and midbrain GABA ($r = 0.81$, $p = 4.8 \times 10^{-2}$). To understand the relationship between these metabolites further, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Ogata et al., 1999) was performed, which revealed an association of all three of these metabolites with energy metabolism. A

modulation of this pathway could be the mechanism of action of OXY, in the context of 6-OHDA induced Parkinsonism.

5.5 Discussion

In this chapter, a combination of GC-MS and LC-MS based metabolomics uncovered a diverse set of metabolic pathways involved in the mechanism of OXY. Two distinct biochemical pathways were revealed as the target of OXY-mediated protection. Metabolomics was performed on midbrain and plasma tissues, to find metabolites associated with the primary site of damage as well as subsequent changes in the plasma that can potentially affect other tissues. Two multivariate models were used in the study to parse metabolites of interest: one between the sham and diseased group, to assess disturbances in metabolic pathways induced by 6-OHDA and the subsequent effects of OXY, and one between the 1 mg/kg OXY treated and diseased group, to further discover targets of OXY.

While PI (40:6) and PI (38:4) increased in the midbrains of 6-OHDA treated rats, DG (16:0/18:1), and DG (18:0/18:1) were reduced. 1 mg/kg OXY treatment prevented accumulation of PI (40:6). OXY also significantly prevented the build-up of lactic acid seen in the midbrain of the 6-OHDA treated rats, while reducing levels of related metabolites: the amino acid alanine, and neurotransmitter GABA. Furthermore, a significant degree of correlation was observed between these three metabolites in the midbrain of the 1 mg/kg OXY treated PD rats. None of these effects were not observed in the cerebellum, indicating their specificity to the 6-OHDA-lesioned MFB.

Phosphoinositides, or the phosphorylated form of phosphatidylinositols are a class of lipids mediating cellular signalling, found in cellular membranes (Falkenburger et al., 2010). One report shows that PIs account for up to 20% of total cellular lipids (Balla, 2013). Here, I found two PIs increased in the entire mesencephalon after 6-OHDA injection. Studies have shown that PI (38:4), presumably with acyl side chains 18:0 and 20:4, is the most abundantly present PI in the rat brain (Traynor-Kaplan et al., 2017) and other tissues (Hicks et al., 2006), while other PI species, such as PI (40:6) contribute to a smaller proportion (Hicks et al., 2006). The finding that PI (38:4) increased after 6-OHDA injection is therefore worth investigating further. OXY was successful in protecting the total PI (40:6) content, and only partially protected PI (38:4) alterations. This might imply that while OXY does have the ability to partially reverse disruptions within this signalling pathway; it is more effective in restoring lipids which are present in lesser quantities.

On the other hand, OXY was not successful in restoring diglyceride levels, but showed a mild trend in bringing them back to control levels. In Chapter 4, I have also shown an imbalance of myo-inositol in this model. Together with PIs and DGs, myo-inositol is another component of the IP₃-DAG pathway (Berridge et al., 1988). PIs undergo a series of enzymatic conversions to ultimately release two secondary messengers, diacylglycerol (DG/DAG), also referred to as diglycerides, and inositol triphosphate (IP₃). IP₃ then releases free inositol, which is recycled (Berridge et al., 1988) (Figure 5.17). This pathway has implications in maintaining the cytoskeleton and cellular adhesion (Shewan et al., 2011), functioning of ion channels (Falkenburger et al., 2010) (Hille et al., 2016), intracellular calcium regulation (Michell, 1975) and cell proliferation (Sawyer & Cohen, 1981). It is possible that

due to enzyme dysfunction, the break-down of PIs to release DAGs and free myoinositol is inhibited. This would also explain a build-up of PIs and diminution of DGs, as observed here. However, this pathway in the context of PD needs to be studied further, by analysing enzymes as well as the intermediary metabolites. A further analysis of PIs and DGs with different side chains is also warranted.

The plasma lipid alteration by 6-OHDA was prevented by low dose RES only. Further analysis of the lipid will give a better indication of its role in the pathology as well as a clearly defined differential mechanism between OXY and RES.

There is some evidence to show an increase of lactic acid in tissues of PD patients. In an Nuclear Magnetic Resonance (NMR) based study, it was observed that lactic acid was significantly reduced in the cerebrospinal fluid (CSF) of PD patients (Öhman et al., 2015). Metabolomics studies on experimental models of PD have also reported similar findings. SK-N-SH cells treated with four different toxins had modified levels of lactic acid, when analysed using NMR. While paraquat (PQ) exposure led to lower levels of lactic acid, 1-methyl-4-phenylpyridium (MPP⁺), 6-OHDA and rotenone all led to increased lactic acid levels (Lei et al., 2014). In a PQ-treated *Drosophila* model, it was also observed that lactic acid accumulation increased after long term exposure, which is in line with the present study (Shukla et al., 2016).

Mechanisms of mitochondrial dysfunction induced by parkinsonian toxins been attributed to an inhibition of complex I, reactive oxygen species (ROS) production and cytochrome *c* mediated mitochondrial apoptosis (Blum et al., 2001). In this study, certain intermediary metabolites of mitochondrial respiration - succinic acid, citric acid and malic acid, remained unchanged. Ketogenesis is a

pathway upregulated during fasting conditions (Guyton & Hall, 2006), especially when the brain is deprived of glucose, whereby fatty acids are broken down in the liver to release ketone bodies like 3-hydroxybutyric acid into the plasma. Ketone bodies are then transported to extrahepatic tissues and incorporated into the mitochondrial respiratory chain (Fukao et al., 2014). However, no changes in midbrain glucose or plasma 3-hydroxybutyric acid were observed here, indicating glycolysis may not have been affected.

The elevated lactate levels observed here, could therefore be a function of either an increase in uptake of lactic acid to compensate for an increased energy demand by neurons, or a dysfunction of the enzyme lactate dehydrogenase (LDH), which is responsible for catalysing the conversion of lactic acid to pyruvic acid. Neurons can take up lactic acid via monocarboxylate transporters (MCTs) directly from the blood or from astrocytes, via the astrocyte-neuron lactate shuttle (Barros & Deitmer, 2010; Mosienko et al., 2015; Riske et al., 2017; Smith et al., 2003) (Figure 5.18). Lactic acid can then either be used in the TCA cycle or converted to alanine via the lactate-alanine shuttle, as demonstrated in glutamatergic (Waagepetersen et al., 2000) and GABAergic neurons (Zwingmann et al., 2000). Alanine aminotransferase (ALAT) is responsible for this conversion to alanine. Increased levels of lactic acid in the brain have also been associated with aging (Ross et al., 2010) and neurological conditions such as schizophrenia (Rowland et al., 2016) and panic disorder (Maddock et al., 2009). OXY was successful in restoring this energy balance in the mesencephalon and preventing a further accumulation of lactic acid, thereby also possibly protecting the mitochondria from overdrive.

Another panel of metabolites that may be involved in OXY mediated neuroprotection alone, was also investigated here. Alanine was significantly reduced in the plasma and midbrain of the 6-OHDA + 1 mg/kg OXY group. Alanine metabolism has been implicated in both animal models of PD as well as human studies. When goldfish were treated with MPTP, a significant increase in alanine levels were observed (Lu et al., 2014). In another longitudinal study on transgenic A53T mice, an increased metabolism of alanine was associated with aging (Chen et al., 2015). Shukla *et al* showed that alanine was also increased in PQ treated *Drosophila*, along with lactate metabolism (Shukla et al., 2016). Furthermore, clinical studies have shown that alanine levels are upregulated in the plasma of PD patients (Trupp et al., 2014), and possibly decreased in the CSF (Öhman et al., 2015). Although in this study, alanine levels were not altered significantly by 6-OHDA, based on the ample evidence in literature related to alanine dysfunction in PD, the ability of OXY to modulate levels of alanine, is a promising effect.

Alanine in the brain is also involved in energy metabolism, and in astrocytes is converted to lactic acid by ALAT, in order to supplement neurons (Waagepetersen et al., 2000; Zwingmann et al., 2000). This is in accordance with the present results which show a high degree of correlation between midbrain lactic acid and alanine levels. There is also evidence to show that RES inhibits ALAT in the liver (Chan et al., 2014; Faghihzadeh et al., 2015). The activity of ALAT would need to be measured in the brain, to assess the effects of OXY on it. It must also be noted that even the sham + 1 mg/kg OXY group showed reduced levels of alanine in the plasma. This inherent activity of OXY, can be particularly be exploited in cases where there is alanine dysfunction, such as PD.

1 mg/kg OXY was also effective in significantly reducing midbrain levels of the inhibitory neurotransmitter, GABA. The role of altered GABA levels has also been demonstrated in *in-vivo* models and patient samples. In *Drosophila* treated with PQ, GABA levels were significantly decreased in the midbrain (Shukla et al., 2016). There is also evidence of altered GABA in the CSF of PD patients on L-dopa therapy (Abbott et al., 1982). In this study, GABA concentrations seemed to increase in the total midbrain, albeit not significantly.

GABAergic neurons play an integral role in signalling of the basal ganglia that controls voluntary movement. They are the site for dopaminergic projections in the striatum and activate signals for initiating or preventing movements (Fisone et al., 2007). Modulation of GABA in the mesencephalon therefore might be a useful strategy to compensate for altered nigrostriatal signalling. There is also evidence supporting the synthesis of GABA from alanine in GABAergic neurons (Schousboe et al., 2003). While alanine provides an amino group for the synthesis of GABA from α -ketoglutarate in these neurons, the corresponding pyruvate enters the TCA cycle. This alanine can be derived from glucose or lactate (Sonnwald et al., 1991), further making a case for the high correlation observed between lactate, alanine and GABA concentrations in the midbrain. It is therefore possible that lowered alanine levels after OXY treatment, result in lowered GABA concentrations in the midbrain. Thus, OXY can exert neuroprotective effects via energy metabolism, not only with respect to mitochondrial proteins (Chao et al., 2008), but by modulating pathways upstream as well. An ideal neuroprotective candidate would have all these properties to effectively minimize progression of the disease.

The neurotransmitters glutamic acid and glycine are also involved in the context of energy metabolism as intermediaries of the lactate-alanine shuttle

(Waagepetersen et al., 2000; Zwingmann et al., 2000). Therefore, these two amino acids were also measured. However, no significant changes were observed with respect to these two metabolites between any of the groups. To understand the effects of OXY on the exact relationship between lactic acid, alanine and GABA shuttle, a thorough investigation of their concentrations and transport between the astrocytes and neurons must be performed. Isotopic labelling studies will further confirm whether the altered levels of all these metabolites in the mesencephalon are indeed related. All the effects seen on pathways in this study, were mediated by 1 mg/kg OXY. This is in accordance with my results in chapter 2, 3 and 4, wherein low dose OXY was also effective in reducing ER stress, motor dysfunction and metabolic disturbances caused by 6-OHDA, respectively. This study also shows an additional role of RES only, on a plasma lipid, which needs to be investigated further. It is possible that the additional effects of OXY seen on midbrain metabolites compared to RES, might be because of its increased blood-brain barrier (BBB) permeability (Breuer et al., 2006).

This study elucidates two very distinct pathways involved in 6-OHDA mediated toxicity and the effects of OXY on them. For further insights into these pathways, intermediary metabolites need to be quantified as well. Additionally, assessing the activity and expression of enzymes involved in both pathways is also necessary. Lastly, there are some discrepancies between the changes in metabolites seen here, compared to other studies. Therefore, these effects of OXY should also be validated in other PD models first, followed by clinical studies.

5.6 Conclusion

This study elucidated a panel of metabolites altered by OXY, in the plasma and midbrain of 6-OHDA treated rats. Two PIs and two DGs lipids were changed in the midbrain after 6-OHDA treatment. Of these, OXY was successful in restoring PI (40:6) levels. RES had no effects on these metabolites, but did show potential in reducing a plasma lipid, which needs further identification. Features related to energy metabolism in the brain, were also altered by low dose OXY treatment. OXY was protective against the build-up of lactic acid in the brain, while reducing levels of alanine and GABA as well. Furthermore, a high correlation was observed between these three metabolites, indicating these changes may be related. Further studies investigating these pathways in detail, will give more information about the exact role of OXY in modulating them.

Figure 5.1. Workflow of metabolomics study. The same metabolomics workflow was used for plasma and midbrain samples individually.

Figure 5.2. PCA plots of LC-MS (reverse phase, positive mode) data. PCA plots for plasma (A) and midbrain (B), indicating a clear clustering of the QC samples.

Figure 5.3. OPLS-DA score plot and S-plot for plasma samples. Plasma OPLS-DA plots (A) showing a separation between the sham + vehicle and 6-OHDA + vehicle groups ($R^2 = 0.79$, $Q^2 = 0.39$), along with their corresponding S-plots (B), from which features were selected based on appropriate thresholds.

A. **B.**

Figure 5.4. OPLS-DA score plot and S-plot for midbrain samples. Midbrain OPLS-DA plots (A) showing a separation between the sham + vehicle and 6-OHDA + vehicle ($R^2 = 0.9$, $Q^2 = 0.82$), along with their corresponding S-plots (B) from which features were selected based on appropriate thresholds.

A.

B.

Figure 5.5. OPLS-DA score plot and S-plot for plasma samples. Plasma OPLS-DA plots (A) showing a separation between the 6-OHDA + vehicle and 6-OHDA + 1 mg/kg OXY groups ($R^2 = 0.83$, $Q^2 = 0.32$), along with their corresponding S-plots (B) from which features were selected based on appropriate thresholds.

Figure 5.6. OPLS-DA score plot and S-plot for midbrain samples. Midbrain OPLS-DA plots (A) showing a separation between the 6-OHDA + vehicle and 6-OHDA + 1 mg/kg OXY groups ($R^2 = 0.99$, $Q^2 = 0.8$), along with their corresponding S-plots (B) from which features were selected based on appropriate thresholds.

Metabolite change in 6-OHDA	Analytical method	Site	p-value	q- value	Fold change	Effects of OXY	Effects of RES
Lactic acid	GC-MS	Mesencephalon	4×10^{-3}	2.4×10^{-2}	1.31	↓	-
LP 928.59_15.93	LC-MS	Mesencephalon	3×10^{-2}	3.5×10^{-2}	1.47	↓	-
LP 887.56_16.05	LC-MS	Mesencephalon	1.7×10^{-2}	4.5×10^{-2}	1.24	-	-
LP 612.56_19.7	LC-MS	Mesencephalon	3×10^{-2}	3.6×10^{-2}	- 1.27	-	-
LP 640.58_21.18	LC-MS	Mesencephalon	4.8×10^{-2}	4.8×10^{-2}	-1.25	-	-
LP 819.66_17.43	LC-MS	Plasma	6.8×10^{-3}	2×10^{-2}	1.84	-	↓

Table 5.1. Summary of metabolic features from S-plot significantly changed between the sham + vehicle and 6-OHDA + vehicle groups and the effects of drugs.

S-plot feature (m/z)	Retention time (minutes)	Annotated metabolite	Precursor (m/z)	Fragment ions (m/z)
928.59 (M + NH ₄ ⁺)	15.9	PI (40:6)	911.57 (M + H ⁺)	261.03 (C ₆ H ₁₄ O ₉ P) 651.53 (C ₄₃ H ₇₁ O ₄)
887.56 (M + H ⁺)	16.05	PI (38:4)	887.56 (M + H ⁺)	261.03 (C ₆ H ₁₄ O ₉ P) 627.55 (C ₄₁ H ₇₁ O ₄)
612.56 (M + NH ₄ ⁺)	19.7	DG (16:0/18:1)	595.56 (M + H ⁺)	339.28 (C ₂₁ H ₃₉ O ₃) 313.27 (C ₁₉ H ₃₇ O ₃)
640.58 (M + NH ₄ ⁺)	21.18	DG (18:0 /18:1)	623.56 (M + H ⁺)	339.28 (C ₂₁ H ₃₉ O ₃) 341.30 (C ₂₁ H ₄₁ O ₃)

Table 5.2. Annotation of the LC-MS midbrain features. Precursor and fragment ions used for annotation for adducts obtained from S-plots. Fragment ions for PIs indicate inositol phosphate and m/z after loss of both acyl chains. Fragment ions for DGs indicate m/z after loss of each side chain.

Figure 5.7. Effects of drug treatment on the levels of phosphatidylinositols changed between the sham and 6-OHDA groups. Changes in midbrain and cerebellar PI (40:6) (A) and (B) and PI (38:4) (C) and (D) respectively. Data represents mean $\pm$ S.D of at least five rats in each group (* indicates $p < 0.05$, compared to sham + vehicle and # indicates $p < 0.05$, compared to 6-OHDA + vehicle, using Kruskal-Wallis test followed by Dunn's *post-hoc* test).

Figure 5.8. Effects of drug treatment on the levels of diglycerides changed between the sham and 6-OHDA groups. Changes in midbrain and cerebellar DG (16:0/18:1) (A) and (B), DG (18:0/18:1) (C) and (D) respectively. Data represents mean $\pm$ S.D of at least five rats in each group (* indicates $p < 0.05$ and compared to sham + vehicle, using Kruskal-Wallis test followed by Dunn's *post-hoc* test).

Figure 5.9. Effects of drug treatment on the levels of lactic acid changed between the sham and 6-OHDA groups. Changes in midbrain and cerebellar lactic acid (A) and (B) levels respectively, along with the effects on the plasma lipid 819.66_17.43 (C). Data represents mean $\pm$ S.D of at least five rats in each group (* indicates $p < 0.05$ and ** $p < 0.01$, compared to sham + vehicle and # indicates $p < 0.05$, ## indicates $p < 0.01$, compared to 6-OHDA + vehicle, using Kruskal-Wallis test followed by Dunn's *post-hoc* test).

Figure 5.10. Effects of 6-OHDA treatment on the levels of energy substrates in the midbrain and plasma. Levels of midbrain glucose (A), midbrain pyruvic acid (B) and plasma 3-hydroxybutyric acid (C) remained unchanged between the sham + vehicle and 6-OHDA + vehicle groups. Data represents mean $\pm$ S.D of at least five rats in each group.

Figure 5.11. Effects of 6-OHDA treatment on the levels of mitochondrial respiratory intermediates in the midbrain. Levels of midbrain succinic acid (A), citric acid (B) and malic acid (C) remained unchanged between the sham + vehicle and 6-OHDA + vehicle groups. Data represents mean $\pm$ S.D of at least five rats in each group.

Metabolite	Analytical method	Site	p-value	q-value	Effects of OXY	Effects of RES
Alanine	GC-MS	Plasma	3.1×10^{-3}	9.3×10^{-3}	↓	-
Alanine	GC-MS	Mesencephalon	8×10^{-3}	4×10^{-2}	↓	-
GABA	GC-MS	Mesencephalon	1.7×10^{-2}	2.8×10^{-2}	↓	-

Table 5.3. Summary of S-plot metabolites changed significantly between the 6-OHDA + vehicle and 6-OHDA + 1 mg/kg OXY groups.

Figure 5.12. Changes in alanine metabolism between the 6-OHDA + vehicle and 6-OHDA + 1 mg/kg groups. Effects of all drugs on midbrain (A), cerebellar alanine (B) and plasma alanine (C) levels. Data represents mean $\pm$ S.D of at least five rats in each group (* indicates $p < 0.05$, ** $p < 0.01$, compared to sham + vehicle, ## indicates $p < 0.01$, compared to 6-OHDA + vehicle using Kruskal-Wallis test followed by Dunn's *post-hoc* test).

Figure 5.13. Changes in GABA metabolism between the 6-OHDA + vehicle and 6-OHDA + 1 mg/kg groups. Effects of all drugs on midbrain (A) and cerebellar (B) GABA. Data represents mean $\pm$ S.D of at least five rats in each group (# indicates $p < 0.05$, compared to 6-OHDA + vehicle using Kruskal-Wallis test followed by Dunn's *post-hoc* test).

Figure 5.14. Effects of drug treatments on the levels of related neurotransmitters in the midbrain. Levels of midbrain glutamic acid (A), and glycine (B) remained unchanged after all drug treatments. Data represents mean $\pm$ S.D of at least five rats in each group.

Figure 5.15. Correlation matrix of metabolites involved in mechanism of OXY and behaviour test scores. Midbrain lactic acid was significantly correlated with midbrain GABA and midbrain alanine (* indicates $p < 0.05$, and *** $p < 0.001$, using Spearman's correlation test).

Figure 5.16. Heat map shows changes in energy-related metabolites between the different groups. Metabolites showing a similar trend among different groups are clustered together. Treatment groups showing similar effects are clustered together. Significant metabolites are marked (* indicates $p < 0.05$, compared to sham + vehicle, # indicates $p < 0.05$ and ## $p < 0.01$, compared to 6-OHDA + vehicle).

Figure 5.17. Summary of IP₃-DAG signalling pathway and the effects of OXY. Myo-inositol (INO) enters the cells through the plasma and is incorporated into the membrane lipid, phosphatidylinositol (PI). PI then undergoes a series of phosphorylation reactions with the help of kinases (K), to yield phosphatidylinositol 4,5-bisphosphate (PIP₂). PIP₂ undergoes receptor mediated hydrolysis wherein phosphodiesterase (PD), catalyses its breakdown into the two secondary messengers: diacylglycerol (DG) and inositol triphosphate (IP₃). DG is further broken down into phosphatidic acid (PA), which is recycled, and monoglycerides (MG) after its signalling function is complete. IP₃, on the other hand, is dephosphorylated with the help of phosphatases, to yield free myo-inositol which is recycled back. Red arrows indicate increase or decrease after 6-OHDA. Red crosses indicate changes prevented by 1 mg/kg OXY treatment. Figure adapted from (Berridge et al., 1988).

Figure 5.18. Summary of energy metabolism in the brain. Lactic acid (LAC) can enter neurons either from the plasma, via the monocarboxylate transporter (MCT) or from astrocytes. LAC is then oxidised to pyruvic acid (PYR) with the help of lactate dehydrogenase (LDH) and enters the TCA cycle. PYR can also further undergo a transamination reaction to yield alanine (ALA) with the help of alanine aminotransferase (ALAT), and α -ketoglutarate (α -keto) from glutamic acid (GLUT). GLUT is also responsible for production of glycine (GLY) in the astrocytes and gamma-Aminobutyric acid (GABA) in the neurons. Red arrows indicate increase after 6-OHDA. Red crosses indicate reduced levels after 1 mg/kg OXY treatment. Figure adapted from (Waagepetersen et al., 2000).

CHAPTER 6

Conclusions and future perspectives

6.1 Conclusions

Parkinson's disease (PD) affects primarily the elderly, with a rapid disease progression resulting in a hampered quality of life. Symptoms that appear in the prodromal stage of the disease, such as constipation and hyposmia often go unnoticed. By the time motor symptoms appear and a diagnosis is possible, pathological damage is often irreversible. Furthermore, treatments currently available for PD aim to supplant lost dopamine levels and prolong its metabolism. These strategies also come with unpleasant side effects but treatment cessation results in reversal of symptomatic relief. Given these challenges with current treatments and a lack of timely intervention, the aim of this study was to investigate the potential of oxyresveratrol (OXY), a nutraceutical, in managing disease progression when administered as a prophylactic. I hypothesized that OXY mitigates pathways altered in PD.

In the first part of the study, I used two cell culture models of PD, which represented different mechanisms of endoplasmic reticulum (ER) stress. The 6-hydroxydopamine (6-OHDA) model induced ER stress by oxidative damage and represented an acute model upregulating apoptotic transcription factors. On the other hand, transfection of neurons with A30P and A53T α -synuclein (α -syn), resulted in oligomer formation and subsequent upregulation of ER chaperones. OXY was successful in mitigating ER stress in both these models, indicated by the downregulation of late and early-stage ER stress markers and A30P- α -syn

oligomerization. These effects are suggestive of its applicability in parkinsonism arising from different insults. Additionally, resveratrol (RES) was only partially protective, whereas pinostilbene (PINO) showed no effects against ER stress.

After confirming the protective effects of OXY in cellular models, I assessed the effects of OXY in an animal model of PD. Sprague-Dawley (SD) rats were exposed to OXY or RES treatment for one week prior to inducing “hemiparkinsonism” using 6-OHDA. After 6-OHDA lesion, drug treatment continued for two weeks. In this model, it was observed that OXY alleviated motor impairment and the intensity of the lesion induced by 6-OHDA. Interestingly, low doses of OXY (1 mg/kg and 10 mg/kg) were more effective in rescuing motor function, whereas all doses of OXY were more effective than RES.

Next, I attempted to uncover unknown cellular pathways implicated in this PD model. Neurodegenerative disorders are multifactorial and affect a vast array of cellular pathways. Using Gas Chromatography-Mass Spectrometry (GC-MS) based untargeted metabolomics, two distinct panels of metabolites altered in 6-OHDA-lesioned rats were discovered. Saturated free fatty acids were upregulated in the plasma and showed a very high correlation with the behavioural test scores used to assess motor symptoms. These fatty acids also showed a high prediction ability and their intensities in the plasma were kept intact in the OXY treated rats, further making a case for their high translational potential. In the midbrain, an imbalance of monoglycerides and myo-inositol was observed. The midbrain metabolite changes were site specific and might be a direct consequence of nigrostriatal damage. Lastly, an unidentified metabolite with very promising prediction ability was found to be severely altered in the lesioned rats which was prevented by OXY treatment.

In the final part of my study, I used a combined Liquid Chromatography-Mass Spectrometry (LC-MS) and GC-MS approach. This approach revealed the role of lipids in experimental PD. The phosphatidylinositols, PI (40:6) and PI (38:4) which are part of the cellular membrane, and their enzymatic products, the diglycerides, DG (16:0/18:1) and DG (18:0/18:1) were found to be altered in the mesencephalon. The combined metabolomics approach was also used to find specific modulatory targets of OXY. Lactic acid, alanine and gamma-Aminobutyric acid (GABA), metabolites related to energy metabolism in the brain, were downregulated in the OXY treated rat midbrains. These three metabolites also significantly correlated with each other, suggesting a similar pattern in their modulation. This finding highlights the importance of maintaining energy homeostasis as a neuroprotective mechanism.

This was the first comprehensive study reporting the potential of OXY as a mild supplement to prolong the damage induced in PD. OXY was protective against a range of pathological and metabolic pathways in PD, which also resulted in alleviation of motor dysfunction. These findings suggest the potential use of OXY as an additive to current treatment strategies, especially in early stages of the disease. While low doses of OXY exerted greater neuroprotection, a wider therapeutic window of OXY compared to RES, was observed. More rigorous concentration response studies are warranted, especially if OXY is considered as a supplement for daily consumption. Comparing the effects of OXY with currently used therapies such as L-dopa, as well as assessing their additive effects will give a better understanding of where OXY might stand in the treatment algorithm for PD. Preclinical and clinical studies should be used for this purpose.

6.2 Future directions

To elucidate the full potential of OXY, larger preclinical and clinical studies are warranted. Given that many pathological pathways are common in causing neurodegeneration, the role of OXY in prolonging damage in Alzheimer's and Huntington's disease is also worth evaluating. With respect the current investigation, additional studies that will aid in giving a holistic understanding of metabolomics and the effects of OXY in experimental PD have been discussed next.

6.2.1 Cell culture study

6.2.1.1 Other ER stress pathways

In this study, only the pathway downstream of PERK was studied. However, there is evidence to support the role of other ER stress pathways in PD as well (Sado et al., 2009). The other downstream pathways are also protective at early stages of ER stress and apoptotic in later stages (Calton et al., 2002; Yamamoto et al., 2007). Therefore, for a full assessment of the role of OXY in modulating ER stress, its effects on all pathways should be assessed. Furthermore, using other toxins and ER stress inducers is an additional consideration.

6.2.1.2 Translation to animal studies

While cell culture models give a clear mechanistic insight of disease, their translational capacity can only be clearly gauged in animal studies. The effects of OXY on ER stress pathways upregulated the SNpc and striatum in such models will give a better understanding of its effects.

6.2.2 Animal model and behaviour

In this study, a one-week treatment of OXY was given prior to inducing lesions. It is worthwhile to investigate the effects of a longer exposure time and

whether it elicits enhanced neuroprotection. For optimal benefits, dietary intervention should be initiated early on. Such a study will also reveal the toxicity of long term OXY treatment. Other genetic and toxin-based animal models of PD can be used to unravel the extent of OXY-mediated protection in PD.

Medial forebrain bundle (MFB) lesions result in an acute model of PD, as discussed in chapter 3. Using sites of injection such as the striatum, which depict a slowly progressing model, will uncover the full potential of OXY as a prophylactic supplement. The striatum is also affected severely by MFB lesions. Therefore, , assessing the effects of OXY in restoring the levels of dopamine and its metabolites in the striatum, can be used as an additional parameter of its neuroprotection. Parkinsonism also consists of symptoms that are non-motor in nature; the effects of OXY against cognitive decline and other non-motor symptoms will determine its neuro-protective potential at later stages of PD. Lastly, experimental models of PD do not accurately represent the entire spectrum of the disease. An accurate depiction of the neuroprotection of OXY can also be achieved with rigorous clinical evidence.

6.2.3 *Metabolomics*

Based on this study, it is evident that metabolomics can be used to successfully investigate disease mechanisms and identify therapeutic targets. There are, however, certain drawbacks that must be accounted for. As an example, every different analytical platform can detect a certain range of metabolites; in this regard, I might have not detected key molecules implicated in the disease. Furthermore, changes in the striatum were not detected in this study. Metabolic changes in the striatum can also be indicative of neuronal damage induced by 6-OHDA.

6.2.3.1 *Pathway investigation:*

To understand the role of metabolites in disease progression, intermediaries and enzymes linked to their pathways should be analysed. For this study in particular, the role of PIs, DGs and myo-inositol dysfunction can be understood better by assessing other intermediary metabolites and phosphatase/kinase activity (Figure 5.17). The exact mechanism of OXY in maintaining energy homeostasis in the brain can also be understood better by evaluating the enzymatic activity of lactate dehydrogenase (LDH) and alanine aminotransferase (ALAT). The energy pathway under question here is a shuttle between astrocytes and neurons (Figure 5.18). While there is evidence of astrocytes being implicated in PD (Teismann & Schulz, 2004), monitoring transport of these metabolites and their levels within both cell types will aid in understanding the role of OXY in this pathway.

6.2.3.2 *Clinical studies:*

To uncover the full translational potential of metabolites with high prediction ability, clinical studies are necessary for validation. Although the metabolome is well conserved between species, human studies have additional considerations such as effects of diet, age, gender and intake of drugs. Large scale human studies will determine the changes of metabolites with biomarker potential revealed here, in clinical PD cases. While cross sectional studies will aid in uncovering the potential of metabolites with high prediction ability, such as the unknown (221_8.15) in the midbrain, longitudinal studies will help determine the ability of plasma free fatty acids in monitoring disease progression.

6.2.3.3 *Metabolite identification*

Metabolite identification has always been a key challenge, especially in untargeted metabolomics, wherein thousands of metabolites with different

properties are analysed. Additionally, GC-MS spectra consist of a highly fragmented pattern, which makes identification gruelling. In chapter 4, an unidentified metabolite in the midbrain showed the best prediction ability. While such information is useful for biomarker development, understanding its nature is key for accurate diagnosis and identification in the clinic. Understanding the nature of the plasma lipid altered by RES treatment, will give insights into the differences in OXY and RES therapeutic targets. Lastly, comparing all metabolite peaks with reference standards is the most conclusive method of identification.

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List of publications

Manuscripts

Shah A., Hiew K. W., Han P., Parsons R.B, Chang R.C.C, Legido-Quigley C. (2017). Alpha-synuclein in Bio Fluids and Tissues as a Potential Biomarker for Parkinson's Disease. *Journal of Alzheimer's Parkinsonism and Dementia* (1) 1-10.

Shah A., Han P., Wong M. Y., Chang R.C.C, Legido-Quigley C. (2019). Palmitate and Stearate are increased in the plasma in a 6-OHDA Model of Parkinson's Disease. *Metabolites* 9(2) 31.

Shah A., Chao J., Legido-Quigley C, Chang R.C.C. (2019). Oxyresveratrol exerts ATF4- and Grp78-mediated neuroprotection against endoplasmic reticulum stress in experimental Parkinson's disease. *Nutritional Neuroscience* (under review).

Conference/symposium abstracts

2015

1. **Shah A.** (2015) The role of oxyresveratrol in mitigating cellular toxicity in Parkinson's disease. *Postgraduate Symposium 2015, Department of Anatomy, The University of Hong Kong.*
2. **Shah A.**, Hung C.H., Cheng S.S.Y. and Chang R.C.C. (2015) Oxyresveratrol mitigates cellular toxicity in Parkinson's disease. *The 9th Pong Ding Yuan International Symposium on Traditional Chinese Medicine, Hong Kong S.A.R.*

2016

3. **Shah A.**, Hung C.H., Cheng S.S.Y. and Chang R.C.C. (2016) The role of oxyresveratrol in mitigating cellular toxicity in Parkinson's Disease. *11th International Symposium on Healthy Aging, Hong Kong S.A.R.*
4. **Shah A.**, Hung C.H., Cheng S.S.Y. and Chang R.C.C. (2016) The effect of oxyresveratrol on endoplasmic reticulum stress in Parkinson's disease. *The Neuroscience Symposium & Annual Scientific Conference of the Hong Kong Society of Neurosciences, Hong Kong S.A.R.*
5. Wong M.Y., **Shah A.**, Cheng S.S.Y. and Chang R.C.C. (2016) Effects of oxyresveratrol on motor and cognitive function in 6-OHDA induced Parkinson's disease. *The 21st Research Postgraduate Symposium, LKS Faculty of Medicine, The University of Hong Kong.*

2017

6. **Shah A.**, Hung C.H., Cheng S.S.Y., Wong M.Y., Legido-Quigley C. and Chang R.C.C. (2017) Oxyresveratrol mitigates cellular toxicity in *in-vitro* and *in-vivo* models of Parkinson's disease. *The 13th International conference on Alzheimer's and Parkinson's Disease, Vienna, Austria.*
7. Wong M.Y., **Shah A.**, and Chang R.C.C. (2017) Protective functions of oxyresveratrol on motor functions in 6-OHDA induced neurodegeneration. *12th International Symposium on Healthy Aging, Hong Kong S.A.R.*

8. Hiew. K.W., **Shah A.**, Han P., Chang R.C.C. and Legido-Quigley C. (2017) Is Alpha-synuclein a biomarker for Parkinson's Disease? *Institute of Pharmaceutical Science M. Pharm Research Symposium, King's College London.*
9. **Shah A.**, Wong M.Y., Legido-Quigley C. and Chang R.C.C. (2017) Investigating the neuroprotective role of oxyresveratrol in *in-vivo* and *in-vitro* models of Parkinson's disease. *Institute of Pharmaceutical Science Postgraduate Research Symposium, King's College London.*
10. **Shah A.**, Han P., Chang R.C.C. and Legido-Quigley C. (2017) Saturated free fatty acids are upregulated in the plasma of an *in-vivo* model of Parkinson's disease. *Emerging Analytical Professionals Conference, Kettering, United Kingdom.*
11. Liu N., **Shah A.**, Han P., and Legido-Quigley C. (2017) The alteration of a plasma lipid in Parkinson's disease. *Institute of Pharmaceutical Science M.Sc. Research Symposium, King's College London.*
12. Cheng Y., Han P., **Shah A.**, and Legido-Quigley C. (2017) The change of small molecules in the brain with Parkinson's disease and semi-quantification. *Institute of Pharmaceutical Science M.Sc. Research Symposium, King's College London.*

2018

13. **Shah A.**, Han P., Chang R.C.C. and Legido-Quigley C. (2018) Oxyresveratrol restores energy homeostasis in a 6-OHDA model of Parkinson's Disease. 1st *AAT-AD/PDTM Focus Meeting on Advances in Alzheimer's and Parkinson's Therapies, Torino, Italy.*
14. Han P., **Shah A.**, Hylands P.J., Chang R.C.C. and Legido-Quigley C. (2018) Can Resveratrol Protect the Brain with Parkinson's Disease via Regulation of Essential Amino Acids? 1st *AAT-AD/PDTM Focus Meeting on Advances in Alzheimer's and Parkinson's Therapies, Torino, Italy.*
15. **Shah A.**, Han P., Chang R.C.C. and Legido-Quigley C. (2018) Association of plasma and mid-brain metabolites with 6-OHDA induced *in-vivo* Parkinsonism. *11th International Conference and Exhibition on Metabolomics & Systems Biology, Tokyo, Japan.*

Awards and bursaries

Young Investigator Award (Poster Category)

11th International Symposium on Healthy Aging "Science and Aging: An Era of Discovery", March 2016, Hong Kong S.A.R

Full bursary to attend the Emerging Analytical Professionals Conference “The Theory of Everything”, May 2017

Separation Sciences Group, The Royal Society of Chemistry, United Kingdom

Partial bursary to attend the 2nd ‘Grass Roots’ Educational Event, September 2017.

The Chromatographic Society, United Kingdom.

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