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Pinnell, Jennifer Rhea

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Investigation of Drp1 inhibition as potential therapy in pesticide-based models of Parkinson's disease

Jennifer Rhea Pinnell

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

Doctor of Philosophy

Peninsula Medical School

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Dedication

I dedicate this thesis to my parents and grandparents, who always nurtured my curiosity and love of science. Their love, support, and encouragement instilled the determination which carried me through this endeavour.

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Authors Declaration

At no time during the registration for the degree of *Doctor of Philosophy* has the author been registered for any other University award without prior agreement of the Doctoral College Graduate Sub-Committee.

Work submitted for this research degree at The University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

This study was financed with the aid of a studentship from *Professor Kim Tieu* and carried out in collaboration with *Florida International University, Miami, FL.*

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Abstract

Jennifer Rhea Pinnell

Investigation of Drp1 inhibition as potential therapy in pesticide-based models of Parkinson's disease

In this study I explored the impact of Drp1 inhibition on pathology in two different pesticide-based models of Parkinson's disease, and found that paraquat induced autophagy blockade *in vitro*, which was abrogated by inhibition of Drp1 function through siRNA knockdown or expression of the $Drp1^{K38A}$ dominant negative. Rotenone and paraquat are two pesticides implicated in the pathophysiology and progression of Parkinson's disease, a hypokinetic neurodegenerative disorder which results in motor and cognitive changes in patients. Previous studies confirmed pesticide-induced loss of dopaminergic neurons in the substantia nigra pars compacta in animal models, however further research was necessary to explore the underlying mechanisms of this pathology. As both pesticides influence mitochondria, and mitochondrial dysfunction has been implicated in Parkinson's disease, I investigated how modulation of the mitochondrial fission factor Drp1 influenced the pesticide-induced pathology. Exploration of rotenone *in vivo* recapitulated dopaminergic neuropathology, motor dysfunction and demonstrated induction of apoptosis, however the systemic toxicity and premature mortality proved it to be an inappropriate model for the exploration of potential therapeutic agents. Paraquat experiments in $Oct3^{-/-}$ mice demonstrated that pharmacological inhibition of Drp1 with mdivi-1 was protective against the paraquat-induced neuropathology, however investigation of genetic Drp1 modulation in the global heterozygous Drp1 knockout mouse model failed to reproduce dopaminergic neuropathology in paraquat-treated wild-type littermate controls, preventing

exploration of any potential protective impact of the Drp1 reduction. Systemic toxicity was much more pronounced in male mice than females, suggestive of gender disparity in their sensitivity to paraquat, with relevance to the human disease as Parkinson's disproportionately affects men. Further investigation of mechanisms of paraquat-induced toxicity *in vitro* implicated autophagy blockade as a mechanism of pathology, with Drp1 inhibition proving effective to abrogate the blockade. Drp1 inhibition in cells co-treated with paraquat and α-synuclein however was insufficient to restore function in tfLC3 HeLa cells. This implicates Drp1 in autophagy, where it was previously considered a mitochondrial fission protein. Whilst further exploration of how Drp1 influences autophagy is required, this work is the first to demonstrate a protective role of Drp1 inhibition against paraquat-induced pathology, including the partial resolution of autophagy blockade. Combined with previous work from our lab, this further supports the involvement of Drp1 in autophagy as a mechanism in Parkinson's disease pathology, which validates the targeting of Drp1 as a potential therapeutic method to alter Parkinson's disease progression.

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Dr Mei Cui contributed her stereology skills for the quantification of dopaminergic neurons in the *Oct3*$^{-/-}$ paraquat and Mdivi-1 experiment [Figure 12].

Dr Martin Helley contributed to the characterisation of Drp1 expression levels in the global heterozygous Drp1 mouse model [Figure 16].

Carolina Sportelli contributed the Calcein AM assay data, paraquat dose-response in tfLC3 Hela cells and assessment of Proteinase K-resistant α-synuclein aggregates in N27 cells in the *in vitro* investigation of paraquat [Figure 22; Figure 23 & Figure 27].

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List of Abbreviations

Abbreviation	Term
2,4-D	2,4-Dichlorophenoxyacetic acid
6-OHDA	6-Hydroxydopamine
AD	Alzheimer's disease
ADo	Autosomal Dominant
ADH/AR	Alcohol dehydrogenase / aldose reductase
ALDH	Aldehyde dehydrogenase
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid-precursor protein
AR	Autosomal Recessive
ATP	Adenosine triphosphate
ATP13A2	ATPase type 13A2
β-HCH	β-Hexachlorocyclohexane
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin-dependent kinases
CHCHD2	Coiled-coil-helix-coiled-coil-helix domain 2
CMC	Carboxymethylcellulose
CNS	Central nervous system
COMT	Catechol-*O*-methyl transferase
COX	Cytochrome C oxidase
COX4I2	Complex IV subunit 4
CSF	Cerebrospinal fluid
CTCF	Corrected total cell fluorescence
CYP2D6	Cytochrome P450 2D6
DAB	3,3'-diaminobenzidine tetrachloride
DAT	Dopamine transporter
DBH	Dopamine beta-hydroxylase
DDT	Dichlorodiphenyltrichloroethane
DLB	Dementia with Lewy Bodies
DMF	Dimethyl fumarate
DMSO	Dimethyl sulfoxide
DMT	Divalent metal transporter 1
DMV	Dorsal motor nucleus of the vagus
DOPAC	3,4-dihydroxyphenylacetic acid
DOPAL	Dihydroxyphenylacetaldehyde
DOPET	3,4-dihydroxyphenyl ethanol
Drp1	Dynamin related protein 1
EL	Encephalitis legargica
ENS	Enteric nervous system
EOPD	Early Onset Parkinson's Disease
ERK	Extracellular-signal regulated kinases
Fpn	Ferroportin
FTD	Frontotemporal Dementia
GABA	Gamma-aminobutyric acid
GCase	Glucocerebrosidase

GD	Gaucher’s disease
GDNF	Glial-derived neurotrophic factor
GI	Gastrointestinal
GSH	Glutathione
GTP	Guanosine triphosphate
GWAS	Genome-wide association studies
HD	Huntington’s disease
HNE	4-Hydroxynonenal
IMM	Inner mitochondrial membrane
IP.	Intraperitoneal
iPSC	Induced pluripotent stem cells
KFR	Kufor-Rakeb syndrome
LAMP1	Lysosome-associated membrane protein 1
LBD	Lewy Body Disease
LBs	Lewy Bodies
LC3	Microtubule-associated protein 1A/1B-light chain 3
LDH	Lactate dehydrogenase
L-DOPA	Levodopa
LoSMoN	Late Onset Spinal Motor Neuronopathy
LOPD	Late Onset Parkinson’s Disease
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
MAM	Mitochondrial-associated endoplasmic reticulum membrane
MAO	Monoamine oxidase
MB	Maneb
MCI	Mild Cognitive Impairment
MDVs	Mitochondria-derived vesicles
Mdivi-1	Mitochondrial division inhibitor 1
MEF	Mouse embryonic fibroblasts
Mff	Mitochondrial fission factor
Mfn1	Mitofusin-1
Mfn2	Mitofusin-2
MiD49	Mitochondrial dynamics protein 49
MiD51	Mitochondrial dynamics protein 51
mHtt	Mutant Huntingtin protein
Mn	Manganese
MnSoD	Manganese superoxide dismutase
MPP	Mitochondrial processing peptidase
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTS	Mitochondrial targeting sites
MUL1	Mitochondrial E3 ubiquitin ligase 1
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NGS	Normal goat serum
OCPs	Organochloride pesticides
OCT3	Organic cation transporter 3
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
PARL	Presenillin-associated rhomboid-like protease

PBS	Phosphate-buffered saline
PCBs	Polychlorinated biphenyls
PD	Parkinson's Disease
PEG	Polyethylene glycol
PEP	Post-encephalitic parkinsonism
PET	Positron emission tomography
PFA	Paraformaldehyde
PGC1α	Proliferator-activated receptor gamma coactivator 1-alpha
PINK1	PTEN-induced putative kinase 1
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PND	Post-natal day
PNMT	Phenylethanolamine N-transferase
PNS	Peripheral nervous system
PonA	Ponasterone A
PQ	Paraquat
PSN	Presenilin
PTM	Post-translational modification
rAAV	Recombinant adeno-associated virus
ROI	Region of interest
ROS	Reactive oxygen species
SLC30A10	Solute carrier family 30 member 10
SLC39A14	Solute carrier family 39 member 14
SN	Substantia nigra
SNP	Single nucleotide polymorphism
SNpc	Substantia nigra pars compacta
SPCA1	Secretory pathway Ca_2^+ ATPase 1
STR	Striatum
TBS	Tris-buffered saline
TCE	Trichloroethylene
tfLC3	Tandem-fluorescent mRFP-GFP-LC3 HeLa cells
TH	Tyrosine-hydroxylase
TIM23	Translocase of the inner membrane 23
TOM20	Translocase of the outer membrane 20
TOM40	Translocate of the outer membrane 40
UPS	Ubiquitin proteasome system
VAMP2	Vesicle-associated membrane protein 2
VDAC	Voltage-dependent anion channel
VMAT2	Vesicular monoamine transporter 2
VMB	Ventral midbrain
VPS35	Vacuolar-sorting protein 35
WT	Wild type
ZIP	Zrt- and Irt-related protein

Chapter 1| Introduction to Parkinson’s Disease

1.1| History, clinical and pathological features

Parkinson's disease (PD) is a debilitating, neurodegenerative motor disorder. Whilst its discovery is attributed to James Parkinson in 1817 (Parkinson, 1817), references to PD symptoms have been found in work as early as 12th Century BC and also in Galen's writing (Lanska, 2010). PD is a hypokinetic neurodegenerative disorder characterised by deterioration of the nigrostriatal dopaminergic pathway; symptomology arises from the loss of dopaminergic innervation of motor nuclei in the extrapyramidal system, causing subsequent dysfunction of their associated pathways (Fahn and Prsedborski, 2010).

PD is clinically characterised by the tetrad of bradykinesia, resting tremor, rigidity, and postural instability. Non-motor symptoms also present in PD, with some appearing decades prior to the onset of motor changes and diagnosis. Non-motor symptoms may include sleep disturbances, depression, olfactory dysfunction, gastrointestinal changes and pain (Drolet *et al.*, 2009; Schrag *et al.*, 2019). Recent work has also suggested that non-motor symptoms may be indicative of symptom severity and co-morbidities; patients who reported more severe pain displayed more severe symptoms and comorbidities (Vila-Chã *et al.*, 2019).

1.2| Aetiology and epidemiology

PD is currently the second most prevalent neurodegenerative disease, affecting approximately ten million individuals worldwide (Dorsey *et al.*, 2007; Marras *et al.*, 2018). In a recent report by Parkinson's UK, it was estimated that over 145,000 individuals were living with PD in the UK alone, with an anticipated increasing incidence of 18% by 2025 (UK., 2017). As a multifactorial disease with complex aetiological interactions, it is impossible to attribute causation to a single genetic or

environmental factor; approximately 10% of cases result from monogenic, familial gene mutations (Ibanez *et al.*, 2009) whilst the majority of cases are considered to be sporadic and likely result from the cumulative effects of genetic and environmental risk factors (Cannon and Greenamyre, 2013). The principal risk factor for PD is age, as incidence increases in aged populations and countries with reduced life expectancies demonstrate comparatively fewer cases (Bridgeman and Arsham, 2017). Gender is also considered a risk, as males are 1.5 times more likely to develop PD than women, with more recent work demonstrating that this risk discrepancy alters with age (Moisan *et al.*, 2016; Wooten *et al.*, 2004).

1.3| Genetic risk factors

There are a number of genetic changes which increase the likelihood of developing PD, including familial monogenic mutations, gene-dosage effects, and risk factor genes [Table 1]. Sir William Gowers was the first neurologist to suggest a genetic contribution to PD, after finding that 15% of his PD patients reported a family history of the disease (Gowers, 1893). In the past few decades, our knowledge of genetic risk factors has grown extensively, with the identification of multiple autosomal dominant and recessive genes, further highlighting the complexity of PD aetiology (Hernandez, Reed and Singleton, 2016; Przedborski, 2017).

Table 1: A summary of current monogenic Parkinson's disease-related genetic mutations and associated risk genes

PARK Locus	Gene Locus	Gene	Inheritance	Mutations
PARK ¼	4q21-22	*SNCA*	ADo	A53T, A30P, E46K, G51D, H50Q, duplications, triplications
PARK 2	1p35-p36	*Parkin*	AR	>50 missense mutations, exon deletions, duplications, triplications
PARK 3	2p13	*Unknown*	ADo	Rs1876487A>C
PARK 5	4p13	*UCHL1*	Risk Factor	I93M risk factor SNP, S18Y protective SNP
PARK 6	6q25.2-q27	*PINK1*	AR	>40 missense mutations, rare exon-deletions
PARK 7	1p36	*DJ-1*	AR	>10 missense mutations, exon deletion (del[Ex1-5])
PARK 8	12q12	*LRRK2*	ADo Risk Factor	>50 missense and nonsense mutations high risk variants, >15 of which are pathogenic
PARK 9	1p36	*ATP13A2*	AR	>10 missense mutations, some produce truncations due to splicing variants, frameshift mutations
PARK 10	1p32	*Unknown*	Risk Factor	Undetermined
PARK 11	2p37.1	*Unknown*	ADo	Undetermined
PARK 12	Xq21-q25	*Unknown*	Risk Factor	Undetermined
PARK 13	2q12	*HTRA2/Omi*	ADo Risk Factor	G339S, A141S
PARK 14	22q13.1	*PLA2G6*	AR	R741Q, R747W, R635Q, D331Y
PARK 15	22q12-q13	*FBX07*	AR	R378G, R498X, T22M
PARK 16	1q32	*Unknown*	Risk Factor	rs823128G>A
PARK 17	16q11.2	*VPS35*	ADo	D620N
PARK 18	3q27.1	*EIF4G1*	ADo	G686C, S1164R, R1197W, R1205H
PARK 19	1p31.3	*DNAJC6*	AR	Missense mutations (Arg927Gly), loss-of-function, knockout
PARK 20	21q22.2	*SYNJ1*	AR	Missense mutations, specifically Arg258Gln, truncations
PARK 21	3q22.1	*DNAJC13*	ADo Risk Factor	Missense mutations (Asn855Ser), GOF
PARK 22	7p11.2	*CHCHD2 (MNRR1)*	ADo	Missense mutations (Thr61Ile), rs10043, rs142444896
PARK 23	15q22.2	*VPS13C*	AR	Loss of function mutation
N/A	22q11.23	*CHCHD10*	Risk Factor	>7 missense mutations, specifically P34S
N/A	11q15.4	*SMPD1*	Risk Factor	L302P mutation
N/A	14q22.2	*GCH1*	Risk Factor	>140 mutations, including N370S and R496H
N/A	11q22.3	*RAB39D*	X-linked Dominant	Missense mutations
N/A	1q21	*GBA*	Risk Factor	>10 missense mutations, L444P and N370S are best studied
N/A	16q24.3	*MC1R*	Risk Factor	Homozygous R151C
N/A	17q21	*MAPT*	Risk Factor	H1 haplotype
N/A	4p16	*GAK/DNAJC 26*	Risk Factor	rs11248051C>T, rs1564282 variant
N/A	6p21	*HLA-DRA*	Risk Factor	rs3129882G>A
N/A	19q13.32	*APOE*	Risk Factor	APOE-E4 allele rs7412-C, rs429358-C, APOE-E2 allele
N/A	17q11.2	*NOS2A*	Risk Factor	rs1060826T>C, rs2255929T>A
N/A	4q23	*ADH1C*	Risk Factor	G78X
N/A	20p12	*TMEM230*	AR	

ADo, Autosomal dominant; AR, Autosomal recessive.

1.3.1| Autosomal dominant genes

SNCA

The first identified PD-causing gene mutation, and the most widely researched, is the autosomal dominant *SNCA* gene, which encodes α-synuclein protein (Polymeropoulos et al., 1997); its discovery prompted increased research to identify other genetic risk factors. Due to its abundance in presynaptic terminals, it is believed to play a role in the regulation of neurotransmitter release via interactions with synaptic vesicle lipids (Larsen *et al.*, 2006; Nemani *et al.*, 2010; Scott *et al.*, 2010; Scott and Roy, 2012; Vargas *et al.*, 2014). Mutations and gene-dosage effects of *SNCA* have been widely explored; a number of point mutations have been identified along with gene duplications and triplications which have been found to increase PD risk in several families (Hernandez, Reed and Singleton, 2016).

In addition to its role in familial PD risk, genome-wide association studies (GWAS) have identified *SNCA* as a genetic risk factor associated with sporadic cases of PD (Campêlo and Silva, 2017; Zhang *et al.*, 2018); therefore, identifying pathogenic mechanisms and effective therapies for α-synuclein toxicity is relevant to both familial and sporadic PD, with further potential to benefit sufferers of other synucleinopathies. Whilst mutations in this gene are rare, the locus is appreciated as a significant variant factor for PD development (Lill *et al.*, 2012).

A small 14kDa protein (Uéda *et al.*, 1993), α-synuclein is considered an intrinsically disordered protein as it doesn't appear to have a stable 3D structure (van Rooijen *et al.*, 2009; Weinreb *et al.*, 1996). Residues 1-60 comprise the amphipathic N-terminal region, which contains four 11-residue repeats with structural alpha-helix propensity. This highly conserved terminal interacts with acidic lipid membranes and

contains all of the identified point mutations of *SNCA* (Bussell and Eliezer, 2003). Residues 61-95 comprise a central hydrophobic region which includes the non-amyloid-beta component (NAC) domain, which is involved in protein aggregation; this domain is unique to α-synuclein among the synuclein family (Uchihara and Giasson, 2016). Residues 96-140 comprise the highly acidic, proline-rich C-terminal region, which is extremely important in the function, solubility and interaction of α-synuclein with other proteins (Burré *et al.*, 2010).

α-synuclein is natively unfolded in solution but possesses the propensity to form aggregates under various conditions. Insoluble, fibrillar α-synuclein aggregates constitute a major component of Lewy bodies, the intracellular proteinaceous inclusions regarded as PD hallmarks (Arima *et al.*, 1999). In pathological conditions, α-synuclein aggregates to form insoluble fibrils in conjunction with ubiquitin, neurofilament, alpha-crystallin B and other proteins (Mezey *et al.*, 1998; Spillantini *et al.*, 1997). The presence of Lewy bodies in both familial and sporadic forms of PD further supports the contribution of α-synuclein to multiple forms of the disease.

Studies suggest that α-synuclein may play a role in restricting the mobility of synaptic vesicles, reducing synaptic vesicle recycling and neurotransmitter release (Larsen *et al.*, 2006; Nemani *et al.*, 2010; Scott *et al.*, 2010; Scott and Roy, 2012; Sun *et al.*, 2019; Vargas *et al.*, 2014; Wang *et al.*, 2014b). An alternative proposed mechanism is that α-synuclein binds to VAMP2 (a synaptobrevin) to stabilise SNARE complexes (Burré, Sharma and Südhof, 2018; Burré, Sharma and Südhof, 2014; Burré, Sharma and Südhof, 2012; Burré *et al.*, 2010; Diao *et al.*, 2013) and to act as a molecular chaperone in SNARE complex assembly (Bonini and Giasson, 2005; Chandra *et al.*, 2005). α-synuclein simultaneously binds to phospholipids of the plasma membrane via the N-terminal domain and to synaptobrevin-2/VAMP-2 via its

C-terminus, which seems to be important for synaptic activity (Burré *et al.*, 2010). Further work has indicated that this α-synuclein-VAMP2 binding is critical for α-synuclein-mediated attenuation of synaptic vesicle recycling, linking the two independently proposed mechanisms to better explain α-synuclein's potential intracellular function (Sun *et al.*, 2019).

Evidence also suggests that α-synuclein may play a role in the function of the neuronal golgi apparatus and vesicle trafficking (Cooper *et al.*, 2006) and, as it has been found to localise to the nucleus of mammalian neurons, it may exhibit an as-yet undefined nuclear function (Yu *et al.*, 2007). It has been demonstrated that α-synuclein is essential for the normal development of cognitive functions, as *SNCA* knockout mice display impaired spatial learning and working memory (Kokhan, Afanasyeva and Van'kin, 2012). It has also been established that α-synuclein significantly interacts with tubulin (Alim *et al.*, 2002) and may therefore function as a microtubule-associated protein, similar to tau (Alim *et al.*, 2004).

A number of missense point mutations have been identified in *SNCA,* all of which confer increased PD risk (Appel-Cresswell *et al.*, 2013; Krüger *et al.*, 1998; Singleton *et al.*, 2003; Zarranz *et al.*, 2004), whilst gene multiplication mutants produce higher levels of wild-type (WT) α-synuclein, which results in autosomal dominant (ADo) PD due to gene dosage effects (Singleton *et al.*, 2003). This observation is important as it indicates that elevated WT α-synuclein alone is sufficient to cause disease. The missense mutations, A53T, A30P, E46K, H50Q and G51D (Polymeropoulos *et al.*, 1997; Krüger *et al.*, 1998; Zarranz *et al.*, 2004; Appel-Cresswell *et al.*, 2013; Lesage *et al.*, 2013) have all been demonstrated to increase PD risk, however the focal mutation in research is the first identified A53T.

A53T is the most common *SNCA* mutation which has been found in seven families worldwide, whilst the other point mutations have only been identified in one family each (Volles and Lansbury, 2003). The replacement of residue 53 from its native alanine to a threonine exerts functional and structural effects on α-synuclein protein; increased fibrilization kinetics and earlier disease onset have been linked to this missense mutation (Conway, Harper and Lansbury, 1998). A53T transgenic mice have been developed and utilised by multiple studies investigating the effects of this mutation (Giasson *et al.*, 2002; Gispert *et al.*, 2003; Martin *et al.*, 2006). Investigation as to whether the protofibril form of α-synuclein may confer toxicity rather than the larger aggregates demonstrated that A53T α-synuclein leads to more rapid formation of protofibrils and increased the protofibril population which, if neurotoxic, would increase the detrimental effects in the brain (Conway *et al.*, 2001; Lashuel *et al.*, 2002; Lee and Lee, 2002; Volles *et al.*, 2001). The A30P mutation has also been shown to accelerate protofibril production, however in the case of this mutation it has not been consistently linked to increased α-synuclein fibrilization (Goedert, 2001; Conway *et al.*, 2000). It has been suggested that the α-synuclein protofibrils produced excessively by these mutants may induce neurotoxicity by binding to and permeabilising vesicular membranes (Volles *et al.*, 2001).

A recent study by Mor et al (2017) investigated the interaction between α-synuclein (WT and A53T) and dopamine (DA); their results clearly indicate that increased dopamine production is insufficient to cause dopaminergic neurodegeneration in the SNpc, even in the presence of endogenous α-synuclein. However, expression of A53T mutant protein accompanied by increased dopamine production, due to overactive tyrosine hydroxylase (TH) which is unresponsive to normal negative feedback, demonstrated a 25% reduction in nissl cells, 62% reduction

in VMAT2 striatal staining and a 55% decline in dopamine transporter (DAT) levels. These results indicate that dopamine toxicity is dependent upon α-synuclein and may be exacerbated by mutant forms; it is thought that dopamine stabilises its oligomerisation. *In vitro* studies suggest that dopamine-induced α-synuclein oligomers can inhibit their own degradation and that of other substrates by chaperone-mediated autophagy and reduce neurotransmitter release by inhibition of SNARE complex formation.

Multiple inter-related pathogenic mechanisms of α-synuclein mutants have been proposed, as α-synuclein can inhibit mitochondrial function at multiple levels; inhibition of the electron transport chain, imbalanced mitochondrial dynamics, impaired mitochondrial import, reduced mitochondrial biogenesis and blockade of autophagy flux (Henderson, Trojanowski and Lee, 2019).

Although the majority of α-synuclein is cytosolic, the protein has been shown to localise to mitochondria and induce dysfunction (Chen *et al.*, 2015; Devi *et al.*, 2008; Di Maio *et al.*, 2016; Liu *et al.*, 2009; Nakamura *et al.*, 2011; Parihar *et al.*, 2008; Subramaniam *et al.*, 2014). Multiple mitochondrial targeting sequences (MTS) have been identified in the N-terminal domain of α-synuclein, conferring its ability to bind to components of the mitochondrial membrane (Devi *et al.*, 2008). α-synuclein has been shown to specifically bind to cardiolipin (Nakamura *et al.*, 2008), TOM20 (Di Maio *et al.*, 2016), TOM40 (Devi *et al.*, 2008) and VDAC (Rostovtseva *et al.*, 2015), either to directly promote dysfunction at the membrane level or to allow import of α-synuclein to other mitochondrial compartments.

A recent study by Di Maio et al (2016) reported that specific forms of post-translationally modified, specifically serine-129 phosphorylated, α-synuclein bind to

the mitochondrial outer membrane protein TOM20 with high affinity, inhibiting mitochondrial protein import in rats expressing virally encoded human α-synuclein. This results in mitochondrial dysfunction, enhanced ROS production and loss of mitochondrial membrane potential. This study also demonstrated an increased association between TOM20 and α-synuclein in PD patient post-mortem brains, confirming relevance of this association to presentation of the human disease. In a similar manner, binding of WT α-synuclein to VDAC was sufficient to induce channel blockade, preventing bi-directional metabolite transfer, thus promoting dysfunction and further demonstrating how interaction of α-synuclein with mitochondrial proteins may impact function (Rostovtseva *et al.*, 2015). α-synuclein has also been shown to enter mitochondria through TOM40, as antibodies against the channel successfully inhibited mitochondrial import of α-synuclein (Devi *et al.*, 2008). Mice with global overexpression of human α-synuclein in the brain, using the Thy1 promoter, exhibited age-dependent mitochondrial accumulation of α-synuclein in the nigrostriatal dopaminergic pathway, impaired electron transport chain function and enhanced oxidative stress (Subramaniam *et al.*, 2014), further supporting the importance of α-synuclein-mitochondria interactions in disease development.

The importance of the N-terminal domain in mitochondrial targeting is emphasised by the discovery that A53T mutant α-synuclein has a greater binding affinity for mitochondria than the WT protein, due to its alanine to threonine substitution in the N-terminal region (Devi *et al.*, 2008). Mice with inducible A53T α-synuclein display severe mitochondrial fragmentation, preceding dopaminergic neurodegeneration (Chen *et al.*, 2015). It has more recently been suggested that α-synuclein specifically targets the mitochondria-associated endoplasmic reticulum (MAM), where it plays a role in regulating mitochondrial morphology and results in

reduced interconnectivity between mitochondria and the endoplasmic reticulum (Guardia-Laguarta *et al.*, 2014).

Evidence of α-synuclein interference with normal mitochondrial dynamics, fission, fusion, and movement [Figure. 1], has been reported by multiple studies. Overexpression of both WT and mutant α-synuclein is associated with severe mitochondrial fragmentation and reduced mitochondrial motility *in vitro* and *in vivo*, through both direct and indirect mechanisms (Bido *et al.*, 2017; Chen *et al.*, 2015; Guardia-Laguarta *et al.*, 2014; Gui *et al.*, 2012; Kamp *et al.*, 2010; Nakamura *et al.*, 2011). α-synuclein reduced mitochondrial biogenesis in multiple studies (Siddiqui *et al.*, 2012; Zheng *et al.*, 2010); following onset of oxidative stress, α-synuclein bound to the promoter region of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), a key component of the mitochondrial biogenesis machinery, thus inhibiting the expression of downstream genes (Siddiqui *et al.*, 2012). Multiple α-synuclein species may further promote the formation of dysfunctional mitochondria by increasing the prevalence of mitochondrial DNA mutations (Bendor, Logan and Edwards, 2013; Martin *et al.*, 2006).

There is increasing evidence that mitochondrial function and autophagy are tightly linked cellular processes. A reduced autophagic capacity has been identified in many neurodegenerative diseases, including PD (Nah, Yuan and Jung, 2015). Multiple genes implicated in familial PD, including *SNCA*, have functions related to autophagy and mitophagy, emphasising the importance of this pathway in the disease. α-synuclein can directly interact with the autophagy pathway; overexpression of WT α-synuclein was shown to directly modulate autophagy via inhibition of Rab1α protein, which is important for autophagosome formation (Winslow *et al.*, 2010). Impaired autophagy has also been demonstrated *in vivo* using mice overexpressing WT α-

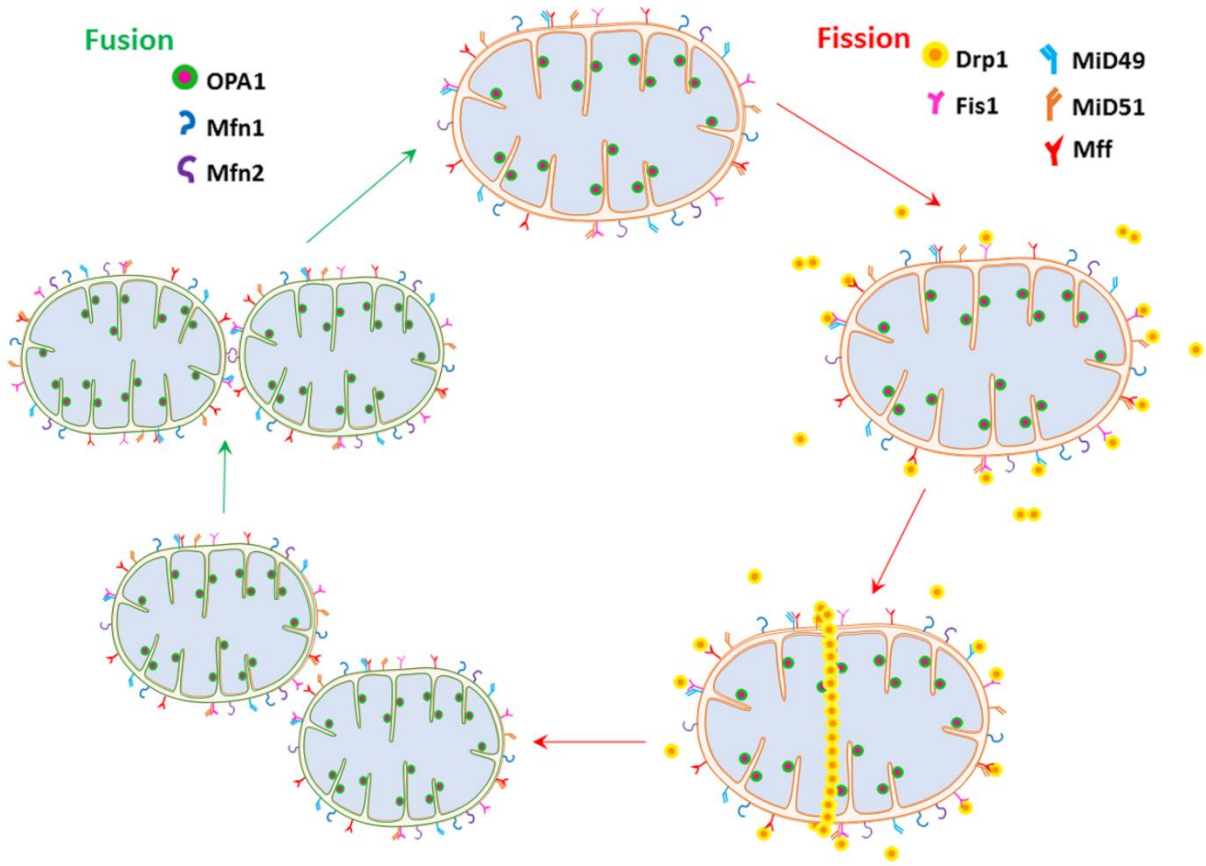


Figure 1: Mitochondrial dynamics. The dynamic mitochondrial processes of fission and fusion work to maintain a healthy, functional population of mitochondria within cells. Fusion is mediated by three specific proteins; Optic atrophy 1 (OPA1), which is localised to mitochondrial cristae in the matrix and mediates inner membrane fusion, and mitofusins (Mfn1 & Mfn2), which are localised to and control fusion of the outer mitochondrial membrane (Chan, 2006; Mecusen *et al.*, 2006). Fission is mediated by Dynamin-related protein 1 (Drp1), a large GTPase which translocates from the cytosol to the mitochondrial outer membrane where it induces mitochondrial division by wrapping around constriction points of dividing mitochondria (Smirnova *et al.*, 2001). Mitochondrial fission factor (Mff), Fission 1 (Fis1) and Mitochondrial dynamic proteins 49 and 51 (MiD49 and MiD51) are all outer mitochondrial membrane-anchored fission proteins to which Drp1 may be recruited. MiD49 and MiD51 are essential for the stabilisation of the multimeric Drp1 structures necessary for mitochondrial fission, and both may further regulate fission through the formation of complexes with Mff and Fis1 (Otera *et al.*, 2010; Palmer *et al.*, 2011; Loson *et al.*, 2012).

synuclein (Ebrahimi-Fakhari *et al.*, 2011) or A53T mutants (Chen *et al.*, 2015; Yu *et al.*, 2009). Our lab recently published data which demonstrates a link between mitochondrial dynamics and autophagy in an α-synuclein *in vitro* PD model; inhibition of the mitochondrial fission factor Drp1 demonstrated neuroprotective effects against α-synuclein-induced mitochondrial fragmentation, mitochondrial dysfunction, α-synuclein aggregation and autophagy blockade (Fan *et al.*, 2019). Furthermore,

impaired autophagy promotes the formation and accumulation of higher order α-synuclein species, thus creating a positive feedback cycle of neurotoxicity (Fan *et al.*, 2019; Xilouri, Brekk and Stefanis, 2016). Mitochondrial dysfunction may lead to inhibition of the autophagy pathway, due to reduced ATP availability and increased ROS. Equally, impaired autophagy can promote mitochondrial dysfunction by reduced clearance of damaged, dysfunctional organelles or toxic α-synuclein oligomers. The complex interplay of these pathways is not fully understood, particularly the query of whether mitochondrial dysfunction precedes autophagy blockade or vice versa. Regardless, the interaction between these two fundamental processes represents a critical aspect of the pathology, which requires further investigation.

Additionally, there is growing evidence that α-synuclein is capable of cell-to-cell transmission. The discovery that α-synuclein is inter-cellularly transmissible, in a prion-like manner, provides a key insight into how PD may develop and may lead to the development of novel therapeutic strategies. Emerging evidence indicates that α-synuclein can spread through multiple mechanisms, including non-classical exocytosis, exosomal release, and via nanotubes, which directly connect distinct cells (Guo and Lee, 2014). According to the Braak staging hypothesis, the olfactory bulb and gut are initial spreading sites of misfolded protein (Hawkes, Del Tredici and Braak, 2007). In line with this hypothesis, α-synuclein has been identified in enteric neurons of patients with early-stage PD (Hilton *et al.*, 2014), with investigations demonstrating its transmission up to the brain. Research involving the transplant of embryonic neurons into patient corpus striatum's further supports this hypothesis, as the cell grafts displayed α-synuclein pathology 10 to 22 years after implant, suggestive that α-synuclein has transmitted from host neurons into the graft tissue (Kordower *et al.*, 2008; Li *et al.*, 2008). Mitochondria have been implicated in this pathological

transmission in both the enteric and central nervous system (CNS); for example, structural mitochondrial abnormalities were identified in enteric neurons of post-mortem tissue from PD patients (Baumuratov *et al.*, 2016). In primary human foetal enteric neurons, recombinant α-synuclein exposure induced mitochondrial dysfunction, reducing complex I activity and impairing mitochondrial respiration (Braidy *et al.*, 2014). In a gene-environment interaction model of α-synuclein transmission, exposure of enteric neurons to the environmental toxicant rotenone was shown to promote the release of α-synuclein species which were subsequently taken up by second-order neurons where they formed aggregates (Pan-Montojo *et al.*, 2010). Exposure of rat ventral midbrain neurons to exogenous pre-formed fibrillar α-synuclein structures, which are often used in the study of transmission, also produced mitochondrial dysfunction (Tapias *et al.*, 2017). Our lab have demonstrated interplay between autophagy and mitochondrial regulation in α-synuclein transmission, as inhibition of Drp1 reduced exosome release and the spread of α-synuclein pathology, from neurons to neurons and also from microglia to neurons (Fan et al, 2019). This recent work further highlights the interplay between autophagy and mitochondrial regulation in an α-synuclein model, representing a potential common therapeutic target in this model.

Whilst these studies support a central role of mitochondria in the spread of α-synuclein from one neuron to another, the mechanism of trans-synaptic transmission is still a topic of debate and requires further investigation to improve our understanding of the complex role this protein plays in PD pathology (Brundin and Melki, 2017; Surmeier, Obeso and Halliday, 2017).

α-synuclein has been at the forefront of genetic PD research and multiple high-profile discoveries have greatly contributed to the understanding of disease pathology,

however the diversity of α-synuclein's mechanisms of contribution to the pathology continue to develop, as we gain a greater understanding of its role in sporadic PD and its interaction with environmental risk factors.

LRRK2

LRRK2 mutations are the most common cause of late-onset familial PD (Cookson, 2015), and have also been found in cases of sporadic PD. *LRRK2* encodes a large multidomain protein known as Leucine-rich repeat kinase 2 (LRRK2) or dardarin, which is largely present in the cytoplasm and also associates with the mitochondrial outer membrane (Biskup *et al.*, 2006; Higashi *et al.*, 2007b; Higashi *et al.*, 2007a). The *LRRK2* locus is 12p11.2-q13.1 (Funayama *et al.*, 2002) and *LRRK2* mutations were originally discovered in 2004 in a Japanese family (Paisán-Ruíz *et al.*, 2004), with subsequent independent confirmation in several other families with different countries of origin (Anfossi *et al.*, 2014; Kachergus *et al.*, 2005; Schlitter *et al.*, 2006). *LRRK2* mutations account for ~40% of familial PD cases and up to 10% of sporadic cases worldwide (Hernandez, Reed and Singleton, 2016), producing a disease phenotype similar to that of classical late-onset idiopathic PD. The identification of heterozygous unaffected carriers of *LRRK2* mutations, with no clinical disease presentation, permitted estimates of penetrance (~17% at age 50). This incomplete penetrance highlights some potential complications associated with pre-symptomatic clinical screening for mutations (Kay *et al.*, 2005).

LRRK2 function is not definitively understood, however research suggests that the protein likely regulates neurite structure, neurite retraction and neuronal survival (Chan *et al.*, 2011a; Heo, Kim and Seol, 2010). It is associated with many cellular

processes, particularly those involving membrane dynamics, such as autophagy, cytoskeletal dynamics, vesicle dynamics and mitochondrial function (Albanese, Novello and Morari, 2019; Juárez-Flores *et al.*, 2018; Ludtmann *et al.*, 2019; Plotegher and Duchen, 2017; Singh, Zhi and Zhang, 2019). Investigations to better understand the contribution of *LRRK2* to PD pathology have been restricted partly by the difficulty recreating *LRRK2* mutants in transgenic models which recapitulate important features of PD pathology, however mutant *LRRK2* has been shown to lead to reduced neurite complexity, formation of tau-positive inclusions, lysosomal abnormalities and apoptotic cell death in rodent and primary cell models (MacLeod *et al.*, 2006).

Despite over 100 *LRRK2* mutations reported, only a subset of point mutations have proven links to PD; N1437H, R1441C, p.R1441G, R1441H, Y1699C, G2019S and I2020T (Funk *et al.*, 2019; Goldwurm *et al.*, 2005; Li, Tan and Yu, 2014). Multiple mutations have also been nominated as PD related-mutants, with G2019S, R1628P and G2385R linked to increased PD risk, whilst R1398H appears to reduce PD risk (Dächsel and Farrer, 2010; Paisán-Ruiz, Lewis and Singleton, 2013; Shu *et al.*, 2019). There also appears to be variation in PD-related *LRRK2* mutants across different populations, with some mutants only contributing to risk in Europeans (Shu *et al.*, 2019). It is possible that the increased risk conferred by these mutants may involve interaction with environmental toxicants, which vary across the world.

G2019S is the most common *LRRK2* mutation, suggested to account for 4% of familial and 1% of sporadic PD cases (Hernandez, Reed and Singleton, 2016; Nalls *et al.*, 2011). Although the exact function of *LRRK2* mutants in the causation of PD is poorly understood, all mutations described to date affect a central enzymatic region of the protein, consisting of a GTPase and kinase domain, conferring gain-of-function (West *et al.*, 2007). Overexpression of pathogenic LRRK2 mutants (R1441C and

G2019S) in cultured neuronal cells caused mitochondrial fragmentation and dysfunction through a Drp1-dependent mechanism. Abnormal mitochondrial morphology and alterations in mitochondrial fission/fusion proteins have also been reported in LRRK2^{G2019S} knock-in mice (Melrose *et al.*, 2010). The majority of *LRRK2* mutant transgenic models fail to display appreciable dopaminergic neurodegeneration, however they demonstrate multiple synaptic deficits, including reduced vesicle endocytosis (Arranz *et al.*, 2015), and altered dopamine release, reuptake and signalling (Beccano-Kelly *et al.*, 2015; Li *et al.*, 2009; Melrose *et al.*, 2010; Tong *et al.*, 2009; Walker *et al.*, 2014; Yue *et al.*, 2015).

LRRK2 has been shown to impair mitochondrial dynamics through direct physical interaction with Drp1, promoting mitochondrial fragmentation associated with enhanced mitochondrial fission, increased ROS generation and impaired autophagy (Esteves and Cardoso, 2015; Niu *et al.*, 2012; Stafa *et al.*, 2014; Wang *et al.*, 2012a). Additional to increased Drp1 expression, concurrent enhanced phosphorylation of Drp1 at Serine-616 has been reported; this is a marker of specific modification for increased mitochondrial-associated activity (Esteves and Cardoso, 2015; Stafa *et al.*, 2014; Wang *et al.*, 2012a). Overexpression of the gain of function mutations G2019S and R1441C result in reduced mitochondrial membrane potential, reduced complex IV activity, increased uncoupling, altered mitochondrial motility and imbalanced calcium signalling (Cherra *et al.*, 2013; Cooper *et al.*, 2012; Gilks *et al.*, 2005; Godena *et al.*, 2014; Papkovskaia *et al.*, 2012). Ultrastructural studies have shown accumulated, condensed, disorganised and damaged mitochondria consistent with defective mitophagy and mitochondrial dynamics (Ramonet *et al.*, 2011; Yue *et al.*, 2015). Increased mutations of mtDNA have also been identified in CSF and iPSCs derived from patients with the G2019S mutation (Podlesniy *et al.*, 2016; Sanders *et al.*, 2014).

LRRK2 interference with mitochondrial function is also linked to impaired autophagy in multiple models; an accumulation of autophagic and lysosomal structures occurs as a result of overexpression of PD-associated mutations both *in vitro* and *in vivo* (Gómez-Suaga *et al.*, 2012; MacLeod *et al.*, 2006; Plowey *et al.*, 2008). Autophagic changes have been demonstrated in patient-derived iPSCs expressing LRRK2^{G2019S} at endogenous levels (Sánchez-Danés *et al.*, 2012); these cells displayed autophagy flux blockade, increased P62 levels and accumulated autophagosomes. Conversely, disrupted mitochondrial motility and increased vulnerability to multiple stressors induced by G2019S and R1441C mutations in PD patient iPSCs was rescued by the autophagy promoter rapamycin (Cooper *et al.*, 2012). Further supporting the bi-directional regulation of mitochondrial function and autophagy is evidence that inhibition of mitochondrial fission by the Drp1 peptide inhibitor P110 increased autophagy and reduced detrimental cellular morphology changes caused by the G2019S mutation in PD patient fibroblasts and dopaminergic neuron-derived iPSCs (Su and Qi, 2013).

The role of LRRK2 in neuronal morphology is also linked to its dysregulation of autophagic processes in *LRRK2* mutants. Expression of mutant LRRK2 causes shortening and simplification of the dendritic tree *in vivo* and in cultured neurons (MacLeod *et al.*, 2006), mediated in part by alterations in macroautophagy (Alegre-Abarrategui *et al.*, 2009; Friedman *et al.*, 2012; Gómez-Suaga *et al.*, 2012; Plowey *et al.*, 2008; Ramonet *et al.*, 2011); This can be prevented by PKA regulation of LC3, a key autophagy protein (Cherra *et al.*, 2010). The G2019S and R1441C mutations induce post-synaptic calcium imbalance, leading to excessive mitochondrial clearance from dendrites by mitophagy (Cherra *et al.*, 2013). LRRK2 is also a substrate for chaperone-mediated autophagy (Orenstein *et al.*, 2013).

LRRK2 mutations have also been linked to other cellular mechanisms, such as apoptosis. Expression of mutant *LRRK2*^{G2019S}, *LRRK2*^{R1441C} and *LRRK2*^{Y1699C} induced apoptotic cell death in neuroblastoma cells and in mouse cortical neurons (Smith *et al.*, 2005) and *LRRK2* has also been associated with Crohn's disease by GWAS, suggesting that Crohn's and PD may share common pathogenic pathways (Manolio, 2010; Nalls *et al.*, 2011).

Incomplete penetrance of multiple mutations led to the identification of *LRRK2* as a risk factor for sporadic PD (Nalls *et al.*, 2014; Hernandez, Reed and Singleton, 2016). Sporadic PD patient-derived cells display slightly increased basal LRRK2 activity, however it is unknown whether this is causative or a consequence of pre-existing pathology (Esteves and Cardoso, 2015). One study demonstrated that variability within the DNM3 gene influences the age of onset in G2019S carriers (Trinh *et al.*, 2016). The presence of other genetic modifiers may therefore influence disease manifestation in the case of *LRRK2* mutants.

Overall, our current knowledge of the role of *LRRK2* in PD supports the implication of mechanisms common to other genetic factors, including dysregulation of mitochondrial and autophagic processes.

VPS35

A third gene associated with autosomal dominant PD is vacuolar protein sorting 35 (*VPS35*); a core subunit of a heteropentameric complex known as the retromer (Hierro *et al.*, 2007), which plays an important role in endosomal transport to golgi and plasma membranes, as well as in the recycling of transmembrane protein cargo (Williams, Chen and Moore, 2017). VPS35 is also involved in mediating the shuttling of cargo from mitochondria to peroxisomes and lysosomes, through the formation of

mitochondria-derived vesicles (MDVs) (Braschi *et al.*, 2010; Sugiura *et al.*, 2014). The pathogenic mechanism of VPS35 and its role in PD development is not yet clear; recent studies have implicated impaired mitochondrial dynamics and function, increased ROS, α-synuclein accumulation and cell death (Tang *et al.*, 2015; Wang *et al.*, 2016). One study revealed that VPS35^{D620N}, the most common mutation, induces mitochondrial fragmentation, dysfunction and neurotoxicity when overexpressed in both cultured neurons and mouse nigral dopaminergic neurons *in vivo*. This single mutation at D260N is sufficient to cause LOPD (Sharma *et al.*, 2012; Vilariño-Güell *et al.*, 2011; Zimprich *et al.*, 2011). Mechanistically, VPS35^{D620N} mutants displayed increased mitochondrial interaction with Drp1, an effect further promoted by oxidative stress, leading to enhanced turnover of mitochondrial-Drp1 complexes via their MDV-dependent trafficking to lysosomes for degradation (Wang *et al.*, 2016). Additionally, dopaminergic neuron specific VPS35 deficiency *in vivo* induced mitochondrial fragmentation by impairing mitochondrial fusion (Tang *et al.*, 2015); using *in vitro* and *in vivo* models, these authors demonstrate that in the absence of VPS35 or the presence of mutant VPS35^{D620N}, mitochondrial E3 ubiquitin ligase 1 (MUL1) is upregulated, leading to increased ubiquitination and proteasomal degradation of mitochondrial fusion protein Mfn2 (Tang *et al.*, 2015). Together this work strongly supports the role of VPS35 in imbalanced mitochondrial dynamics, contributing to PD risk.

Dopaminergic neurodegeneration has been further demonstrated in multiple models which explored *VPS35* mutations; aged (60 day old) *Drosophila melanogaster* VPS35^{D620N} mutants demonstrated a significant loss of dopaminergic neurons compared to age-matched controls (Wang *et al.*, 2014a). Tang et al (2015) determined ~20% loss of dopaminergic neurons in 12 month old VPS35$^{+/-}$ mice compared to

VPS35$^{+/+}$ controls, indicative of age-dependent dopaminergic neuron loss, which was accompanied by the accumulation of α-synuclein. Further investigation of the role of VPS35 in dopaminergic neuron loss in rats using AAV2-VPS35^{D20N} supported VPS35-mediated dopaminergic cell loss, with a detected 32% loss 12-weeks post-stereotactic injection (Tsika *et al.*, 2014).

Mutations in *VPS35* are rare and account for only ~1% of familial PD cases and 0.2% of sporadic PD cases; although other mutations have been identified, D620N remains the most researched and the pathological relevance of other mutants remains to be determined. Our current understanding of the pathological contribution of this gene to PD risk supports the role of mitochondrial changes in disease pathogenesis, although through distinct mechanisms compared to the other genes already discussed.

CHCHD2

The most recently identified gene linked to autosomal dominant cases of PD is coiled-coil-helix-coiled-coil-helix domain 2 (CHCHD2); with several genetic mutations identified, it is linked to late-onset familial PD and dementia with Lewy bodies (DLB) (Funayama *et al.*, 2015; Koschmidder *et al.*, 2016; Ogaki *et al.*, 2015). Interestingly, the mutations reported to date are ethnicity specific, with some detected only in Caucasian or Asian populations. CHCHD2 protein contains an MTS and has been detected in the inter-membrane space where it binds Complex IV (cytochrome C oxidase, COX). This binding is required for COX activity (Aras *et al.*, 2015) and is therefore necessary for oxidative phosphorylation (Baughman *et al.*, 2009). This critical role is consistent with the observation that loss of function mutations result in

impaired electron transport chain flux, reduced mitochondrial membrane potential, increased ROS levels and mitochondrial fragmentation (Aras *et al.*, 2015), which results in cell death in *Drosophila melanogaster* (Meng *et al.*, 2017). *CHCHD2* mutant flies display chronic oxidative stress, reduced oxygen consumption, reduced ATP production, increased cytochrome C release and mitochondria with abnormal ultrastructure, leading to degeneration of dopaminergic neurons. This study also suggests that CHCHD2 may be an important mitochondrial stress response protein, with the ability to modulate cell death signalling through interactions with cytochrome C and MICS1, a member of the Bax inhibitor-1 superfamily (Meng *et al.*, 2017). CHCHD2 predominantly localises to mitochondria and it is also functions as a transcription factor of the complex IV subunit 4 isoform COX4I2 under stress conditions, in which it translocates from the mitochondrial intermembrane space to the nucleus, where it binds to a highly conserved 13 nucleotide oxygen responsive element in the promoter of cytochrome oxidase 4I2 to influence transcription (Aras *et al.*, 2015). Therefore, loss of CHCHD2 function negatively affects complex IV both functionally and structurally.

Despite limited work to investigate the impact of *CHCHD2* mutants on mitochondrial dynamics, given its dramatic influence of the electron transport chain, it is possible that mitochondrial morphology and movement may also be affected.

GBA

Glucocerebrosidase (GCase), encoded by the *GBA* gene, exhibits pseudo-autosomal-dominant inheritance and interest in *GBA* as a PD gene stems from the observation that some Gaucher's disease patients developed Parkinsonian symptoms

(Neudorfer *et al.*, 1996), with unaffected non-Gaucher relatives of these patients displaying increased risk of developing PD (Halperin, Elstein and Zimran, 2006).

Gaucher's disease (GD) is the most common lysosomal storage disorder (Tsuji *et al.*, 1987) caused by homozygous and compound heterozygous mutations in the *GBA* gene. GD is characterised by the accumulation of glucocerebrosides in macrophages which infiltrate many vital organs (Mucci and Rozenfeld, 2015; Stirnemann *et al.*, 2017). GCAse is a lysosomal hydrolase, which is localised to and specifically acts in the lysosomal membrane to convert sphingolipid glucosylceramide to glucose and ceramide (Rijnboutt *et al.*, 1991). Approximately 300 different *GBA* mutations have been identified, with many shown to reduce GCase activity. Such a reduction has been identified in PD cases with *GBA* mutations (Kalia and Lang, 2015) and importantly in the substantia nigra of sporadic PD brains (Gegg *et al.*, 2012). More severe mutations, such as L444P, increase PD risk, promote earlier disease onset, and increase cognitive decline when compared with milder mutations, such as N370S. However, overall there is no significant difference in the mortality rate between these two mutations (Arkadir *et al.*, 2016; Cilia *et al.*, 2016; Gan-Or *et al.*, 2015); this suggests that even a partial loss of function is sufficient to cause PD. *GBA* mutation prevalence varies in different PD populations; although rare in PD patients overall, it has been found in up to 31% of PD patients with Ashkenazi Jewish ancestry (Sidransky and Lopez, 2012).

Reduced GCase activity has been associated with dysfunctional mitochondria; inhibition of GCase, whether pharmacological or genetic, results in reduced membrane potential and ATP production, along with increased oxidative stress and mitochondrial fragmentation *in vitro* (Cleeter *et al.*, 2013). Such findings have been confirmed in GD-derived patient fibroblasts and mouse models of GD. Fibroblasts from *GBA*^{L444P}

mutant patients exhibit reduced complex I-III activity, co-enzyme Q10 expression, membrane potential and ATP production, along with increased oxidative stress (de la Mata *et al.*, 2015). Mouse models have demonstrated reduced mitochondrial membrane potential, reduced complex I-III activity, reduced oxygen consumption and increased mitochondrial fragmentation in neurons and glia (Osellame *et al.*, 2013; Xu *et al.*, 2014). Ultrastructural analysis of mitochondrial morphology displayed impaired cristae organisation, with mitochondria appearing more rounded and denser (Xu *et al.*, 2014).

Mechanisms of *GBA*-associated mitochondrial dysfunction are unclear, although it has been suggested that impaired mitophagy may play a role. This is unsurprising given the role of GCase in the cell and further strengthens the link between mitochondria and autophagy in PD. Mouse models and primary fibroblast experiments demonstrate that decreased mitophagy flux accompanied the observed mitochondrial dysfunction in GBA mutants (de la Mata *et al.*, 2015). It remains unclear, however, whether impaired mitophagy responses may cause the observed mitochondrial dysfunction in these models or whether it is a consequence of upstream effects.

Neuroinflammation is another contributing factor, as demonstrated in *GBA* knockout mouse models where astrocyte and microglial activation precede disease manifestation (Enquist *et al.*, 2007; Farfel-Becker *et al.*, 2011). Astrogliosis has also been identified in some GD patients who exhibit parkinsonian symptoms (Wong *et al.*, 2004).

It is important to note however that most studies related to *GBA* included here have been undertaken in the context of GD rather than PD, and therefore conclusions

made from this work may not be directly transferable to cases of PD with *GBA* mutations, despite their relevance.

GBA expression has also been linked to the expression and aggregation of α-synuclein. In *GBA* mutant mice, accumulated aggregated α-synuclein, β-amyloid and amyloid precursor protein (APP) was observed in the cortex, hippocampus, striatum and substantia nigra (Xu *et al.*, 2014), consistent with the role of GCase in autophagy. Inhibition of *GBA* expression using zinc-finger techniques promotes the intercellular transmission of α-synuclein species, contributing toward the spread of PD pathology (Bae *et al.*, 2014). This further supports the investigation of α-synuclein intercellular transmission and also raises the possibility that targeting GCase function to enhance it may be efficacious to reduce α-synuclein spread. As increased α-synuclein has also been found to inhibit GCase function, it seems there is a feedback loop in which changes in *GBA* which mediate increased α-synuclein accumulation further increase toxicity by contributing to inhibition of the autophagic clearance of α-synuclein (Gegg *et al.*, 2012; Mazzulli *et al.*, 2011; Murphy *et al.*, 2014).

1.3.2| Autosomal recessive genes

PINK1 and *Parkin*

The most well researched autosomal recessive mutations linked to PD are *PINK1* and *Parkin*, which cause almost identical forms of autosomal recessive-early onset PD (AR-EO-PD), which may present with or without Lewy body pathology (Kitada *et al.*, 1998; Samaranch *et al.*, 2010; Sasaki *et al.*, 2004; Valente *et al.*, 2004a; Valente *et al.*, 2004b). *Parkin* mutations were first discovered in a Japanese family (Kitada *et al.*, 1998; Matsumine *et al.*, 1997) and have now been shown to encompass

a wide range of variations spanning the entire length of the gene to confer loss of function changes (Klein and Westenberger, 2012). *Parkin* mutations are considered the most prevalent AR mutation in PD, accounting for ~77% of familial EOPD and 10-20% EOPD in general (Kilarski *et al.*, 2012; Klein and Lohmann-Hedrich, 2007; Klein and Westenberger, 2012). *PINK1* mutations were first identified in an Italian family (Valente *et al.*, 2001) and are the second most common cause of AR-EO-PD, accounting for up to 9% of cases (Klein and Westenberger, 2012). Mutations span the length of the gene, with the majority presenting as missense or nonsense mutations, conferring loss of function, similarly to *Parkin*. Heterozygous *PINK1* and *Parkin* mutations may also be considered as crucial risk factors for PD (Criscuolo *et al.*, 2006; Hedrich *et al.*, 2006; Hiller *et al.*, 2007; Khan *et al.*, 2005).

PINK1, which encodes the PINK1 serine/threonine kinase localised to mitochondria, is one of the most common genes to display mutations in recessive cases of PD (Hatano *et al.*, 2004; Valente *et al.*, 2004a; Zhou *et al.*, 2008). PINK1 binds to the outer mitochondrial membrane (OMM) of normal, healthy mitochondria and is then imported to the inner mitochondrial membrane (IMM) via the translocase of the outer membrane (TOM) complex and the translocase of the inner membrane 23 (TIM23) in a membrane potential-dependent manner. Full length PINK1 is then rapidly cleaved by mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL). Cleaved PINK1 is released into the cytosol for non-mitochondrial processes (Dagda *et al.*, 2014) and can be degraded by the proteasome (Meissner *et al.*, 2011; Yamano and Youle, 2013), hence the low levels of PINK1 maintained in healthy mitochondria. The cytosolic distribution of non-mitochondrial-bound PINK1 has been suggested to function as a scout for defective mitochondria to target them for autophagic/mitophagic disposal (Narendra, Walker and Youle, 2012)

and promote neurite outgrowth (Dagda *et al.*, 2014; Das Banerjee *et al.*, 2017; Tieu and Xia, 2014; Wang *et al.*, 2018).

Upon mitochondrial damage and loss of membrane potential, mitochondrial PINK1 import is blocked, causing it to accumulate at the OMM (Jin *et al.*, 2010; Lazarou *et al.*, 2012; Okatsu *et al.*, 2013) where PINK1's C-terminal kinase domain faces the cytosol, enabling it to phosphorylate both mitochondrial and cytosolic proteins (Zhou *et al.*, 2008). PINK1 has been observed to phosphorylate ubiquitin and ubiquitin-like (Ubl) domains of Parkin, a ubiquitin E3 ligase, at a conserved Ser65 residue. This phosphorylation initiates the cascade which subsequently activates and recruits Parkin to mitochondria (Durcan and Fon, 2015; Roberts *et al.*, 2016). Active Parkin promotes ubiquitination of multiple OMM proteins, such as mitochondrial fusion proteins Mfn1/2 (Youle and van der Bliek, 2012) and TOM complexes (TOM20 and TOM70), targeting them for degradation via the proteasomal pathway (Chan *et al.*, 2011b; Gegg *et al.*, 2010; Kazlauskaite *et al.*, 2014; Seirafi, Kozlov and Gehring, 2015; Yoshii *et al.*, 2011) as well as the recruitment of autophagy adaptor proteins (Roberts *et al.*, 2016). PINK1 and Parkin participate in a positive feedback cycle in mitophagy initiation; PINK1's role in the phosphorylation of ubiquitin and Parkin at Ser65 potentiates Parkin mobilisation and substrate ubiquitination (Seirafi, Kozlov and Gehring, 2015). The PINK1/Parkin axis is thereby an important mediator of mitochondrial quality control and mediates selective removal of damaged organelles by mitophagy (Geisler *et al.*, 2010; Narendra *et al.*, 2010; Narendra, Walker and Youle, 2012; Vives-Bauza *et al.*, 2010).

Mutations in *Parkin* are associated with mitochondrial dysfunction, leading to neuronal death in PD (Dawson and Dawson, 2010) . *In vivo* and *in vitro* models have investigated the effect of PINK1 and Parkin mutants on mitochondrial abnormalities.

PINK1 deficient and PD-associated mutant models display reduced mitochondrial membrane potential, reduced ATP levels, reduced respiratory capacity via complex I and IV activity, increased mitochondrial calcium levels, sensitised mitochondrial permeability transition pore opening and increased ROS production (Cui *et al.*, 2010; Dagda *et al.*, 2009; Gandhi *et al.*, 2009; Gautier, Kitada and Shen, 2008; Gegg *et al.*, 2009; Heeman *et al.*, 2011; Liu *et al.*, 2009; Morais *et al.*, 2009; Morais *et al.*, 2014; Piccoli *et al.*, 2008). Isolated mitochondria from the brains of aged *PINK1* null mice displayed complex I deficits (Gautier, Kitada and Shen, 2008; Morais *et al.*, 2014) and reduced calcium buffering capacity, whilst aged *Parkin* null brains showed alterations in the expression of a wide range of mitochondrial proteins, with many related to reduced respiratory capacity and increased oxidative damage (Palacino *et al.*, 2004; Periquet *et al.*, 2005; Stichel *et al.*, 2007).

PINK1 and Parkin also play regulatory roles in mitochondrial dynamics through mechanisms including the turnover of fusion and fission proteins, such as Mfn1/2 and Drp1 (Buhlman *et al.*, 2014; Clark *et al.*, 2006; Cui *et al.*, 2010; Deng *et al.*, 2008; Exner *et al.*, 2012; Gegg *et al.*, 2010; Lutz *et al.*, 2009; Poole *et al.*, 2008; Pryde *et al.*, 2016; Tanaka *et al.*, 2010; Yang *et al.*, 2008; Ziviani, Tao and Whitworth, 2010). It remains a topic of debate whether PINK1 and Parkin are pro-fission or pro-fusion in their contribution to the regulation of mitochondrial dynamics (Exner *et al.*, 2012; Pilsl and Winklhofer, 2012). Loss of PINK1 function, or expression of human relevant *PINK1* mutants, has been demonstrated to increase mitochondrial fission in mammalian cell models. Parkin has been reported to ubiquitinate Mfn2, leading to enhanced mitochondrial fission which facilitates mitophagy; the functional role of Parkin in regulating mitochondrial dynamics is as yet incompletely understood (Basso *et al.*, 2018; Pickrell and Youle, 2015). Overexpression of Drp1 can rescue subjects

deficient in *PINK1* or *Parkin*, suggestive that mitochondrial fission initiated by Drp1 recreates the same effects of the PINK1-Parkin pathway (Poole *et al.*, 2008). Parkin targeting of Miro (Narendra, Walker and Youle, 2012) produces a marked reduction in mitochondrial motility, facilitating spatial separation of defective mitochondria from their healthy counterparts (Shlevkov *et al.*, 2016) and PINK1 may also influence mitochondrial movement by arresting mitochondrial motility to enhance degradation.

PINK1 has an MTS at the N-terminus and a serine/threonine kinase domain at the C-terminus, which faces the cytosol. Although best known for its role in mitophagy, a portion of PINK1 is also associated with the cytosol to promote dendritic outgrowth by enhancing mitochondrial transport to dendrites (Dagda *et al.*, 2014; Das Banerjee *et al.*, 2017; Tieu and Xia, 2014; Wang *et al.*, 2018).

PINK1 and Parkin have also been shown to mediate mitochondrial biogenesis through indirect interaction with PGC1α, the master regulator of mitochondrial biogenesis (Han, Kim and Son, 2014; Sanchis-Gomar *et al.*, 2014; Valero, 2014; Zheng *et al.*, 2017). PGC1α-repressing PARIS (ZNF746) is increased in the striatum and substantia nigra of PD patients with AR and sporadic PD, as well as in *Parkin* KO mice (Shin *et al.*, 2011).

As Parkin is a ubiquitin protein ligase, thought to be involved in the degradation of abnormal proteins by the proteasome (McNaught *et al.*, 2001), evidence suggests it may have a role in the degradation of glycosylated α-synuclein, as well as other known neurotoxic proteins to reduce their accumulation (Haass and Kahle, 2001; Shimura *et al.*, 2001). This evidence has contributed to the hypothesis that defects in protein degradation may be a common aetiopathogenic factor unifying different PD causes (McNaught *et al.*, 2001). It has also been suggested that deficiency of ATP

production attributable to mitochondrial dysfunction may lead to failure of the proteasomal proteolytic system (DeMartino and Slaughter, 1999), which is further relevant in the context of *Parkin* and *PINK1* mutants which contribute to mitochondrial regulation, as described above. Evidence suggests that PINK1 may also play a role in the creation of MDVs, which can separate ROS and shuttle them towards lysosomes for degradation (McLelland *et al.*, 2014).

Various studies have pharmacologically targeted PINK1 as a potential PD therapy; a 2013 study demonstrated that kinetin was an effective PINK1 activator (Hertz *et al.*, 2013) and subsequent work with a nucleoside kinetin derivative (kinetin riboside) demonstrated significant activation of PINK1 in cells (Osgerby *et al.*, 2017). Monophosphate prodrugs of kinetin riboside (ProTides) have also shown activation of PINK1 (Osgerby *et al.*, 2017). Recent work has also identified niclosamide (an anthelmintic drug) as a potent PINK1 activator (Barini *et al.*, 2018).

Parkin also functions in cell survival pathways by activation of NF-KB signalling, enhancing survival and protecting cells from stress-induced apoptosis by indirectly potentiating transcription of the mitochondrial GTPase OPA1 (Laforge *et al.*, 2016); Increased OPA1 translation maintains mitochondrial cristae structure and reduces mitochondrial cytochrome C release, thereby inhibiting caspase-mediated apoptosis. Importantly, Parkins indirect activation of NF-KB for this increased transcription has greater potency than other associated factors, demonstrating that Parkin mobilisation significantly enhances moderate stressor tolerance (Laforge *et al.*, 2016). Parkin can also produce a dose-dependent reduction in the transcription and activity of pro-apoptotic factor p53 by direct binding of Parkin to the p53 promoter via its RING1 domain (da Costa *et al.*, 2009). Parkin aids p53 in maintaining mitochondrial

respiration while limiting glucose uptake and lactate production as part of its role in mitochondrial homeostasis (Matoba *et al.*, 2006).

Overall, current research demonstrates a strong link between Parkin and PINK1 function in relation to processes affected by other PD-related genetic risk factors, including influence in a number of mitochondrial activities.

DJ-1

DJ-1 mutations in PD were identified by homozygosity mapping in two consanguineous families from Italy and the Netherlands with early onset PD (Bonifati *et al.*, 2003). Mutations in *DJ-1* collectively account for approximately 1-2% of EO-AR-PD, including deletions, homozygous and heterozygous point mutations, and truncations (Bonifati *et al.*, 2003). A small cysteine protease of 183 residues, DJ-1 localises to the nucleus, mitochondria and cytoplasm (Canet-Avilés *et al.*, 2004; Junn *et al.*, 2009; Li *et al.*, 2005). DJ-1 is involved in regulating mitochondrial activity (Hayashi *et al.*, 2009; Junn *et al.*, 2009) and protecting against oxidative stress (Taira *et al.*, 2004).

DJ-1 interacts with mitochondrial subunits of complex I and is translocated to mitochondria under stress conditions (Hayashi *et al.*, 2009). The WT protein and its mutant forms can associate with HSP70, a chaperone protein; this association is strengthened under oxidative stress conditions, which is suggestive that DJ-1 translocation to mitochondria may occur with the aid of mitochondrial chaperone proteins (Li *et al.*, 2005). Dimeric DJ-1 may be required for oxidative stress reactions, whilst monomeric mutant forms are cellularly toxic (Maita *et al.*, 2013; Zhang *et al.*, 2005).

DJ-1 has a critical role in the oxidative stress response, mitochondrial function, and basal autophagy processes. Studies have found *DJ-1* knockout mice and flies display mitochondrial dysfunction and reduced membrane potential (Giaime *et al.*, 2012; Hao, Giasson and Bonini, 2010). *DJ-1* KO mice are more vulnerable to the mitochondrial toxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Kim *et al.*, 2005; Manning-Boğ *et al.*, 2007). This suggests that DJ-1 may confer some protection against mitochondrial toxicants when functional.

Proper balance of mitochondrial dynamics is also influenced by DJ-1; primary cortical neurons and embryonic fibroblasts cultured from *DJ-1* KO mice and human dopaminergic neurons with *DJ-1* knockdown exhibit mitochondrial fragmentation and reduced Mfn1 levels (Irrcher *et al.*, 2010; Krebiehl *et al.*, 2010; Thomas *et al.*, 2011). Promotion of mitochondrial fusion or blockade of mitochondrial fission attenuates development of this abnormal morphology (Irrcher *et al.*, 2010; Thomas *et al.*, 2011).

Loss of DJ-1 function has also been linked to impaired mitochondrial function and defective autophagy (Irrcher *et al.*, 2010; Krebiehl *et al.*, 2010; Thomas *et al.*, 2011), in which *PINK1* and *Parkin* overexpression is protective (Irrcher *et al.*, 2010; Thomas *et al.*, 2011). Results are consistent with the suggestion that DJ-1 may aid the clearance of misfolded proteins and damaged mitochondria by PINK1 and Parkin (Thomas *et al.*, 2011; Xiong *et al.*, 2009a).

Under oxidative conditions, DJ-1 inhibits aggregation of α-synuclein via chaperone activity (Shendelman *et al.*, 2004), thus functioning as a redox-sensitive chaperone and an oxidative stress sensor. DJ-1 apparently protects neurons against oxidative stress and cell death (*PARK7 Gene*, 2019), and has also been shown to bind

metals and protect against metal-induced cytotoxicity from copper and mercury (Björkblom *et al.*, 2013).

Overall results from *DJ-1* studies to date support its role in PD risk and its common mechanisms of potential pathology, including mitochondrial dysfunction, autophagy dysregulation and α-synuclein aggregation.

ATP13A2

ATP13A2 encodes ATPase type 13A2 (ATP13A2), which is usually localised to lysosomes (*ATP13A2 Gene*, 2019). Mutations in this gene were originally reported in Kufor-Rakeb syndrome (KRS) (Ramirez *et al.*, 2006) which is a recessively inherited disease that manifests as juvenile onset parkinsonism with pyramidal degeneration, supranuclear gaze palsy and dementia (Williams *et al.*, 2005). KRS patient-derived fibroblasts display reduced ATP synthesis, increased mtDNA mutations, impaired oxygen consumption and increased mitochondrial fragmentation (Grünewald *et al.*, 2012). *ATP13A2* mutations are also found in non-KRS early-onset parkinsonism (Di Fonzo *et al.*, 2007; Yang and Xu, 2014), suggestive that mutation of this gene also affects broader cases of PD.

ATP13A2 is a multifunctional protein linked to endosome-lysosome dynamics (Dehay *et al.*, 2012; Usenovic and Krainc, 2012), mitochondrial function (Grünewald *et al.*, 2012; Gusdon *et al.*, 2012) and divalent cation metal-induced (eg. Mn^{2+} and Zn^{2+}) toxicity (Gitler *et al.*, 2009; Kong *et al.*, 2014). ATP13A2 is a cation-transporting ATPase involved in the transport of divalent transition metal cations (Ramirez *et al.*, 2006; Schultheis *et al.*, 2004). It appears to confer cellular protection against manganese (Mn) (Tan *et al.*, 2011) and zinc (Tsunemi and Krainc, 2014) toxicity, possibly by inducing cellular efflux and/or lysosomal sequestration of the cations. It

has also been shown to have effects on iron toxicity, possibly by preserving lysosome integrity against iron-induced lipid peroxidation (Rinaldi *et al.*, 2015), and it seems to potentiate the toxicity of cadmium and nickel on developing neurites (Podhajska *et al.*, 2012). Loss of function of this protein has been reported to reduce the ability of vesicular structures, such as lysosomes, to regulate divalent metal cations and thereby sensitise cells to metal toxicity, α-synuclein accumulation and mitochondrial dysfunction (Gitler *et al.*, 2009; Kong *et al.*, 2014). These are most likely downstream events resulting from autophagy defects, leading to the accumulation of misfolded proteins and defective mitochondria. Research has demonstrated that overexpression of *ATP13A2* is protective against α-synuclein-induced toxicity (Gitler *et al.*, 2009; Kong *et al.*, 2014).

Overall, ATP13A2 may exert indirect, protective functions on mitochondria and the cell as a whole through its roles in the regulation of endosome-lysosome dynamics, mitochondrial health, and metal cation toxicity.

Whilst not an exhaustive exploration of all that is known about PD-related genes, all of the PD-linked genes and risk genes discussed above have been linked to crucial cellular homeostatic processes, including autophagy, protein misfolding and accumulation, and mitochondrial regulation. These seem to be key aspects of cellular health which present with defects in PD pathogenesis, and as such represent important targets for research to further understand their significance for therapeutic targeting.

1.4| Environmental risk factors

Prior to the identification of genetic PD risk factors, environmental factors were the major candidates in the search to better understand the aetiology of PD pathogenesis. The rationale for this dominant environmental theory was largely based on observations of infection and toxicant-exposure induced parkinsonism (Calne *et al.*, 1994; Kwakye *et al.*, 2015; Langston *et al.*, 1983; Rail, Scholtz and Swash, 1981). Epidemiological studies and meta-analyses have implicated a number of environmental factors, including exposure to pesticides, herbicides, insecticides, heavy metals and infection, with increased PD risk [Table 2] (Gorrell, DiMonte and Graham, 1996; Gorrell *et al.*, 1997; Kamel, 2013; Tanner *et al.*, 2011; Wang *et al.*, 2011). Among these factors, pesticides and heavy metals have been the most widely studied (Priyadarshi *et al.*, 2000; Van der Mark *et al.*, 2012); their ability to persist in the environment long after application increases the likelihood of human exposure to the contaminants. As our understanding of the genetics of PD has increased over the past three decades, the low incidence of genetically linked PD cases highlights the necessity for research to delineate the role of environmental risk factors in PD risk and development, especially as our understanding of the interplay between genetics and environmental factors to confer PD risk improves.

Table 2: A summary of some environmental chemical risk factors for Parkinson's disease

Risk	Applications	Exposure Route	Clinical Phenotype and Neuropathology	Mechanism of Action
2,4-D	Herbicide	Ingestion, inhalation, occupational	α-synuclein oligomerisation and Lewy body pathology	Dysfunctional mitochondrial energetics and calcium flux
β-HCH	Insecticide	Ingestion	Risk factor for PD and AD elevated in PD patients, also carcinogenic	Increased oxidative stress
Benomyl	Fungicide	Occupational and agricultural	Risk factor for PD, diabetes and various cancers, including non-Hodgkin's lymphoma	Prevents toxin breakdown and interferes with intracellular transport
DDT	Pesticide specific for mosquitos	Ingestion and agricultural	Links to both PD and AD, some studies report stronger links to AD	Opens sodium channels in neurons, causing spontaneous firing
Dieldrin	Pesticide specific for termites	Ingestion	Risk factor for PD α -synuclein aggregation, intracellular DA depletion	Initiates apoptosis, oxidative stress and GSH depletion
Lindane (γ-HCH)	Pesticide, insecticide	Ingestion and agricultural	Risk factor for PD	Interferes with GABA function, interacting with $GABA_A$ receptor-chloride channel complex at the picrotoxin biding site
Maneb	Fungicide	Agricultural and occupational	Loss of SNpc neurons and Lewy body pathology	Disrupts mitochondrial function, increases oxidative stress and inhibits proteasomal function
Manganese	Numerous industrial uses	Inhalation, ingestion and occupational	Causes Manganism, risk factor for PD and results in motor abnormalities	Inhibits tyrosine hydroxylation, thereby inhibiting dopamine production
Paraquat	Herbicide	Ingestion, agricultural and occupational	Loss of SNpc neurons and Lewy body pathology	Increased ROS, specifically superoxide
PCBs	Coolants for electrical equipment	Ingestion and dermal	Increased PD risk and unspecified neurotoxicity	Increased ROS and elevated oxidative stress
Permethrin	Insecticide	Dermal	Reduced dopamine levels, muscle rigidity, rhythmic tremor and shuffling gait	Unknown
Pyrethroid	Insecticide	Inhalation, ingestion and dermal	Widely used household insecticide, used in insect repellents	Increased oxidative stress, apoptosis and neuroinflammation
Rotenone	Pesticide, insecticide and piscicide	Agricultural and occupational	Loss of SNpc dopaminergic cell bodies and STR terminals, reduced striatal DA content, Lewy body pathology, L-DOPA responsive motor deficits in animal models	Complex I inhibition, increased ROS generation and inhibited dopamine production
TCE	Solvent	Ingestion, dermal and industrial	Risk factor for PD	Unknown
Ziram	Fungicide	Dermal and occupational	α -synuclein pathology	Inhibits UPS function

2,4-D, 2,4-Dichlorophenoxyacetic acid; β-HCH, β-Hexachlorocyclohexane; DDT, Dichlorodiphenyltrichloroethane; GSH, glutathione; PD, Parkinson's disease; PCBs, polychlorinated biphenyls; ROS, reactive oxygen species; TCE, Trichloroethylene; UPS, Ubiquitin proteasome system

1.4.1| Pesticides

Rotenone

Rotenone is a pesticide, piscicide and herbicide widely used in organic farming due to its derivation from the seeds, stems and roots of several plants of the *Leguminosa* family (Soloway, 1976). The first reports of rotenone-containing plants application to control pests come from 1848 and for centuries the same plants were used as piscicides by indigenous people to catch fish (Metcalf, 1948; Schmidt, 2010). Rotenone was first licensed for piscicide use in the United States in 1952, however it is not licensed for use in any of its applications in the European Union (Database, Accessed 2018; Schmidt, 2010). Rotenone is classed by the World Health Organisation as moderately hazardous (International-Programme-on-Chemical-Safety, 2009); Whilst it is considered only mildly toxic to humans and other mammals, it is extremely toxic to insects and aquatic life. Accidental poisoning with rotenone is rare in humans due to its irritant property rapidly inducing vomiting, however deliberate ingestion can be fatal, with both deliberate ingestion for suicides and accidental deaths reported (De Wilde, Heyndrickx and Carton, 1986; Holland, 1938; Wood *et al.*, 2005).

In 2011, a case-control NIH study reported that rotenone exposure increased the risk of PD (Tanner *et al.*, 2011), though prior to this report rotenone was already under investigation as a means to model PD in research, both *in vitro* and *in vivo*. *In vivo,* rotenone has been used to model PD in multiple species, including rodents, zebrafish, *Drosophila melanogaster, C. Elegans* and *L. Stagnalis* (Betarbet *et al.*, 2000; Bretaud, Lee and Guo, 2004; Ray *et al.*, 2014; St Laurent, O'Brien and Ahmad, 2013). It was first applied systemically to investigate the role of systemic mitochondrial toxin administration by Ferrante (1997), however it wasn't until later work following titration of the rotenone concentration that selective nigrostriatal degeneration was

reported (Betarbet *et al.*, 2000). In the rotenone rodent models of PD, the observed neurodegeneration is comparable to clinical PD, with damage apparent in dopaminergic nerve terminals of the striatum and cell bodies in the substantia nigra (Bai *et al.*, 2016; Betarbet *et al.*, 2000; Cannon *et al.*, 2009); evidence of α-synuclein aggregation in neurons of the brain and GI tract has also been reported (Drolet *et al.*, 2009; Bove *et al.*, 2005; Pan-Montojo *et al.*, 2010). Motor changes are sometimes discernible in these models, indicative that rotenone may reproduce both the behavioural and associated neuropathological changes distinguished in PD (Drolet *et al.*, 2009; Bai *et al.*, 2016). Various methods of rotenone administration have been utilised to model PD *in vivo*, including subcutaneous, intraperitoneal (IP), intragastric and intrastriatal stereotactic delivery (Bai *et al.*, 2016; Betarbet *et al.*, 2000; Carriere, Kang and Niles, 2014; Caboni *et al.*, 2004; Drolet *et al.*, 2009; Heikkila *et al.*, 1985; Mulcahy *et al.*, 2011), with variability in the pathogenic changes reported. IP rotenone treatment, at doses ranging from 1-3mg/kg, has been demonstrated to reproduce several parkinsonian features in rodents, including the phosphorylation and aggregation of α-synuclein in the SNpc. Adverse health effects caused by systemic administration limit the use of this model for an extended time at high doses (Cannon *et al.*, 2009). Intrastriatal stereotactic delivery of rotenone has been utilised to avoid the systemic effects of IP delivery, however the environmental relevance of the model is then compromised, as it bypasses the body's natural physical and metabolic defences, removing the opportunity to investigate peripheral changes which develop during the pre-motor stages of the disease. Stereotactic delivery to the medial forebrain bundle depletes both striatal dopamine and serotonin, although acute toxicity has been observed (Rojas *et al.*, 2009). However, α-synuclein pathology is absent in these animals, demonstrating that direct rotenone delivery to the brain fails to

recapitulate this key neuropathological PD feature. Oral rotenone administration has been used to induce gastric α-synuclein pathology, demonstrating that gut-initiated protein pathology may potentiate neuronal changes, as proposed by Braak et al (2003); along with gastric changes, intragastric rotenone administration has also been shown to cause a loss of TH-positive neurons in the SNpc (Drolet *et al.*, 2009; Inden *et al.*, 2011; Pan-Montojo *et al.*, 2010). A more recent environment-contact rotenone mouse model has also been investigated, which explored the potential role of dermal contact and inhalation as concurrent exposure routes, removing the difficulties of systemic toxicity whilst successfully demonstrating rotenone-induced impairment of motor function, nigrostriatal degeneration and depletion of striatal dopamine (Liu *et al.*, 2015). To date only one study has published results from this model however, so further work is necessary to ascertain its corroboration of previously reported mechanisms of rotenone-induced pathology.

In terms of human exposure, the relatively short half-life (~3 days) (Hisata, 2002) limits the risk of rotenone exposure via consumption of exposed food products, with occupational exposure more likely. Epidemiological studies have surmised that rotenone increases PD risk by ~1.5-3 fold (Nandipati and Litvan, 2016; Tanner *et al.*, 2011). The high lipophilicity of rotenone allows it to easily pass through the blood brain barrier without requirement for specific transporter interactions; once inside cells it can directly alter mitochondrial function by impairing oxidative phosphorylation through inhibition of NADH-ubiquinone reductive activity via binding to the PSST subunit of complex I (Schuler and Casida, 2001). This results in a back-up of electrons in the mitochondrial matrix which then reduce cellular oxygen to produce superoxide and other ROS that can further potentiate rotenone-induced cellular damage. This complex

I inhibition is consistent with the observation of reduced complex I activity in PD patient mitochondria (Parker, Boyson and Parks, 1989; Schapira *et al.*, 1989).

Rotenone has been shown to interfere with multiple cellular functions, including mitochondrial homeostasis. It was demonstrated to enhance mitochondrial fission in rat-derived primary cortical neurons, where it induced rapid mitochondrial fragmentation within two hours of treatment, prior to any cellular changes indicative of cytotoxicity (Barsoum *et al.*, 2006); this suggests that mitochondrial dysfunction is an early consequence of rotenone toxicity, upstream of other cellular effects. Investigation of mitochondrial dynamics as a protective target against rotenone-induced neurotoxicity provided promising results *in vitro*; Barsoum et al (2006) promoted mitochondrial fusion with Mfn2 overexpression and blocked mitochondrial fission with expression of Drp1-K38A, a dominant negative mutation, to demonstrate protection against rotenone-induced mitochondrial fission *in vitro*. To date there is no published exploration of the potential neuroprotective effects of Drp1 inhibition or mitochondrial dynamics regulation in *in vivo* rotenone models of PD.

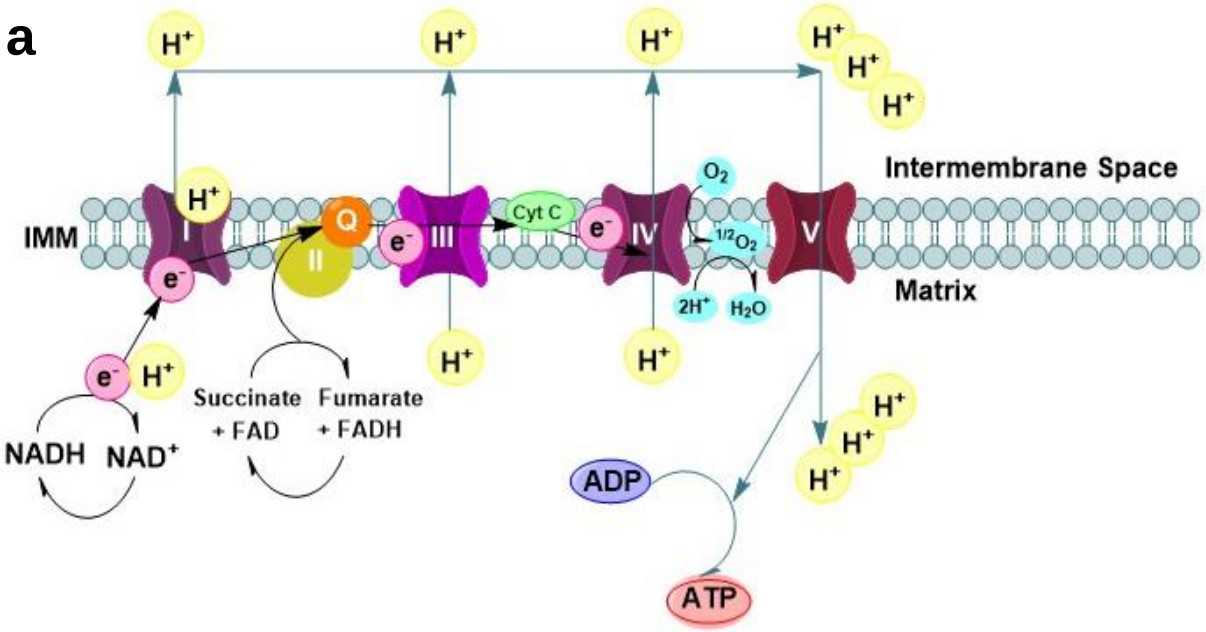
Chernivec, Cooper and Naylor (2018) demonstrated that rotenone reduced mitochondrial fusion and altered cytoskeletal protein interactions by disrupting the actin and microtubule cytoskeleton; increasing concentrations of rotenone drove microtubules to less-assembled shorter structures, with similar results demonstrated upon analysis of actin filaments with decreased complexity of the network and reduced length of individual actin fibres. Rotenone was also demonstrated to inhibit mitochondrial fusion in this model, with no detected effect on mitochondrial fission. However, as the only currently published study of rotenone in *Dictyostelium discoideum*, further verification of their findings would be beneficial to support their conclusions. In 2011, Choi et al (2011) demonstrated that rotenone caused

microtubule depolymerisation, accumulation of cytosolic dopamine and increased reactive oxygen species in mesencephalic cultures. The identification of cytoskeletal changes in these rotenone-models could contribute to changes in mitochondrial movement and thereby contribute to mitochondrial abnormalities in rotenone toxicity.

Rotenone has also been shown to interact with multiple genetic risk factors for PD. It was demonstrated to increase the interaction of α-synuclein with the mitochondrial outer membrane protein TOM20 in the dopaminergic neurons of rats (Di Maio *et al.*, 2016), by which it contributes to mitochondrial dysfunction indirectly by increasing α-synuclein accumulation in mitochondria. Rotenone also contributes to the release of α-synuclein by enteric neurons in an intragastric rotenone exposure model; this research demonstrated that chronic rotenone treatment (1.5-3 months) induced α-synuclein accumulation in the enteric nervous system (ENS), dorsal motor nucleus of the vagus (DMV), intermediolateral nucleus of the spinal cord and the substantia nigra. The results also exhibited inflammatory changes in the ENS and DMV, alongside α-synuclein phosphorylation (Pan-Montojo *et al.*, 2010). This publication supports the proposed gut-brain axis in PD development and suggests that chronic gastric exposure to the pesticide may induce neuropathological changes, which are potentiated by *SNCA* expression as rotenone enhances α-synuclein mediated neurotoxicity. Rotenone has similarly been shown to interact with the $LRRK2^{R1441G}$ mutant in mice, enhancing their susceptibility to rotenone-induced ATP-deficiency and death. Investigation of striatal synaptosome function following rotenone treatment resulted in reduced synaptosomal dopamine uptake as a result of reduced mitochondrial content and synaptic vesicular proton pump protein (V-ATPase H) levels. $LRRK2^{R1441G}$ mice presented with more pronounced locomotor deficits than WT controls following chronic intermittent oral rotenone exposure (Liu *et al.*, 2017).

Primary neuronal cultures from WT or *Parkin* knockout mice treated with different rotenone concentrations demonstrated enhanced rotenone sensitivity in *Parkin* KO cultures (Casarejos *et al.*, 2006). As Parkin functions in mitophagy, the cumulative effects of Parkin defective cells with rotenone may relate to reduced clearance of defective mitochondria from the neuronal mitochondrial population, inhibiting effective cellular respiration and calcium homeostasis. Rotenone may thereby enhance the likelihood of disease development in individuals with Parkin mutations, or it may decrease the age of disease onset by potentiating pathogenic processes. Increased vulnerability of *VPS35* mutants in primary neuronal cultures treated with rotenone has also been described (Tsika et al, 2014), suggestive that increased PD susceptibility results from the cumulative impact of rotenone and genetic mutations.

These studies highlight the importance of rotenone as a risk factor for PD, both independently and as a contributing factor to individuals with increased genetic susceptibility, further highlighting the importance of investigating the role which rotenone plays in PD aetiology and pathogenesis.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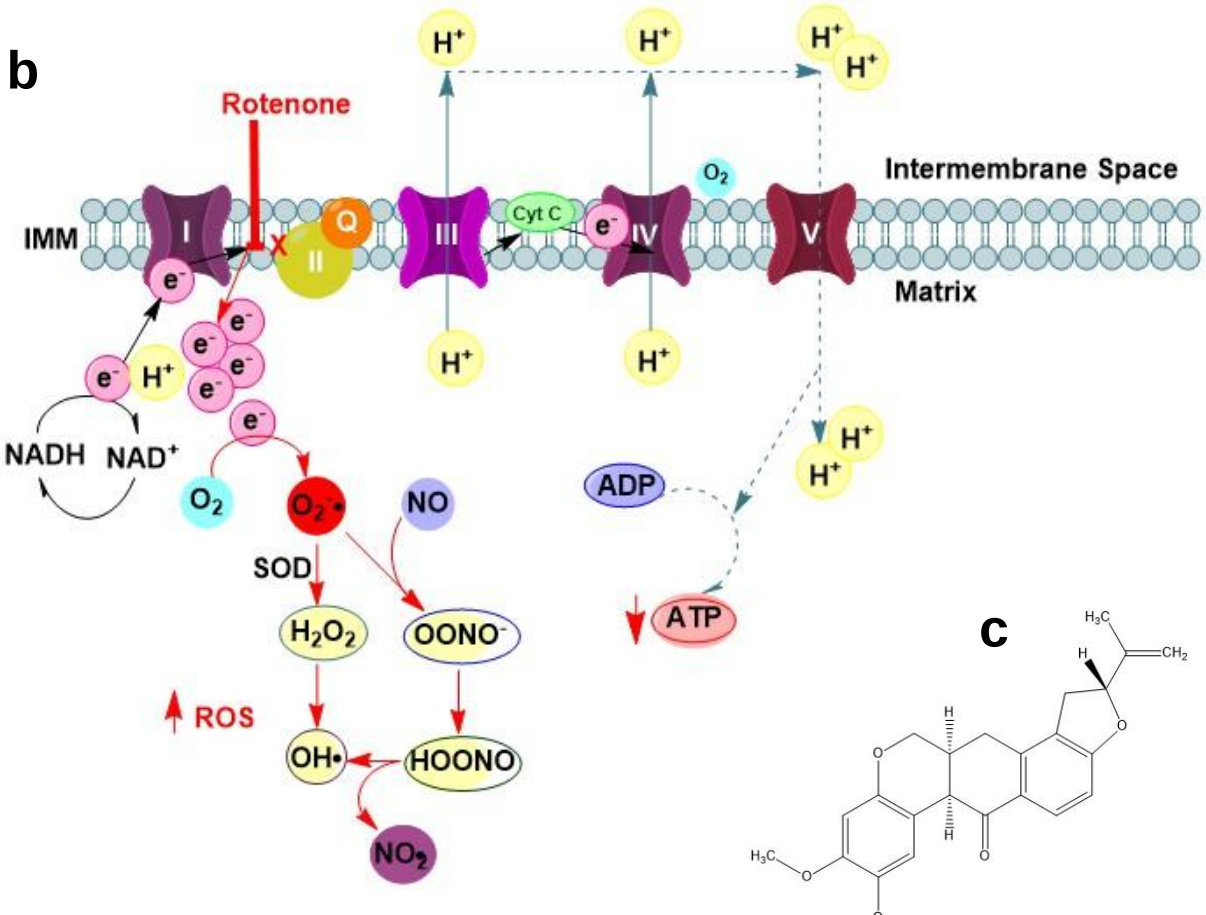
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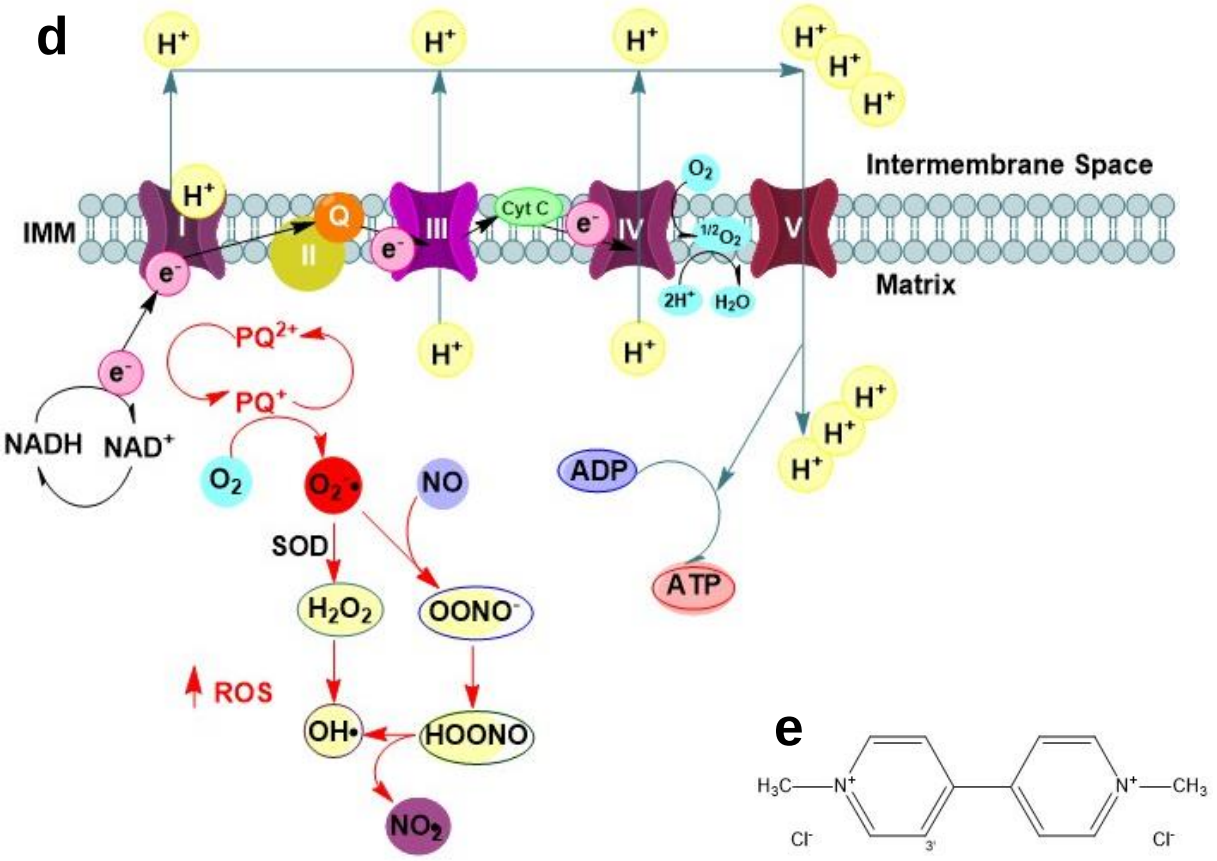


Figure 2: Rotenone and paraquat induce distinct mechanisms of mitochondrial dysfunction. (**a**) Functional mitochondrial electron transport chain. (**b**) Rotenone causes a two-hit change to mitochondrial electron transport chain function; first by inhibition of electron transfer from Complex I to ubiquinone, reducing electron flow to reduce ATP production; secondly by generation of superoxide and downstream ROS as a result of the electron accumulation in the mitochondrial matrix. (**c**) Rotenone structure. (**d**) Paraquat redox cycling, including by NADH at electron transport chain Complex I, causes ROS generation which induce its toxic effects. Regeneration of di-cationic paraquat facilitates continuation of this process and the downstream effects of ROS on cellular function. (**e**) Paraquat structure.

Paraquat

Paraquat (*N-N'*-methyl-4,4'-bipyridinium dichloride; PQ) is a herbicide which has also been implicated in PD development; it interferes with photosynthesis and oxygen free radical production, resulting in damage to plant membranes (Breckenridge *et al.*, 2013; Tanner *et al.*, 2011). As a cheap, non-selective, rapid-acting herbicide PQ is one of the most widely used pesticides worldwide (CDC, 2019). First synthesised in 1882, the herbicidal properties were not identified until 1955, with its primary production for agricultural use initiated in the early 1960's (Health, 2018). There are

several distinguishing features of PQ which make it an attractive choice of herbicide; it is non-selective so it rapidly kills a broad range plants on contact, it is rain-fast within minutes and it partially inactivates upon contact with soil (Coats *et al.*, 1966; Revkin, 1983). PQ played a major role in the development of 'no-till farming' (Huggins and Reganold, 2008), a conservative method which reduces loss of top soil and contributes to long-term protection of farming land. As no-till farming methods are adopted more widely, PQ use may concurrently increase as application of the herbicide helps to control weed populations which are more persistent in this method of farming.

PQ is currently classed as a Category II toxin, however there is active campaigning for reclassification due to the acute toxicity and high mortality in cases of accidental exposure (PubChem, 2019). Accidental ingestion in humans typically leads to acute respiratory distress syndrome, and with no specific antidotes available, death due to asphyxiation can result between a few hours and ~30 days post-ingestion. Guidance for treating PQ-exposed patients includes warnings against oxygen administration which may potentiate toxicity (Pratt, Keeling and Smith, 1980). A major danger of PQ is the low dose required to induce the catastrophic pathophysiological changes which precede death; The LD_{50} of PQ in humans is only 3-5mg/kg (Roberts and Reigart, 2013), whereas the LD_{50} in mice is as high as 196mg/kg (National Library of Medicine, 1992). Although PQ is less acutely toxic than some other pesticides, such as rotenone, there have been multiple reports of its lethality (Hsu, Chang and Lin, 2003; Smith, 1988; Zhou *et al.*, 2013), including the use of PQ in some developing countries as a method of suicide, due to its low cost and the small dose required (Daisley and Hutchinson, 1998; Wilks, 1999).

A member of the viologen chemical family, PQ can exist in mono- and di-cationic forms (Bockman and Kochi, 1990). It contributes to the production of

intracellular reactive oxygen species due to its capacity for rapid electron transfer, which can increase intracellular ROS that then initiate oxidative stress cascades, particularly in cells where protective mechanisms are overwhelmed or ineffective. PQ has been shown to contribute to superoxide production in the mitochondrial matrix following uptake of the dicationic form, which is then reduced by complex I to the radical monocation, that reacts with oxygen to form superoxide (Cochemé and Murphy, 2008). The membrane-dependent carrier-mediated uptake of PQ^{2+} is distinct from that of the lipophilic rotenone transition across membranes to inhibit complex I and induce superoxide production; PQ-mediated superoxide production does not directly inhibit mitochondrial complex I function. This may in part relate to the requirement of the mitochondrial membrane to enhance PQ uptake into the mitochondrial matrix or to assist in its reduction by the respiratory chain.

PQs herbicidal method of action relies on its redox capacity; in light-exposed plants, PQ interferes with photosynthesis by accepting electrons from ferredoxin of photosystem I and transferring them to molecular oxygen to produce superoxide ions. This results in the downstream production of destructive oxygen species, which increasingly oxidatively stress the cells leading to cytotoxicity. The continual regeneration of divalent PQ enables the perpetuation of this reductive cycle, leading to the destruction of cell membranes and cell death. In plants, this process appears as rapid yellowing and desiccation of the leaves (Bus *et al.*, 1976; Bus and Gibson, 1984; Fukushima *et al.*, 2002). PQs distinct mechanism of action has led to its use targeting herbicide-resistant crops, particularly plants resistant to the widely-used glyphosate (Beckie, 2011). Glyphosate, most commonly known as Roundup, inhibits the production of key amino acids in plant growth, however dual-treatment regimens

utilising both Roundup and PQ demonstrate greater efficacy of weed control (Eubank *et al.*, 2008).

In 2011, an NIH study of farm workers demonstrated a link between PQ and PD (Tanner *et al.*, 2011) and further studies have supported the conclusion that PQ increases the risk of developing PD, with a two-fold increase in risk suggested by meta-analyses (Goldman *et al.*, 2012; Kamel, 2013; Pezzoli and Cereda, 2013). Further epidemiology studies linked PQ to PD, both independently and in conjunction with other pesticides (Costello *et al.*, 2009; Ritz *et al.*, 2009; Tanner *et al.*, 2011). Investigation of the effect of PQ in various laboratory models has confirmed its induction of oxidative stress in *Drosophila melanogaster* leading to early mortality, and the development of PD-like neurological degeneration in rats (Ossowska *et al.*, 2006; Rzeznicak *et al.*, 2011). PQ primarily induces toxicity through redox cycling with cellular diaphorases such as NADPH oxidase and nitric oxide synthase (Day *et al.*, 1999), leading to superoxide generation. Superoxide propagates the downstream production of further ROS which facilitate lipid peroxidation and cellular damage (Bus and Gibson, 1984; Bus *et al.*, 1976). Despite structural similarities to MPP+ (Snyder and D'Amato, 1985), PQ is not a complex I inhibitor (Richardson *et al.*, 2005), however it does produce superoxide from mitochondrial redox cycling (Cochemé and Murphy, 2008) and Complex III has also been implicated in PQ-induced superoxide production (Castello, Drechsel and Patel, 2007).

Experimental use of PQ to model PD has furthered our understanding of PQs mechanisms of DA toxicity (Tieu, 2011). It has been demonstrated that PQ relies on the L-amino acid transporter to cross the blood brain barrier (McCormack and Di Monte, 2003; Shimizu *et al.*, 2001) and once in the brain it enters cells through the

DAT and organic cation transporter 3 (Oct3) (Rappold *et al.*, 2011). The mechanism of PQ entry to mitochondria is currently unreported.

A number of pathogenic mechanisms of PQ within neurons have been demonstrated, primarily stemming from the initial finding that PQ increases PD risk by augmenting superoxide production. mtDNA damage has been reported in PD patients with a history of PQ exposure (Sanders *et al.*, 2017), suggestive of PQ-induced ROS-production contributing to DNA damage and increasing the susceptibility of individuals with defective DNA repair pathways.

PQ has also been shown to exert a number of mitochondrial effects, including increased mitochondrial fission via reduction of mitochondrial fusion protein Mfn1 and Mfn2 levels (Tanaka *et al.*, 2010). Research has demonstrated that PQ causes neuroinflammation, alongside α-synuclein upregulation and aggregation in rodent models (Fernagut *et al.*, 2007; Manning-Bog *et al.*, 2002; Purisai *et al.*, 2007; Wu *et al.*, 2005).

There have been conflicting results from *in vivo* PQ studies, with a loss of nigral dopaminergic cell bodies but intact striatal dopaminergic terminals reported in some models (Tieu, 2011). This is suggested to be the result of compensatory sprouting from surviving dopaminergic neurons and uptake of PQ into non-dopaminergic cells, as PQ uptake is mediated by DAT and Oct3, which is expressed on neurons, astrocytes and microglia (Rappold et al, 2011). It has therefore been suggested that Oct3 may mediate uptake of PQ into astrocytes and microglia, which can act as sinks and sources of the toxin, reducing the acute neuronal effects by absorbing some of the chemical from the extracellular space, and also potentially contributing to its chronic effects as glia may possess efflux mechanisms which can redistribute PQ to

increase availability for later uptake by dopaminergic neurons. Despite the lack of striatal degeneration in some PQ models, they still demonstrate a range of PD-relevant neuropathology's and PQ has been widely published with various treatment regimes demonstrating success in modelling the disease.

PQ has a number of associated genetic modifications linked to increased PD risk, including *ATP13A2, CHCHD2*, *DJ-1 and PINK1 (Gegg et al., 2009; Meng et al., 2017)*. The PD risk gene *ATP13A2* was implicated in PQ toxicity as expression of the gene in CHO cells demonstrated increased sensitivity to PQ toxicity (Pinto *et al.*, 2012), which may be linked to upregulation of *ATP13A2* under oxidative stress conditions, contributing to further oxidative stress and mitochondrial dysfunction. *CHCHD2* has a role binding COX for oxidative phosphorylation and C*HCHD2* null *Drosophila* demonstrated increased sensitivity to PQ-induced oxidative stress, suggestive that mutations which reduce the ability of cells to scavenge ROS are likely to enhance their susceptibility to PD-related neurotoxicants (Meng *et al.*, 2017). *DJ-1* has been linked to PQ sensitivity, as siRNA silencing of *DJ-1* enhanced PQ sensitivity and exacerbation of apoptotic cell death (González-Polo *et al.*, 2009), however Kwon et al (2011) reported that *DJ-1* null cells showed increased resistance to PQ-mediated apoptosis and reduced superoxide production, indicative that the risk relationship between these two factors requires further investigation. *PINK1* null cells display reduced viability following PQ treatment compared to control cells (Gegg *et al.*, 2009); this enhanced vulnerability is likely a combination of reduced ATP production and increased intracellular oxidative stress. Increased mitochondrial dysfunction and susceptibility to apoptosis or oxidative stress over time was reported in brain cells with a loss of PINK1 activity (Gautier, Kitada and Shen, 2008; Wood-Kaczmar *et al.*, 2008). Overall, the currently identified gene-environment interactions with PQ seem to

enhance susceptibility to the toxic insult, which could suggest that some individuals with ‘mild’ PD risk mutations may face enhanced risk in conjunction with toxic insult from exposure to environmental risk factors.

Current evidence supports the role of PQ exposure in enhancing PD risk, in both epidemiological studies and lab-based animal models. Further work to elucidate the exact mechanisms of toxicity could better inform environmental agencies and producers of the herbicide in their classification of PQ toxicity, to limit its use and to protect workers from future exposure.

Maneb

Maneb (MB) is a polymeric manganese-based fungicide associated with increased PD risk, primarily when used in conjunction with PQ; co-exposure to PQ and MB has been found to increase PD risk by up to 75% and exposure to either pesticide, alone or in combination, was demonstrated to enhance risk for those diagnosed at 60≤ years old (Costello *et al.*, 2009). MB acts as a redox modulator through alkylation of protein thiols and previous characterisation has implicated it in the inhibition of complex III of the mitochondrial transport chain and uncoupling of the mitochondrial proton gradient (Domico *et al.*, 2006; Zhang *et al.*, 2003). An early study demonstrated that MB treatment inhibited locomotor activity in mice (Morato, Lemos and Takahashi, 1989), although most investigation of MB has focused on co-exposure with PQ.

PQ and MB are often used in combination in agriculture as PQ is used to treat crops whilst growing, and MB treatment post-harvest helps to prevent spoiling (Soderstrom, Baum and Kordower, 2009). To date there is limited research into the effect of MB alone, with most studies focusing on its effects in conjunction with other

toxins. An early study (Takahashi, Rogerio and Zanin, 1989) demonstrated that acute administration of MB, in conjunction with MPTP treatment, exacerbated the effects on locomotion and catalepsy in mice, compared to those treated with MPTP alone.

The combinatory effects of MB and PQ have been examined in the context of early-life exposure followed by a repeat exposure in adulthood, to explore how early insult may sensitise individuals to later toxicity. Recent work by Colle et al (2020) investigated the effect of early postnatal exposure to PQ with MB, and demonstrated increased nigrostriatal dopaminergic susceptibility for re-challenge with the same pesticide in adulthood. Despite the limitations of this study, it supports the earlier conclusion that developmental exposure to environmental risk factors may contribute to the onset of later-life neurodegeneration.

MB has also been investigated in other pathologies, with Desplats et al (2012) investigating the impact of MB-PQ exposure on adult hippocampal neurogenesis. In *SNCA* and *LRRK2* transgenic mouse models, combined PQ-MB exposure enhanced the significant reduction in neuronal precursors and proliferating cells seen in WT animals. This work supports not only the interaction of MB and PQ in toxicity, but also implicates gene-environment interactions as the two PD-related transgenic mouse lines displayed greater sensitivity to MB-PQ-induced toxic insult. Hou et al (2017) investigated the role of MB-PQ exposure in the noradrenergic locus coeruleus to demonstrate that neurodegeneration occurred through NADPH oxidase-mediated microglial activation. These two studies highlight the importance of exploring beyond the SNpc to discover neurodegenerative mechanisms, as microglial activation and gene-environment interactions may have relevant effects beyond the nigrostriatal system.

Recent work by Cao et al (2019) explored the role of MB alone; utilising a range of MB doses (0.1, 1, 5, 10μM), they explored the impact of treating 6-hour post-fertilisation zebrafish embryos for 96 hours to investigate whether MB conferred developmental neurotoxicity. Results demonstrated dose-dependent effects on mortality, hatching rate, and notochord deformity rate, with 50-60% reduction in basal oxygen consumption observed in 10μM MB treated embryos following 24hr exposure. This work supports a role of MB-toxicity in other aspects of neuronal health, suggesting detrimental impacts on development, whilst supporting the implication of respiratory processes in its toxicity.

To date there is limited research to elucidate the mechanism of action of MB in these models, however one study demonstrated that MB mediated reduced baseline oxygen consumption and maximal respiration following acute, subtoxic treatment of SK-N-AS neuroblastoma cells. They also found that MB-treatment inhibited ATP synthesis and coupling efficiency, implicating mitochondrial dysfunction in the detrimental effects of MB (Anderson *et al.*, 2018).

Overall, the study of MB in relation to PD highlights the potential for enhanced toxicity in mixed-exposure models, along with the potential of gene-environment interactions to further potentiate toxicity. MB shares induction of mitochondrial dysfunction as a mechanism of toxicity common to other environmental and genetic PD risk factors.

Organochloride Pesticides

Organochloride pesticides (OCP), such as dieldrin and aldrin, have been implicated in the aetiology of PD as potential environmental risk factors (Chhillar *et al.*,

2013; Corrigan, French and Murray, 1996; Fleming *et al.*, 1994; Kanthasamy *et al.*, 2005). Dieldrin was one of the first OCPs commercially available, produced in 1948 by J.Hyman & Co, Denver as an insecticide. It launched in 1950 and was widely used until the late 1980s (Jorgenson, 2001). Dieldrin was not banned in the UK until 1998, with ongoing effects suspected due to bio-persistence and bioaccumulation common with OCPs. OCPs are still extensively used in many developing countries despite research into their potential risks (Beiras, 2018).

Dieldrin has high lipophilicity which contributes to the risk of dermal exposure, facilitating its rapid absorption through the skin (Costa, 2015). The long half-life (~25 years) contributes to high exposure risk, even decades after use has ceased, and human exposure may be mediated by the consumption of contaminated meat, dairy and fish products (Kanthasamy *et al.*, 2005); the cumulative result of multiple low exposures is likely to pose greater risk to health than with shorter lived pesticides.

Once in the circulatory system, dieldrin can readily pass through the blood brain barrier and amass in the brain (Hatcher *et al.*, 2007). Diedrin was detected in ~30% of post-mortem brain tissue from PD patients in one study, with none detected in age-matched controls (Fleming *et al.*, 1994). Another study found dieldrin in both patient and control brains, with higher levels in the caudate nucleus of PD patients (Corrigan, French and Murray, 1996), supporting its implication as a risk factor for this disease.

Dieldrin's primary mechanisms of injury are via increased ROS production (Chun *et al.*, 2001) and stimulation of mitochondria-mediated apoptosis (Kanthasamy *et al.*, 2008; Kitazawa, Anantharam and Kanthasamy, 2003). Dopaminergic neurons exhibit increased dieldrin sensitivity compared to other cell types *in vitro* (Kitazawa, Anantharam and Kanthasamy, 2001; Sanchez-Ramos *et al.*, 1998) and *in vivo* (Heinz,

Hill and Contrera, 1980; Sharma, Winn and Low, 1976; Wagner and Greene, 1978). Dieldrin blocks mitochondrial aldehyde dehydrogenase function (ALDH) (Fitzmaurice *et al.*, 2013; Fitzmaurice *et al.*, 2014), which has implications for neuronal health as ALDH is responsible for the metabolism of toxic aldehydes including 3,4-Dihydroxyphenylacetaldehyde (DOPAL), a reactive neurotoxic dopamine metabolite. Investigation of ALDH2 levels in the putamen and frontal cortex of PD patient post-mortem samples revealed a significant increase in mitochondrial ALDH2 activity in the putamen compared to controls (Michel *et al.*, 2014). Dieldrin exposure in ALDH2 variants has been associated with a 2-6 fold increase in PD risk (Fitzmaurice *et al.*, 2014); ALDH2 inhibition has thereby been suggested as a pathogenic mechanism in PD.

Dieldrin was demonstrated to cause a concentration-dependent increase in ROS in a murine microglial cell line, in a time-dependent manner. Inhibition of NADPH oxidase, gene transcription and protein synthesis significantly reduced this dieldrin-induced microglial ROS generation. In primary microglia, dieldrin also demonstrated concentration-dependent ROS generation, however this was not replicated in primary astrocytes. Results demonstrate that dieldrin can stimulate microglial-derived ROS generation which may contribute to dopaminergic cell degeneration (Mao *et al.*, 2007).

Proteomic investigation has identified 18 respiratory chain proteins which were affected by dieldrin treatment (Cowie *et al.*, 2017), further supporting the effect of dieldrin on mitochondrial function. Dieldrin was also shown to directly stimulate α-synuclein fibril formation *in vitro*, via induction of a conformational change which may serve as a precursor or seed for the aggregation of further protein (Uversky, Li and Fink, 2001b). Prolonged exposure to this pesticide has also been associated with

inhibition of the ubiquitin-proteosome protein degradation system (Sun, Anantharam and Kanthasamy, 2004), further promoting protein aggregation.

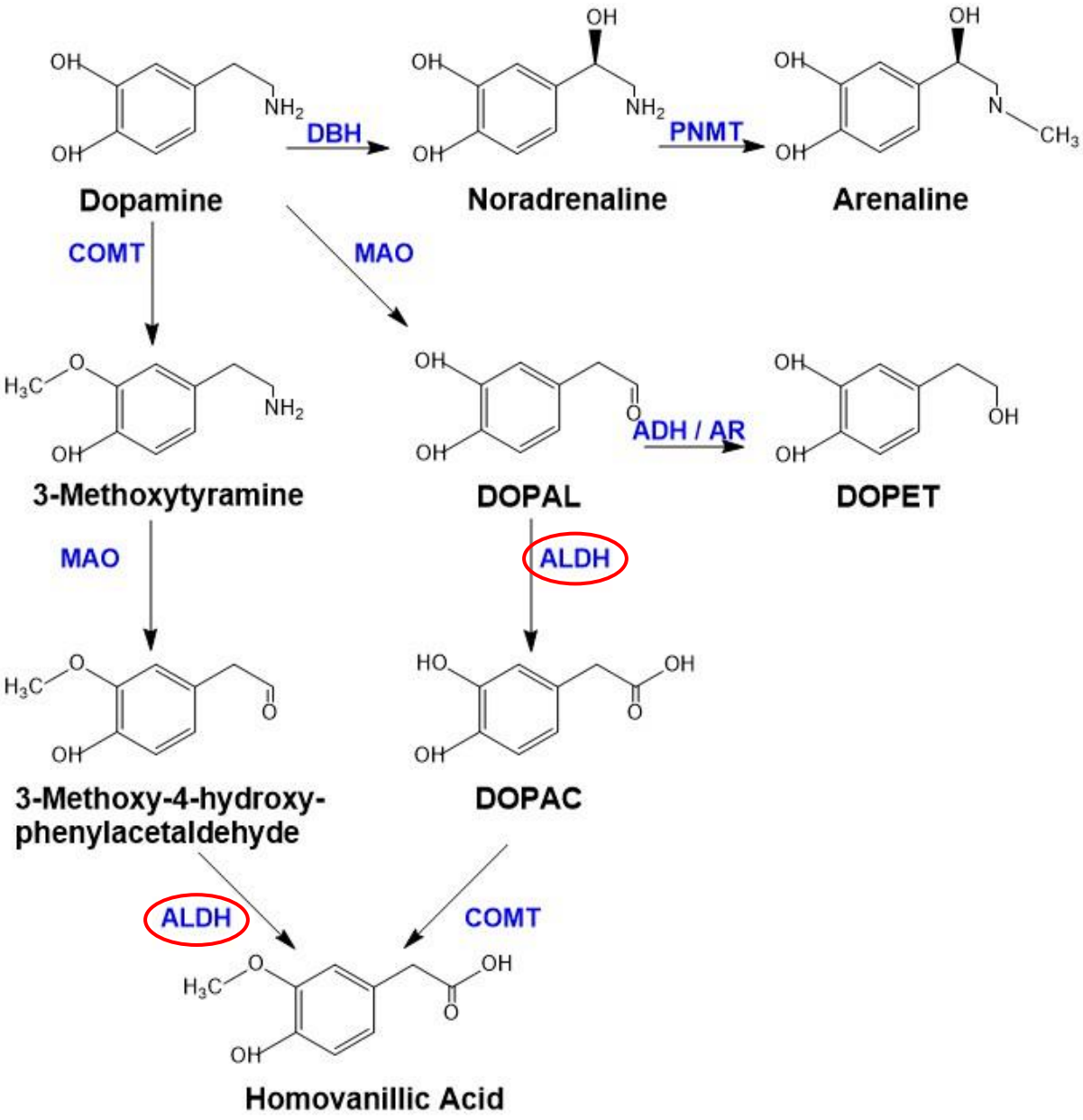


Figure 3: Aldehyde dehydrogenase is necessary for two branches of the dopamine metabolism pathway. Dysfunctional ALDH2, potentially caused by the organochloride pesticide dieldrin as demonstrated in multiple studies, impacts the metabolism of DOPAL and 3-methoxy-4-hydroxyphenylaldehyde; DOPAL is a highly reactive toxic metabolite which covalently alters α-synuclein to induce oligomerisation, contributing to α-synuclein-induced PD pathology (Masato *et al.*, 2019; Plotegher *et al.*, 2017; Werner-Allen *et al.*, 2016).

Our current understanding of the function of dieldrin as a PD risk factor clearly demonstrates the inclusion of previously identified mechanisms of toxicity, including mitochondrial dysfunction and α-synuclein pathology.

Pyrethroids

Pyrethroids are synthetic derivatives of the naturally occurring pyrethrins from chrysanthemum flowers (Metcalf and Horowitz, 2014). These pesticides are widely used both agriculturally and in household applications (Elwan *et al.*, 2006). They are classified as safe substances (Casida *et al.*, 1983) despite evidence of human intoxication (Chen *et al.*, 1991). Permethrin and deltamethrin are two examples of pyrethroid pesticides, which can readily cross the blood brain barrier to accumulate and concentrate in the brain (Anadón *et al.*, 1996). These two pesticides have been shown to increase DAT-mediated dopamine uptake by up to 30% in the mouse striatum and to induce apoptosis in dopaminergic neuroblastoma cells (Elwan *et al.*, 2006). As DAT can mediate the neurotoxicity of some chemicals, such as PQ as described above, pyrethroids may enhance dopaminergic neuron vulnerability to other toxic compounds by increased DAT activity (Donovan *et al.*, 1999; Gainetdinov *et al.*, 1997; Rappold *et al.*, 2011).

Permethrin has been shown to potently inhibit mitochondrial complex I function in isolated rat liver-derived mitochondria (Gassner *et al.*, 1997). However, a subsequent study demonstrated that deltamethrin's induction of apoptosis was independent of mitochondria, suggestive of distinct mechanisms of different pyrethroid pesticides (Hossain and Richardson, 2011); they demonstrated that deltamethrin induced calcium overload through interactions with Na^+ channels, causing calcium

influx and endoplasmic reticulum stress, as cytochrome C was not released which indicates that the mitochondrial apoptosis pathway was not activated.

Recent work investigated the potential use of permethrin to model PD, in a progressive model of early life exposure to the pesticide (Nasuti *et al.*, 2017). Treatment of Wistar rat pups with permethrin from post-natal days (PND) 6-21 induced cognitive impairments, reduced striatal dopamine levels, and reduced dopaminergic neurons in the substantia nigra. The recapitulation of these key neuropathological changes commonly found in PD patients was first evident at PND 60, although motor changes were not apparent until PND 150. This research potentially adds a new pesticide model to the repertoire currently used for research, and highlights an important consideration as early life exposure to environmental risk factors, including pesticides, may predispose individuals to pathogenic changes later in life, or they may initiate slow progressive changes which culminate in later development of the clinical phenotype. However, despite strong experimental evidence of pyrethroid pesticides inducing PD-like changes in experimental models, the evidence of human exposure resulting in disease still remains limited (Costa, 2015).

The pesticides summarised above constitute those with the strongest association with PD risk. There are a number of others for which the associations are less robust or have been less investigated. Rotenone and PQ are the best characterised and comprise the major models for my research.

1.4.2| Heavy Metals

Metals are essential for many physiological processes however excessive levels of heavy metals, such as iron and manganese, have been found to be detrimental to health and in some cases confer increased disease risk (Martinez-Finley *et al.*, 2013; Park *et al.*, 2015). Despite some mixed literature regarding the association of heavy metals and PD aetiology, with a range of studies denying (Cheng *et al.*, 2015; Jiménez-Jiménez *et al.*, 1992; Meamar *et al.*, 2016; Semchuk, Love and Lee, 1993) or supporting (Cruces-Sande, Méndez-Álvarez and Soto-Otero, 2017; Fukushima *et al.*, 2010; Fukushima *et al.*, 2011; Rose, Hodak and Bernholc, 2011) the proposed association, the consensus sustains their role as disease risk factors.

Metals can induce mitochondrial dysfunction, oxidative stress, DNA damage and interactions with neurotoxic proteins such as α-synuclein and β-amyloid to cause structural cellular changes (Cervantes-Cervantes *et al.*, 2005; Chen, Miah and Aschner, 2016; Ha, Ryu and Park, 2007; Martinez-Finley *et al.*, 2013; Park *et al.*, 2015; Salvador, Uranga and Giusto, 2010); these changes can all increase the risk of neurological damage.

Manganese

Manganese (Mn) is the heavy metal most investigated in relation to PD; Mn is an essential dietary element, with vital roles in homeostasis and brain development (Aschner and Aschner, 2005), including functioning as a cofactor for glutamine synthase in astrocytes and as a component of superoxide dismutase (MnSoD) as part of the mitochondrial antioxidant defence (Borgstahl *et al.*, 1992). Mn is widely distributed in the environment, in part due to industrial activity such as use in steel additives, alloying agents, batteries, and chemicals, such as the pesticide maneb. Mn

is also found in various foods, such as beans, legumes and leafy green vegetables (Keen and Zidenberg-Cherr, 2003). Mn can increase PD risk through dietary, occupational and environmental exposure routes (Caudle *et al.*, 2012; Kwakye *et al.*, 2015).

Mn is essential for various homeostatic processes within the body and can exist in multiple oxidation states (Aurora, 1977). Mn metabolism is closely linked to other heavy metals, especially iron due to their analogous redox behaviour (Claus Henn *et al.*, 2011; Kim, Buckett and Wessling-Resnick, 2013; Kwakye *et al.*, 2015). Some functions and localisations of Mn are dependent on its oxidative state; it can be localised to the CNS via import through separate transmembrane importers, such as divalent metal transporter 1 (DMT1), which is highly expressed in the basal ganglia, DAT, calcium channels, choline and citrate transporters, transferrin and the ZIP (Zrt- and Irt- related protein) family of metal transporters (Gunter *et al.*, 2013; Huang, Ong and Connor, 2004; Tuschl, Mills and Clayton, 2013). Mn efflux is equally important when considering the maintenance of homeostatic intracellular Mn levels and exporters include ferroportin (Fpn), SLC30A10 (solute carrier family 30 member 10), secretory pathway Ca2+ ATPase 1 (SPCA1) and ATP13A2, a PD risk-gene-encoded transporter (Tuschl, Mills and Clayton, 2013). Recent studies have demonstrated that *ATP13A2*-deficient mice display increased sensitivity to Mn toxicity *in vivo* (Fleming *et al.*, 2018) and that *ATP13A2* overexpression confers protection against manganese toxicity in HeLa cells and primary *C.elegans*-derived dopaminergic neurons (Ugolino *et al.*, 2019). Mutations in the *SLC30A10* gene, the first identified human Mn transporter, can result in the accumulation of Mn in the basal ganglia and liver, resulting in hypermanganesemia, childhood onset dystonia and adult onset parkinsonism (Quadri *et al.*, 2012; Tuschl *et al.*, 2012). In 2016, a novel Mn transporter

defect was identified, the autosomal recessive *SLC39A14*-encoded divalent metal transporter. It was discovered in children affected by hypermanganesemia and progressive parkinsonism-dystonia. SLC39A14 is a Mn-specific transporter, as mutations in this gene impair Mn import without impacting the levels of other metals (Tuschl *et al.*, 2016).

Occupational or chronic Mn exposure induces symptomatic changes characterised as Manganism (Couper, 1837), a parkinsonian syndrome which includes the development of motor impairments, behavioural changes and cognitive alterations. Despite the symptomatic similarities with PD (Aschner *et al.*, 2009), manganism is a distinct disorder with divergent changes, including a lack of L-DOPA responsiveness, presentation of a masked face, dysphonia and drooling (Calne *et al.*, 1994; Couper, 1837; Guilarte and Gonzales, 2015). Research suggests that manganism is primarily a disorder of the neurotransmitter GABA, produced by globus pallidus neurons; Mn has a predilection to accumulate in the palladium and striatum as opposed to the substantia nigra (Olanow, 2004) and in manganism the number of dopaminergic neurons in the substantia nigra remain relatively unaffected (Guilarte *et al.*, 2006; Guilarte and Gonzales, 2015); this was recently confirmed by stereology (Harischandra *et al.*, 2019)

Despite the clinical distinction between manganism and PD, Mn exposure is still believed to confer increased risk of PD development, in conjunction with other risk factors such as genetic susceptibility. Epidemiological findings have demonstrated Mn accumulation in PD patients (Fukushima *et al.*, 2010) and chronic Mn exposure results in significantly increased Mn in the dopaminergic neurons of rodent brains compared to non-exposed rodents (Robison *et al.*, 2015). Mn has also been shown to suppress striatal DA release, a deficit commonly observed in animal models with PD gene

mutations (Roth *et al.*, 2013). PET showed that Mn-exposed monkeys exhibited reduced dopamine release in the dorsal striatum, demonstrative of an impaired nigrostriatal dopamine system (Guilarte *et al.*, 2008). It is possible that Mn may interact with these proteins at the presynaptic level to inhibit neurotransmitter release.

One possible mechanism of toxicity relates to the ability of Mn to interact with mitochondria; divalent Mn is imported by the calcium uniporter channel and its slow export is facilitated by the sodium-independent calcium channel (Gavin, Gunter and Gunter, 1999). High levels of Mn can accumulate in the mitochondrial matrix and promote sodium-dependent and -independent calcium efflux through direct competitive inhibition, which subsequently results in increased mitochondrial calcium levels which interfere with oxidative respiration and induce oxidative stress (Gavin, Gunter and Gunter, 1990). Investigation of ATP production to improve understanding of the effect of Mn on inhibition of the complex oxidative phosphorylation systems in multiple organs (liver, heart and brain mitochondria) demonstrated that Mn^{2+} inhibits ATP production in different manners, which are distinct across tissue types (Gunter *et al.*, 2010). In the brain, Mn^{2+} appears to inhibit two independent sites on mitochondria; primarily the electron transport chain complex II, with secondary inhibition of either the glutamate/aspartate exchanger or the aspartate aminotransferase (Gunter *et al.*, 2010).

Alaimo et al (2014) showed that Mn may shift the balance of mitochondrial dynamics towards fission by increasing Drp1 translocation to mitochondria and Drp1 inhibition prevented Mn-induced apoptosis (Alaimo *et al.*, 2014). Supporting this finding, Mn has been reported to induce mitochondrial fragmentation, consistent with its well-documented role in impairing mitochondrial function and the requirement of mitochondrial function for synaptic neurotransmitter release (Malecki, 2001). In one

study, the authors showed that Mn increased mitochondrial fission in cultured rat astrocytoma C6 cells by increasing Drp1 translocation to mitochondria from the cytosol; the addition of mito-apocynin, a mitochondrial-targeted antioxidant, significantly reduced Mn-induced inflammation, supporting the neuroprotective effects of restoring mitochondrial health (Sarkar *et al.*, 2017a). Concomitantly, Mn impaired mitochondrial fusion by reducing OPA-1 levels; overexpression of OPA-1 or inhibition of Drp1 function using Drp1-siRNA or a small molecule Drp1 inhibitor prevented this excessive fission and the subsequent neurotoxicity (Alaimo *et al.*, 2014).

Generation of ROS is another toxic process in which Mn plays a role; superoxide, which may result from Mn interference with the electron transport chain, can be converted to hydrogen peroxide by the Mn and Cu/Zn superoxide dismutases, which can then be reduced to form hydroxyl radicals in the presence of Mn or other transition metals (Goldstein, Meyerstein and Czapski, 1993; Martinez-Finley *et al.*, 2013). Increased ROS production promotes opening of the mitochondrial permeability transition pore, causing a loss of membrane potential, impaired ATP synthesis and mitochondrial swelling, which contribute to cellular apoptosis (Gavin, Gunter and Gunter, 1990; Gavin, Gunter and Gunter, 1992). Hydroxyl radicals also react with DNA, membrane lipids and cellular proteins, leading to dysfunction and potential mutations (Jenkins, 1966).

The implication of the PD risk gene *ATP13A2* in Mn transport also highlights a potential gene-environment interaction, as *ATP13A2* mutants may display differential sensitivity to Mn intoxication. *ATP13A2* has been shown to be protective against Mn toxicity (Gitler *et al.*, 2009) however this protective effect is lost in cases of *ATP13A2* mutation, which may enhance the detrimental effects of Mn on motor coordination in Mn-exposed humans (Rentschler *et al.*, 2012). Further proposed gene-environment

interactions include *DJ-1*, *PINK1, Parkin*, *SNCA*, *LRRK2* and *VPS35*; Parkin has been reported to modulate Mn transport (Roth *et al.*, 2010) through its role in the ubiquitination and subsequent proteasomal degradation of one DMT1 isoform involved in Mn uptake. Therefore, a loss in Parkin function could facilitate increased Mn uptake and accumulation in the basal ganglia, due to insufficient degradation of this DMT1 isoform. Mn has been shown to reduce both RNA and protein levels of DJ-1, inducing effects similar to that of *DJ-1* mutants (Lee *et al.*, 2012). As both Mn and *DJ-1* mutants independently impact mitochondrial function, this could present a mechanism for exacerbation of the detrimental influence. *SNCA* encodes α-synuclein, which has three Mn binding sites present in the C-terminal domain: Asp121, Asp122 and Glu123 (Uversky, Li and Fink, 2001a). There are currently conflicting views on Mn interactions with α-synuclein, and the downstream effects on cells, with some studies reporting neurotoxic results of this interaction, with enhanced α-synuclein aggregation (Peres *et al.*, 2016), whilst others report that α-synuclein confers protection against Mn-induced toxicity in *C elegans* (Bornhorst *et al.*, 2014) and rat dopaminergic neuronal cells (Harischandra *et al.*, 2015); the latter of these studies only found protective effects in the earlier stages of Mn exposure, suggestive that Mn accumulation may eventually induce protein aggregation and neurotoxicity. Dučić et al (2015) suggested that α-synuclein may act as an intracellular anchor or store for Mn as they reported increased intracellular Mn content in α-synuclein overexpressing rat primary midbrain neurons, suggestive that Mn may play a role in protein localisation under normal protein homeostasis; however in the case of mutated or excessive α-synuclein expression, Mn could potentiate α-synuclein aggregation if it facilitates its localisation to distinct cellular sites.

Although our understanding of the differences in the role of Mn exposure to confer PD risk and to cause manganism remain incomplete, research strongly supports a role of Mn as an environmental risk factor for PD, with shared pathological mechanisms with other environmental toxicants, genetic risk factors and interactions with several genetic risk factors to further promote PD risk.

Iron

Iron has been implicated in PD risk due to its role in oxidative stress, leading to neuronal death through the Fenton-Haber-Weiss reaction (Hellman and Gitlin, 2002). Whilst meta-analysis of five studies found no significant link between dietary iron and increased risk of PD (Cheng *et al.*, 2015), the meta-analysis was limited by differences in the methodology of the independent studies; four used recent recall techniques whereas the fifth used a past-history approach which may have resulted in recall bias. Further research has identified elevated levels of iron in the brain of PD patients (Chen *et al.*, 2019) and it has been suggested that iron impairs lysosomal function, facilitating the release of iron from lysosomes into neurons where it contributes to oxidative stress (Rajagopalan *et al.*, 2016). More recent meta-analyses suggested that changes in nigral iron levels may be associated with PD, however the variations in study quality and design contribute to complications in interpretation, highlighting additional difficulties interpreting the current research into the role of iron in PD risk (Genoud *et al.*, 2019).

A very recent publication investigated the effect of *IRP2*, an iron regulatory protein, as *IRP2* knockout-induced iron overload; $IRP2^{-/-}$ mice displayed enhanced sensitivity to MPTP treatment, with increased cell apoptosis and decreased dopamine levels reported (Ci *et al.*, 2020). MPTP treatment in these mice significantly increased

iron accumulation in both neurons and the surrounding astrocytes; this has implications for the ability of astrocytes to confer protection to neurons. Overall, this paper sheds light on a potential mechanism by which iron toxicity causes damage to dopaminergic nigral neurons to contribute to PD pathogenesis.

Iron is also involved in α-synuclein toxicity (Jenkins, 1966), as α-synuclein has been reported to regulate dopamine and iron transport, with PD-related associations disturbing these processes to contribute to further iron accumulation and toxicity (Duce *et al.*, 2017). Dysregulated α-synuclein oxidation and phosphorylation also mediates iron-dependent oxidative stress, suggesting that other environmental PD risk factors which disrupt α-synuclein oxidation or phosphorylation may further exacerbate iron toxicity (Duce *et al.*, 2017). Iron has been shown to increase α-synuclein and ROS in SH-SY5Y cells (Wan *et al.*, 2017) and to post-transcriptionally regulate α-synuclein synthesis and iron interactions with proteolytic pathways, further facilitating regulation of α-synuclein via its degradation pathways (Chen *et al.*, 2019). Iron has also been shown to promote α-synuclein aggregation by inhibition of autophagy flux (Xiao *et al.*, 2018), a mechanism shared by several genetic PD risk factors.

Investigation into the potential use of iron chelation to reduce iron accumulation has demonstrated some promise; study of deferiprone, a prototype chelator, found that iron could be scavenged from labile iron complexes and transferred to high affinity cellular acceptors or extracellular transferrin (Devos *et al.*, 2020), leading to pre-clinical studies which seek to establish the safety and efficacy of such a possible therapy, as targeting iron requires control to avoid induction of iron depletion and anaemia.

Whilst there are limitations in our current understanding of the exact mechanisms of iron toxicity contributing to PD risk, research to date demonstrates

important interactions with α-synuclein, ROS and autophagy to mediate the influence of iron on PD risk.

There is increasing evidence for a role of multiple metals contributing increased risk of PD development, and as heavy metal intoxication of water sources or residential areas is a risk from some industrial processes it may impact people without the limitation of occupational exposure, which is the most likely route for many of the PD-implicated pesticides.

1.4.3| Infection

The first suggestion of a relationship between viral infections and PD arose from the 1920-30s flu epidemic, which was associated with post-encephalitic parkinsonism (PEP), following an outbreak of encephalitis lethargica (EL). Following the initial acute infection and EL, some patients developed PEP, which was at the time untreatable, leading to the institutionalisation of many. Treatment of these patients with L-DOPA in the 1960's by Dr Oliver Sacks confirmed a role of dopamine depletion in this pathology, although the longer-term L-DOPA responsiveness was variable (Sacks, 1973). Dr Sacks' work supported a prior study by Birkmayer and Hornykiewicz (1961) to demonstrate efficacy of L-DOPA in PD treatment.

Although some uncertainty persists around the causation of PEP, with diverse clinical encephalitic presentation between patients (Rail, Scholtz and Swash, 1981), newer research into the potential role of other viruses in PD risk supports viral infection as a potential risk factor for PD development. A number of studies have investigated the contribution of viral infections to PD risk, with two studies determining an increased

risk of PD in Hepatitis C patients (Goldstein, Fogel-Grinvald and Steiner, 2019; Lin *et al.*, 2019) and implication of other viral infections (Vlajinac *et al.*, 2013).

Recent work by Sadasivan et al (2017) investigated how infections can sensitise animals to later toxic insults. Their model utilised the H1N1 influenza virus, associated with the 2009 swine flu pandemic, in the MPTP PD mouse model. H1N1-infected animals administered MPTP exhibited a 20% greater loss of SNpc dopaminergic neurons than from MPTP treatment alone. This synergistic impact was not detected in assessment of SNpc microglial activation. Treatment of mice with influenza vaccination or oseltamivir carboxylate, an antiviral drug, eliminated the additive neuropathology demonstrated in the H1N1-MPTP dual treated group. This research supports the proposed 'multi-hit hypothesis' of PD development, demonstrating the synergistic impact of toxic insult following influenza infection. The successful abrogation of the enhanced neuropathology through use of protective influenza treatments also suggests their potential efficacy in reducing PD risk or minimising the severity of onset, by attenuating the impact of one risk factor.

Examination of neuroinvasive infections, such as the western equine encephalitis virus (WEEV), has also demonstrated increased PD risk; Bantle et al (2019) found that this mosquito-borne virus could induce post-encephalitic parkinsonism. In outbred CD-1 mice, non-lethal WEEV infection induced neuroinflammatory changes, neurobehavioural abnormalities and selective loss of dopaminergic neurons in the SNpc. Surviving mice 8 weeks post-infection displayed continued dopaminergic neuron loss, persistent glial activation and phospho-Ser129 α-synuclein-positive proteinase-K resistant aggregates in the entorhinal cortex, basal midbrain and hippocampus. This further supports the proposal that viral infection may induce lasting neurological changes, which may contribute to PD development.

As genetic variance between mouse substrains has been implicated in differential susceptibility to viral infection and subsequent inflammatory changes, the interplay of genetic risk with infections should also be considered (Eisfeld *et al.*, 2018). Further research into infections as a risk factor for PD is required to improve our understanding of how to limit this risk and the cellular mechanisms involved; current findings suggests that infections which induce peripheral or central neuroinflammatory changes could contribute to PD risk and patient pathology.

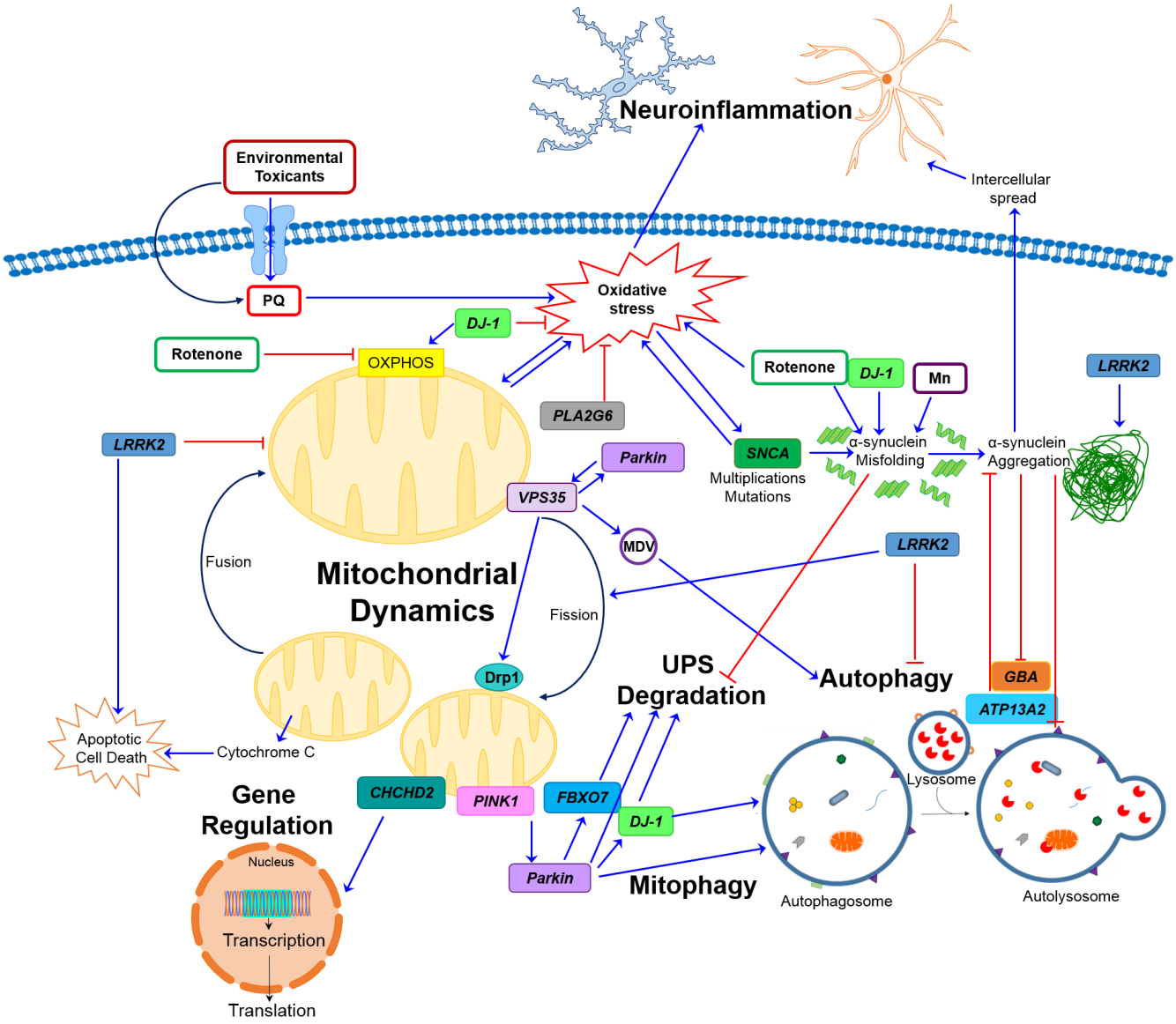


Figure 4: Interactions between Parkinson's disease risk factors and cellular pathogenic mechanisms. As detailed in the text, both genetic and environmental risk factors for Parkinson's disease share common pathogenic mechanisms including mitochondrial dysfunction, dysregulated mitochondrial dynamics, apoptosis, autophagy and ubiquintin-proteosome system disruption, α-synuclein accumulation and aggregation, reactive oxygen species generation, DNA damage and neuroinflammation. Cross-talk between these mechanisms and combinatory effects of risk factors culminate in neurodegenerative processes in Parkinson's disease.

1.5| Gene-environment interactions in Parkinson's disease

As our understanding of the roles which genetic and environmental risk factors play in PD has developed, so has interest in the interplay between these factors. Though less than 30% of PD cases are attributed to genetic risk (Foltynie *et al.*, 2002; Pang *et al.*, 2019), environmental factors alone are insufficient to account for the other cases, and thus it was proposed that combinatory effects may be responsible. Gene-environment interactions also became a topic of interest as curiosity increased regarding the identification of PD-related mutations in sporadic PD, such as *LRRK2* mutations (Gilks *et al.*, 2005), and the identification of PD in heterozygous *PINK1* and *Parkin* mutants, when these are autosomal recessive genes which typically require expression of two mutant alleles to induce pathology (Klein and Lohmann-Hedrich, 2007). Additional familial PD genes have been found among the risk loci identified for sporadic PD (Nalls *et al.*, 2014). As demonstrated in Figure 5, multiple genetic risk factors interact with environmental factors and it is possible that currently unidentified genes may increase susceptibility to environmental factors to confer increased PD risk.

Some recent epidemiological studies have identified specific interactions which may be relevant to PD (Cannon and Greenamyre, 2013), however most research into this topic is derived from genetically modified experimental models which evaluate the sensitivity of neurons to toxicants. Genetic susceptibility, which impacts enzymatic systems involved in the clearance of neurotoxins, may be one gene-environment link in the puzzle of PD pathogenesis. A number of known PD-risk genes have been linked to currently identified chemical risk factors, as well as PD-risk gene-gene interactions demonstrated. Some of these interactions have been described in the above

environmental risk factor sections, with rotenone, PQ, dieldrin and several heavy metals shown to interact with PD risk genes.

Additionally, studies have identified non-PD-related genes, specific variants of which can increase sensitivity to environmental risk factors; in a study of 100 participants who all met clinical PD criteria, increased risk was demonstrated in patients with a poor *CYP2D6* genotype who had been exposed to solvents (De Palma *et al.*, 1998). *CYP2D6* encodes the Cytochrome P450 2D6 (CYP2D6) enzyme which is highly expressed in the substantia nigra; CYP2D6 is important for the metabolism of a range of xenobiotics, thereby contributing to the detoxification of the brain. In individuals with slow-acting CYP2D6, the risk of toxicity may be higher as clearance of chemicals will be limited by the enzyme's maximal rate. *CYP2D6* is not currently considered a risk gene for PD, however this study demonstrates that a non-risk gene may still confer increased PD-risk in conjunction with environmental risk factors. Involvement of the detoxification enzyme GSTP1 in the metabolism of pesticides and the development of PD has also been investigated (Menegon *et al.*, 1998); heterozygosity at the *GSTP1* locus was found to be significantly associated with PD but only in patients exposed to pesticides, demonstrating that genes which are not identified as PD-risk genes may confer risk in the circumstance of specific toxic exposures.

Gene-environment interactions may also be pertinent to supposed protective interactions, in which genotype can play a role. The protective effects of cigarette smoking which have been previously reported (Morens *et al.*, 1995) may be lost in patients with the GSTM1*0 detoxification enzyme phenotype (De Palma *et al.*, 1998), and may actually increase PD risk in patients with the 'A' polymorphism at the MAO-

B locus, with protection limited to those with the 'G' polymorphism at that locus (Checkoway *et al.*, 1998).

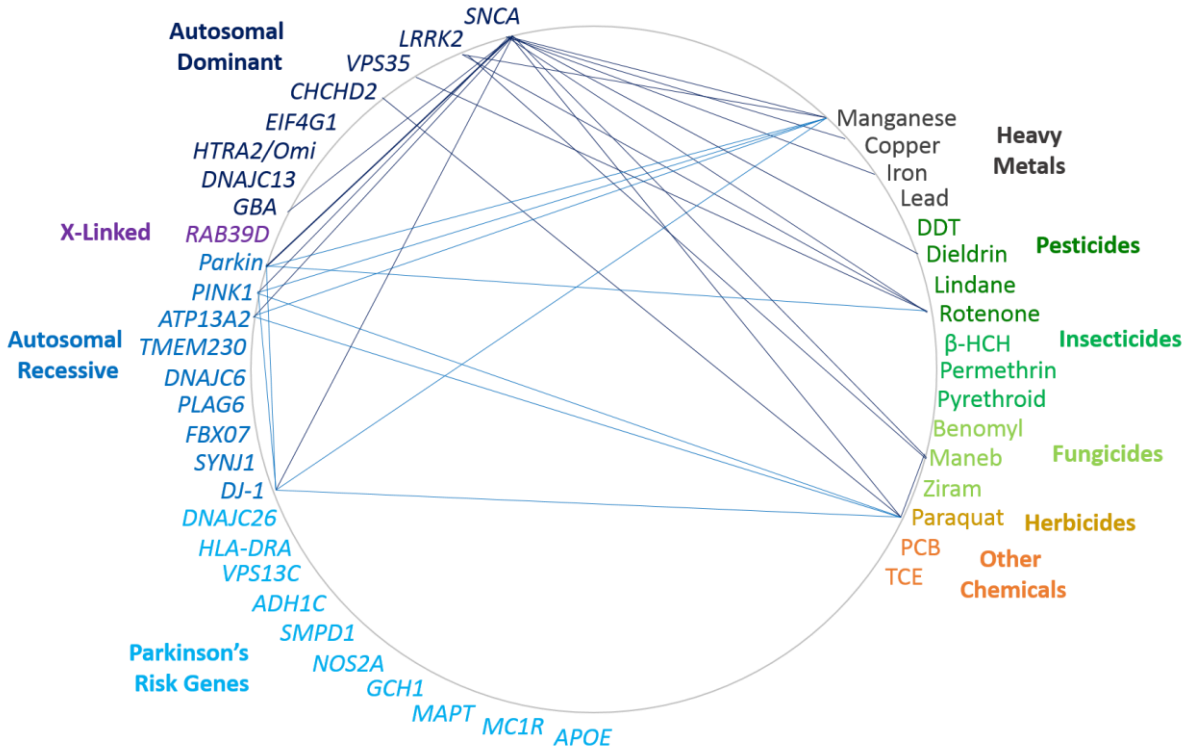


Figure 5: Map of currently demonstrated interactions between genetic and environmental risks factors. Our current understanding of these interactions clearly shows that the genetic and environmental risk factors which have been most researched also demonstrate the most interactions. Some of these interactions are more completely understood than others, and with the common mechanisms of pathology which present across risk factors, there is a high likelihood that further interactions may be reported in the future.

1.6| Mitochondria and their role in Parkinson's disease

Although there are numerous mechanisms of pathophysiology in PD [Figure 4], a shared target of contributing risk factors seems to be mitochondria, the small organelles essential for the maintenance of neuronal health and functionality. Mitochondrial dysfunction has been implicated in PD pathogenesis since the accidental self-administration of MPTP-contaminated meperidine caused drug users to present with parkinsonian symptoms in 1982 (Langston *et al.*, 1983). MPTP is a mitochondrial toxicant, as oxidation in the brain produces MPP^+ which potently inhibits mitochondrial complex I, impeding energy production and resulting in neuronal abnormalities (Dauer and Przedborski, 2003). Further support for the role of mitochondrial changes in the pathogenesis of PD is garnered from extensive research into monogenic mutations of *PINK1, Parkin, DJ-1, LRRK2 , CHCHD2* and *SNCA*, all of which either function to support normal mitochondrial function in neurons, or present mutations which negatively influence mitochondrial function (Aras *et al.*, 2015; Chen *et al.*, 2015; Gui *et al.*, 2012; Irrcher *et al.*, 2010; Narendra *et al.*, 2008; Polito, Greco and Seripa, 2016; Youle and Narendra, 2011), and the identification of dysfunctional mitochondria in PD patient platelets and brain tissue (Parker, Boyson and Parks, 1989; Schapira *et al.*, 1989). Rotenone and MPP^+ share their mechanism of mitochondrial impairment, and the structural similarity between MPP^+ and PQ prompted early research into PQ's role in PD; as has been widely explored, however, PQ's mechanism of toxicity is distinct to that of MPP^+ and rotenone (Richardson *et al.*, 2005).

The dynamic nature of mitochondria facilitates regulation of the organelle population in response to metabolic and environmental stress. Mitochondrial dynamics encompasses the processes of fission, fusion, and movement [Figure 1], to maintain

a healthy population of mitochondria (Chen and Chan, 2009; Onyango *et al.*, 2010; Xie and Cheng, 2012). Fusion facilitates mitigation of cellular stress effects by combining the contents of damaged or defective mitochondria, allowing complementation of the undamaged components in the resultant organelle. It is mediated by three specific fusion proteins; Optic atrophy 1 (OPA1), which is localised to mitochondrial cristae in the matrix and mediates inner membrane fusion, and mitofusins (Mfn1 & Mfn2), which are localised to and control fusion of the outer mitochondrial membrane (Chan, 2006; Mecusen *et al.*, 2006).

Fission is the opposite process by which mitochondria divide; this is beneficial as it facilitates the elimination of destructive mitochondrial mutations and components from the mitochondrial pool. Prior to fission, damaged DNA and proteins are segregated to one side of the mitochondrion, such that only one daughter mitochondrion contains damaged molecules and can be targeted for mitophagy, whilst the other daughter mitochondrion remains healthy (Mattson, Gleichmann and Cheng, 2008). Fission is mediated by Dynamin related protein 1 (Drp1), a large GTPase which translocates from the cytosol to the mitochondrial outer membrane where it induces mitochondrial division by oligomerising and wrapping around constriction points of dividing mitochondria (Smirnova *et al.*, 2001). Mitochondrial fission factor (Mff), Fission 1 (Fis1) and Mitochondrial dynamic proteins 49 and 51 (MiD49 and MiD51) are all outer mitochondrial membrane-anchored fission proteins to which Drp1 may be recruited. MiD49 and MiD51 are essential for the stabilisation of the multimeric Drp1 structures necessary for mitochondrial fission, and both may further regulate fission through the formation of complexes with Mff and Fis1 (Loson *et al.*, 2012; Otera *et al.*, 2010; Palmer *et al.*, 2011). Fission is also important for the induction of cellular

apoptosis, and thus excessive mitochondrial fission can be destructive (Youle and van der Bliek, 2012).

Together these processes regulate the mitochondrial population within cells and manage their response to metabolic stress. Dysregulation of these processes, as has been shown in several PD models, would cause disruption to mitochondrial function and thereby contribute to neuronal death. As Drp1 is crucial for successful regulation of mitochondrial fission, it has been suggested as a potential therapeutic target for the treatment of neurodegenerative diseases, including PD. The development of small molecule inhibitors of Drp1 facilitates pharmacological investigation as to the potential benefits of targeting Drp1 in PD models. Mitochondrial division inhibitor 1 (mdivi-1) is a small-molecule inhibitor of Drp1, developed by Cassidy-Stone et al (2008) following screening of several chemical libraries using a yeast two-hybrid system. Mdivi-1 has been shown to inhibit Drp1 function by directly binding to the fission protein, inhibiting its assembly into oligomeric ring structures and the subsequent GTPase activity required for fission (Xie *et al.*, 2013). Mdivi-1 application in neurodegenerative models suggests promise as a neuroprotective and potentially neurotherapeutic agent; in 2010 our lab demonstrated that abnormal mitochondrial morphology induced by $PINK1^{L347P}$ in N27 cells can be prevented by mdivi-1 (Cui *et al.*, 2010). Further work *in vivo* supports the protective effects of mdivi-1 against aberrant mitochondrial changes in PD models (Rappold *et al.*, 2014), ischemic brain injury (Zhao *et al.*, 2014) and epilepsy (Qiu *et al.*, 2013; Xie *et al.*, 2013), distinguishing its promise as a potential therapeutic with multiple clinical applications.

1.7| Hypothesis and specific aims

Much of the current research into potential therapeutic targeting of pathogenic mechanisms in PD utilised genetic models, which are representative of only a small proportion of PD cases, or experimental models such as MPTP and 6-OHDA, which do not recapitulate environmental risk as they are purely lab-based models. Using two previously established pesticide-based models of PD (Cannon *et al.*, 2009; Cui *et al.*, 2009), I proposed investigation of the potential neuroprotective effects of targeting Drp1, utilising both pharmacological and genetic interventions.

I focused on addressing three key aims within my project; Aim 1, establish the neurotoxic effects of these pesticides, including evaluating their effects on motor function in addition to neuropathology in the rodent models; Aim 2, determine the impact of blocking mitochondrial fission on motor function and neuropathology within these models and; Aim 3, investigate the mechanisms by which targeting Drp1 may confer protection. As our lab very recently demonstrated that targeting Drp1 in an α-synuclein PD model conferred neuroprotection though restoration of both mitochondrial and autophagic pathways, further insight as to whether this may be a shared mechanism across PD models, and thereby risk factors, will inform the development of future therapeutics. Through this research I hoped to improve our understanding of the mechanisms through which pesticide exposure may confer increased PD risk, whilst improving our understanding of the role which Drp1 may play in this process.

Chapter 2| Examining the potential of rotenone as a neurotoxic model of Parkinson's disease for the investigation of potential therapeutic interventions

2.1| Introduction

Chapter 1.4.1 introduces rotenone as a PD risk factor, and the wide variety of experimental paradigms in which it has been utilised to model aspects of the disease pathology. In the past, there has been some contention around the use of rotenone to model PD experimentally, and thus choosing a rotenone model for my work required careful consideration.

Some controversy around the rotenone model of PD arose following a Nature Neuroscience paper (Betarbet *et al.*, 2000), which reported successful characterisation of a rotenone rat model of PD: however, multiple groups failed to reproduce the findings (Höglinger *et al.*, 2003; Lapointe *et al.*, 2004; Sherer *et al.*, 2003; Zhu *et al.*, 2004) which called the reproducibility of the model into question. In 2009, a further refined model by the same authors was published, which is the origin of my rotenone model. In this paradigm, the authors successfully recapitulated several key hallmarks of PD, including motor dysfunction, loss of TH-positive neurons in the SNpc and dopamine depletion in the striatum. This paper also reported α-synuclein and poly-ubiquitin positive proteinaceous neuronal inclusions in brain samples, which some rotenone models have failed to establish (Cannon *et al.*, 2009).

Rotenone is a potent non-competitive complex I inhibitor, which directly alters mitochondrial function. As a highly lipophilic chemical, rotenone passes through the blood brain barrier and cellular membranes without any requirement for transporter interactions (Martinez and Greenamyre, 2012). Once inside cells, rotenone can directly alter mitochondrial function; it impairs oxidative phosphorylation by inhibiting NADH-ubiquinone reductive activity via binding to the PSST subunit of complex I of the electron transport chain (Di Maio *et al.*, 2016; Schuler and Casida, 2001). As previous reports demonstrate oxidative damage of mitochondrial complex I subunits

in Parkinson's brain samples, resulting in mis-assembly and functional impairment of the complex, it is a relevant source of mitochondrial damage in PD (Keeney *et al.*, 2006). Complex I inhibition also feeds into further destructive processes as it results in ATP depletion and electron leakage, which can produce superoxide, cause reduced glutathione levels and increased oxidative stress (Duty and Jenner, 2011; Martinez and Greenamyre, 2012); thus rotenone mediates both direct and indirect damage of mitochondria. Rotenone-induced complex I inhibition also contributes to the accumulation and oligomerisation of α-synuclein (Betarbet *et al.*, 2000; Betarbet *et al.*, 2006; Cannon *et al.*, 2009; Greenamyre *et al.*, 2010), a common pathological hallmark of PD. As α-synuclein has been shown to directly interact with and disrupt the function of mitochondria, including direct impairment of complex I function (Devi *et al.*, 2008), rotenone-induced accumulation of this protein may further exacerbate mitochondrial dysfunction to contribute to neuronal death (Choubey *et al.*, 2011; Nakamura *et al.*, 2011; Orth *et al.*, 2003).

In addition to the direct and indirect effects of rotenone on mitochondrial function, it has been shown to alter mitochondrial transport and protein interactions within neurons. In 2011, it was revealed that rotenone stimulated microtubule depolymerisation, thereby interfering with the intracellular transport of vesicles and organelles, including mitochondria (Choi, Palmiter and Xia, 2011). Recent work has demonstrated increased α-synuclein association with mitochondria, specifically outer membrane protein TOM20, in nigrostriatal dopaminergic neurons of rotenone-treated rats relative to controls (Di Maio *et al.*, 2016). This suggests that rotenone exposure may promote α-synuclein-mediated changes in mitochondrial function and morphology to further promote PD pathology. Rotenone has also been shown to alter vesicular monoamine transporter 2 (VMAT2) distribution and increase intracellular

dopamine levels, indicative that it may play a role in dopamine toxicity as a further mechanism of cellular damage (Watabe and Nakaki, 2007; Watabe and Nakaki, 2008).

The current results from rotenone studies implicate the chemical in multiple mechanisms of neurodegeneration, but its influence on mitochondrial dynamics has only been investigated *in vitro* (Barsoum et al., 2006), and the implications of targeting mitochondrial dynamics therapeutically in *in vivo* rotenone models of PD remains to be established. In this portion of the project I aimed to establish whether this rotenone rat model (Cannon *et al.*, 2009) could reproduce PD-like pathology in my hands and to assess its suitability for use to study the therapeutic potential of Drp1 manipulation.

2.2| Materials and Methods

2.2.1 | Animal care

Three-month-old male Lewis rats were purchased from Charles River, UK and aged in-house to adulthood (425-500g; ~seven months of age). All rat care and experimental procedures were approved and conducted in accordance with the Home Office Animals for Scientific Procedures Act (ASPA) 1986 guidelines (PPL 30/3088; PIL IC7E2094D). Rats were maintained on a 12-hour light/dark cycle, with access to food and water *ad libitum*.

2.2.2 | Chemical preparation and animal treatment

Rotenone (Sigma) was dissolved in DMSO to create a 150mg/mL stock, stored at -20^{o}C, and then diluted in Miglyol 812N, a medium chain triglyceride, to a working solution of 3mg/ml; this was stored at 4^{o}C and prepared fresh every three days. Prior to chemical administration, the weight of each rodent was recorded in a tracking chart to enable weight monitoring throughout the duration of the treatment. For each injection, the dose was calculated from the weight of the animal immediately prior to administration. Based on the protocol developed by the laboratory of T.Greenamyre (Cannon *et al.*, 2009) ~7 month old rats were injected with rotenone intraperitoneally at a dose of 3mg/kg every 24 hours for a total of eight doses, using a 23G needle and a 3ml syringe. Animals were supplemented with hydrogel and mash food throughout treatment to assist with hydration and nutrition to minimise weight loss. Rats were sacrificed 24 hours after the final injection. Rats were monitored twice daily for the appearance of parkinsonian features, such as bradykinesia, postural instability, gait disturbances and rigidity. If the behavioural phenotype became debilitating, limiting

mobility, grooming, or feeding, the animal was euthanised prior to endpoint and, when possible, samples collected.

2.2.3 | Assessment of locomotor function

An automated chamber (Med Associates) was used to quantify locomotor activity in the rotenone study. The chamber (17" L X 17" W X 12" H), with sound isolation cubicle, is equipped with infrared photobeams (3mm diameter) that project across the open field along three axes: X, Y and Z. The software, Activity Monitor 7, detects when and where the photobeams are disrupted by the presence of an animal. Photobeam breaks were recorded for 20 minutes in each session to quantify the ambulatory activity of the animal, including the distance travelled, resting time and rearing behaviour, amongst other parameters. All animals were assessed twice prior to treatment to acclimate to the open field chamber, once at baseline and then 14-24 hours after every second dose of treatment (DMSO vehicle or rotenone).

2.2.4 | Sample collection and preparation

For immunohistochemistry, brain tissue was collected from adult rats following conclusion of the rotenone treatment regime. Animals were anaesthetised with pentobarbitol (Euthatal®) and transcardially perfused with cold 0.1M PBS followed by 4% (w/v) paraformaldehyde (PFA; Sigma) in 0.1M PBS (pH 7.4). The brains were removed, post-fixed in 4% PFA overnight and subsequently cryoprotected in sequential 15% and 30% sucrose phosphate buffer solutions for 2-3 days at 4^{o}C. The brains were then frozen in chilled methyl butane (approximately -50^{o}C) and stored at -80^{o}C until sectioning. Serial coronal sections (30µM) spanning the entire midbrain were collected free-floating in 0.1M TBS (Leica CM3050S cryostat; -20^{o}C) and stored at 4^{o}C.

2.2.5 | Immunohistochemistry

For stereological assessment of dopaminergic cell loss in the substantia nigra, every 6th midbrain section, spanning caudal to rostral in the midbrain, was prepared with immunohistochemistry. Sections were treated with 10% methanol-3% hydrogen peroxide solution for 10 minutes, background reactivity blocked by incubation in 5% natural goat serum in 0.1M TBS for 1 hour, then incubated with anti-tyrosine hydroxylase (1:4000 SNpc, Calbiochem #657012) in 0.1M TBS with 2% normal goat serum (NGS) for 24hrs at 4^{o}C. Sections were then washed in 0.1M TBS and incubated in biotinylated goat anti-rabbit IgG (1:200 dilution; Vector BA-1000) for 1 hour at room temperature, followed by washing in 0.1M TBS before 1 hour incubation in ABC solution (Vectastain® ABC HRP Kit; 1:5 dilution in 0.1M TBS; Vector Laboratories). Brief washes with 0.1M TBS removed excess ABC solution and immunoreactivity was visualised by use of the DAB Generation System; sections were incubated with 3,3'-diaminobenzidine tetrachloride (DAB; 0.4mg/ml: Sigma Aldrich D-5905), ammonium dichloride (200mg/ml; Sigma), glucose (250mg/ml; Sigma) and glucose oxidase (3mg/ml; Sigma) in 0.1M Tris GN for 8-15 minutes. Following final rinses with 0.1M TBS, sections were mounted in approximate rostro-caudal order on Superfrost™ Plus microscope slides and air dried for 48hrs. Sections for stereological assessment of dopaminergic neuron loss were nissl stained with thionin and formalin acetic acid solutions, dehydrated and cover slipped with DPX Mountant.

2.2.6 | Stereology

Stereological microscope set up

An Olympus BX51 light microscope fitted with an Olympus DP73 digital camera (0.5x), an IK220 encoder and an automated stage for imaging in the X, Y and Z planes (Proscan II, Prior Scientific, UK) was used to analyse the TH-labelled tissue sections. The microscope system was calibrated with an Olympus calibration slide and the alignment of the camera and stage monitored throughout counts. The computer was equipped with Visiopharm stereological software (Version 6.7.0.2590, Denmark) and two 21-inch monitors (Dell) with screen resolutions of 1920 x 1200 pixels. The entire system was mounted on a vibration isolation table (Vision IsoStation, Newport Inc., CA, USA) to allow stable high magnification imaging.

Stereological Methods

Optical fractionator stereological cell counting was utilised to investigate whether pesticide treatment caused any significant loss of dopaminergic neurons in the SNpc of the rodents. Prior to assessment of the full experimental cohort, a pilot study was performed to validate the antibody penetration and to determine the most appropriate counting parameters for the study. TH-immunostaining was used to delineate the dopaminergic SNpc neurons from the surrounding regions, with morphological characterisation between the VTA and SNpc, using The Mouse Brain (Franklin and Paxinos, 2008) for additional guidance. Neurons which displayed clear TH-positive staining with a visible nucleolus were counted. To ensure consistency, all stereological tasks, including delineation of the ROIs in all of the animals, were performed by myself in a blinded fashion. Unbiased stereological assessment of the total number of TH-labelled neurons in the animals was computed using the optical

fractionator principle (Gundersen, 1986) with a uniform random sampling scheme in the region of interest and a reciprocal sampling fraction [Equation 1]. The bsf, ssf, asf and hsf settings were constant throughout the stereological analyses. A sampling percentage of 30% of the ROI was counted, using a counting frame area of 6400µm (80µm x 80µm). A guard zone of 1µm was utilised at the upper and lower limits of the dissector. The CE values were <0.1 for all animals.

Equation 1:

$$Estimated\ Total\ Number: N_{Total} = \frac{1}{bsf} \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{hsf} \sum Q^{-}$$

bsf = block sampling fraction (1 for the whole SNpc region)

ssf = section sampling fraction (0.25 in mice, 0.165 in rats)

asf = area sampling fraction (0.5 for unilateral counts; see Equation 2)

hsf = height sampling fraction (see Equation 3, automatically calculated by Visiopharm)

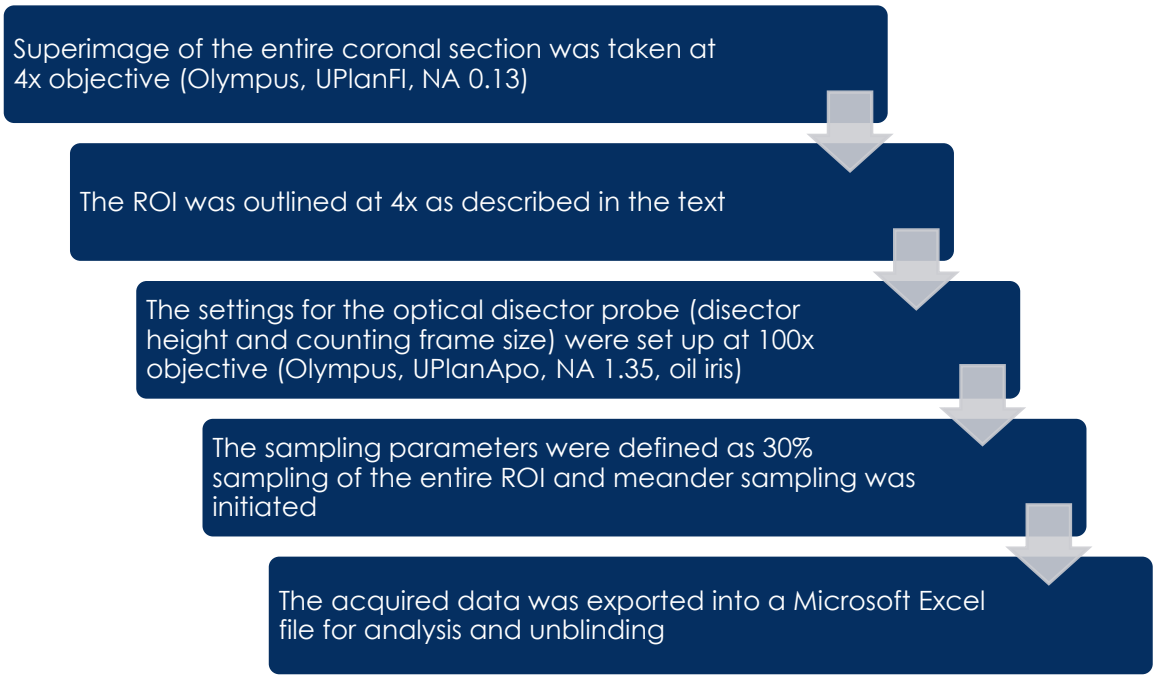
Equation 2:

$$asf = \frac{a(frame)}{A}$$

Equation 3:

$$hsf = \frac{h}{t_Q -},\ t_Q = \frac{\sum_i (t_i q_i)}{\sum_i q}$$

Stereological Procedure



Stereology Pilot.

Multiple sections (typically 3 per animal), uniformly distributed through the rostro-caudal axis of the region, were selected from just one or two animals per group. These nigra were then counted using multiple counting-frame and sampling percentage parameters. Antibody penetration was validated by plotting a histogram of the neurons counted and the depth within the tissue slice at which they were marked; production of a uniform curve indicated successful penetration whereas two distinct peaks with a dip in the centre would suggest that the antibody was unsuccessful in staining the neurons in the greatest depth of tissue. Calculation of the CE for results from each counting parameter combination for each treatment group allowed optimisation of the parameters for the study.

2.2.7 | Protein sample fractionation

Samples for western blotting were collected from rats euthanised by CO_2-exposure, followed by cervical dislocation and decapitation. The brain was rapidly dissected on ice and the striatum (STR) and ventral midbrain (VMB) were dissected and processed for isolation of mitochondrial and cytosolic fractions, as described by Guegan et al (2001). In brief, samples were homogenised in ice cold dounce homogenisers with 10x (w/v) homogenisation buffer (250mM sucrose; 10mM KCl; 1.5mM $MgCl_2$; 2mM EDTA; 20mM HEPES; 1:100 Protease-phosphatase inhibitor cocktail, PI78443, Thermo Scientific; distilled H_2O). The homogenate was centrifuged at 500x *g* for 5 minutes at room temperature, following which the supernatant was collected in a fresh tube whilst the pellet was discarded. The collected supernatant was centrifuged at 13,000x *g* for 20 minutes at 4^oC, after which the pellet was resuspended in homogenisation buffer to provide a mitochondrial fraction whilst the supernatant was transferred to an ultracentrifuge and spun at 100,000x *g* for 1 hour at 4^oC to provide a cytosolic fraction.

2.2.8 | BCA assay

Prior to assessment of protein expression by western blot, the protein levels of each sample were determined using the BCA assay (Pierce™, Thermo Scientific) to enable calculation of appropriate loading volumes. Briefly, serial dilutions of BSA were created in duplicate as protein standards (0 - 2000mg/ml). Experimental protein samples were diluted 1:20 in milliQ water in duplicate (25µL/well) and 200µL AB solution (Solution A: Solution B, 50:1 ratio) added to each well of protein sample, BSA standard and blank (AB solution alone). Following 30-minute incubation at 37^oC, sample absorbance was measured at 562nm to determine the optical density of the

BCA signal. The blank values were subtracted from all data and the BSA protein standard curve used to estimate the protein content of the experimental samples.

2.2.9 | Western blotting

Protein samples were run on a 12% resolving gel with a 4% stacking gel, using a 1mm mould with 15 wells per gel. The protein ladder (Odyssey® Protein Molecular Weight Marker, 928-40000, Li-Cor) was included in the first well of each gel. 30µg of each sample was mixed with 6x Laemmeli buffer and boiled for 10 minutes, spun down briefly, and loaded into the wells. Gels ran at 95V for 30 minutes, then 130V for an hour. Samples were transferred to EMD Millipore™ Immobilon™ PVDF (PSQ) at 100V for 1 hour, using the Biorad wet transfer system, and blocked with 5% NGS in TBS-T for 1 hour. Following probing with the appropriate antibodies [Table 3], using β-actin as a protein for normalisation, and incubation with IRDye® secondary antibodies, the blot was imaged and analysed using a Licor Odyssey® CLx with Image Studio™ software.

Table 3: **Primary antibodies utilised for immunoblotting during characterisation of the rotenone model**

Target	Host	Supplier	Catalogue #	Dilution
Anti-β-Actin	Mouse monoclonal	Sigma	A5441	1:20,000
Anti-Cytochrome C	Mouse monoclonal	BD Phamingen	556433	1:500 Cytosolic 1:10,000 Mitochondrial

2.2.10 | Statistical analysis

All values are expressed as mean ± SEM or absolute values where specified. Differences between means were analysed using students T-test or one-way ANOVA

with repeated measures. All analyses were performed in GraphPad Prism 5 (GraphPad Software Inc. California). The null hypothesis was rejected when *P*-value <0.05.

2.3| Results

2.3.1 | Rotenone induces acute weight loss and early mortality

As rotenone is a highly lipophilic molecule, it can easily cross the blood brain barrier and enter different cell types within the brain, to block mitochondrial function. Before adopting this PD model for my further studies, I wanted to investigate the side effects and toxicity of this model which utilises systemic administration of rotenone. I decided to use monitoring of body weight, locomotor function and mortality as measures of systemic toxicity. Adult male Lewis rats treated with rotenone daily for 8 days, following the published model by Cannon et al (2009), demonstrated acute systemic side effects. These included rapid weight loss [Figure 6a; 5-12% start weight lost by endpoint, compared to <5% in the Vehicle group, $*P<0.05$] and increased incidence of early mortality [Figure 6b]. In the rotenone group, 50% of animals were sacrificed prior to endpoint due to the severity of their side effects under the protocol limitations, whereas the vehicle group displayed no changes in feeding behaviour. Based on these observations, it is clear that systemic injection of rotenone can cause severe weight loss and increased mortality; these severe effects should be taken into consideration for adjustment of dosage and treatment duration for future use of rotenone to model PD.

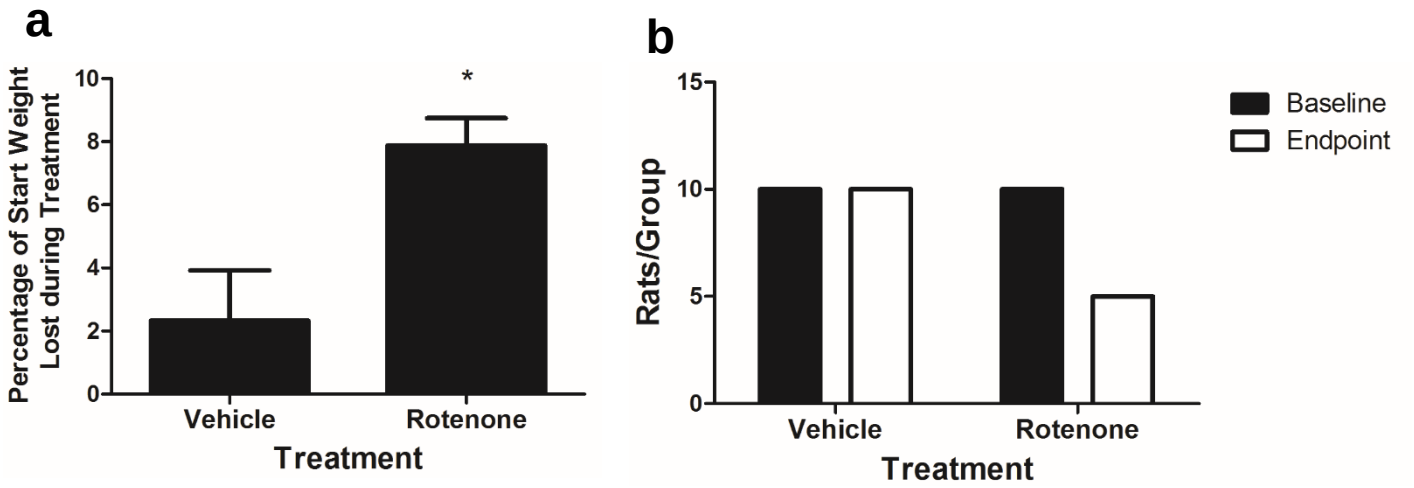


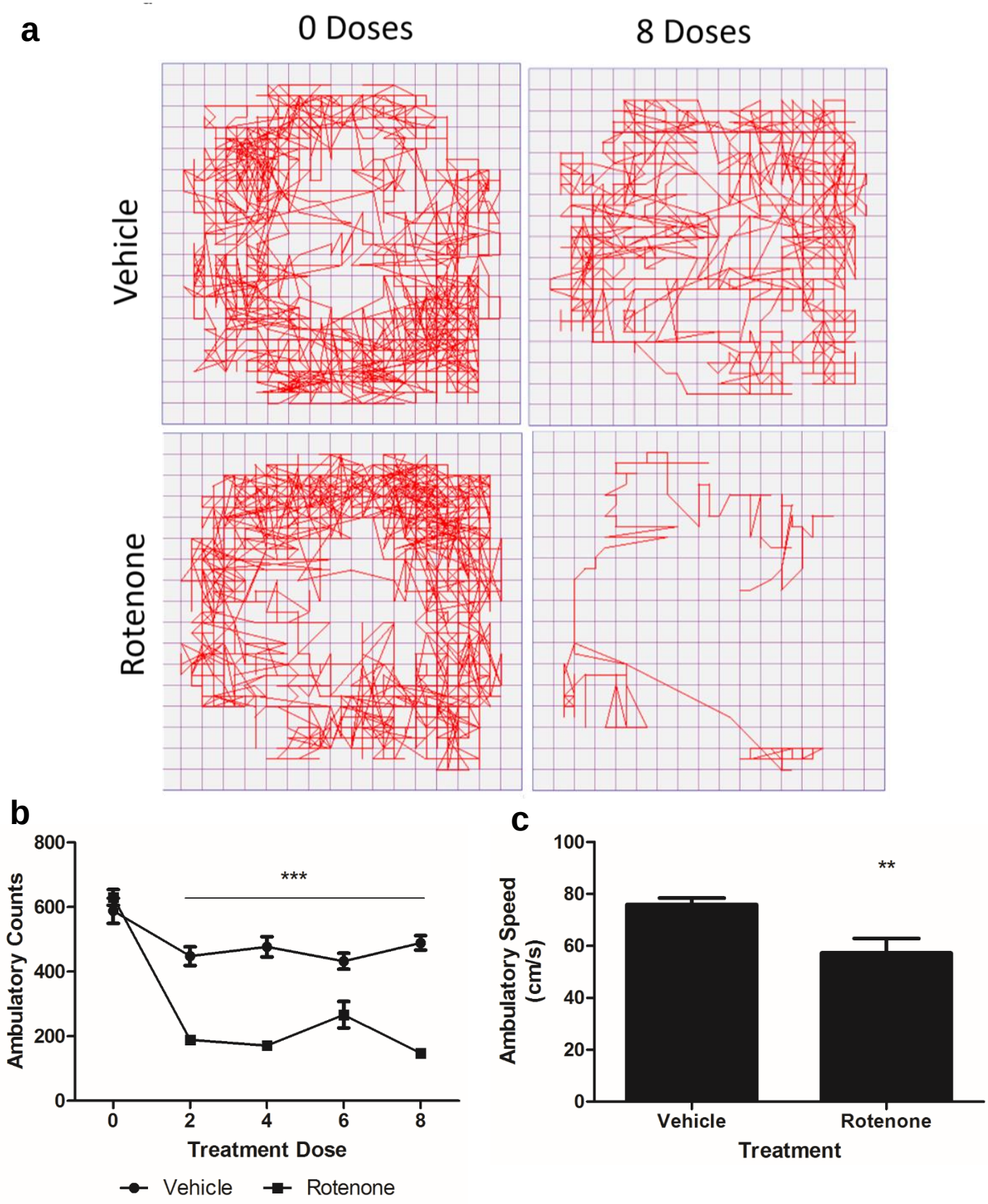
Figure 6: Rotenone treatment induces weight loss and early mortality. Male Lewis rats were treated with 2% DMSO in Miglyol 812N vehicle or rotenone (3mg/kg, IP) daily for eight days. Daily monitoring of the rat's weight for injection demonstrated an increase in weight loss (**a**) in the rotenone group compared to the vehicle group. The vehicle group displayed <5% weight loss whilst the rotenone treated group presented with weight loss between 5-12% in all animals. Overall, the treatment paradigm caused a loss of 50% of rotenone-treated animals prior to endpoint (**b**) with a 0% loss in the vehicle group. Data (**a**) represents mean percentage of the start weight lost ± SEM, n=10 rats per group, analysed by t-test, **P*<0.05. Data (**b**) represent absolute values.

2.3.2 | Rotenone induces locomotor depression

Regular assessment of locomotion throughout the treatment regime provided quantitative data of the impairment in spontaneous locomotion observed in the rotenone-treated rats. Baseline measurements were established prior to treatment and then repeat measures were recorded after every second treatment dose. Open field chamber assessment of locomotor activity revealed changes in motor activity; movement path maps provide clear visualisation of the overall depression of movement in the rotenone group compared to baseline measurements and the vehicle group [Figure 7a]. Mild depression in movement is visible in the paths of the vehicle group, however this is likely the result of discomfort caused by the daily intraperitoneal injections and habituation to the open field chamber. There is no significant difference between the locomotor activity of vehicle-treated rats at baseline and after eight doses, whereas significant changes in motor activity are detectable in rotenone-treated rats from two doses onwards. A significant reduction in multiple factors, including ambulatory counts (the rectilinear distance travelled, measured in infrared beam

breaks) is present from two doses onwards [Figure 7b; ***P<0.001], suggestive that the reduction in spontaneous movement resulted from the systemic side effects of rotenone treatment, rather than rotenone-induced neuropathology which was not established this early in the treatment paradigm. Average ambulatory speed was unaffected until later in treatment, so it is unclear whether it may better reflect a change resulting from the rotenone-induced neuropathology, or if it is merely a delayed effect of the earlier-established locomotor depression due to increasing illness of the animals. Rotenone-treated rats displayed a significant slowing of the speed of their movement at eight doses of treatment, whereas vehicle-treated rats displayed no change [Figure 7c; **P<0.01], which indicates that rotenone treatment may recapitulate the bradykinesia, or slowing of movement, of PD patients.

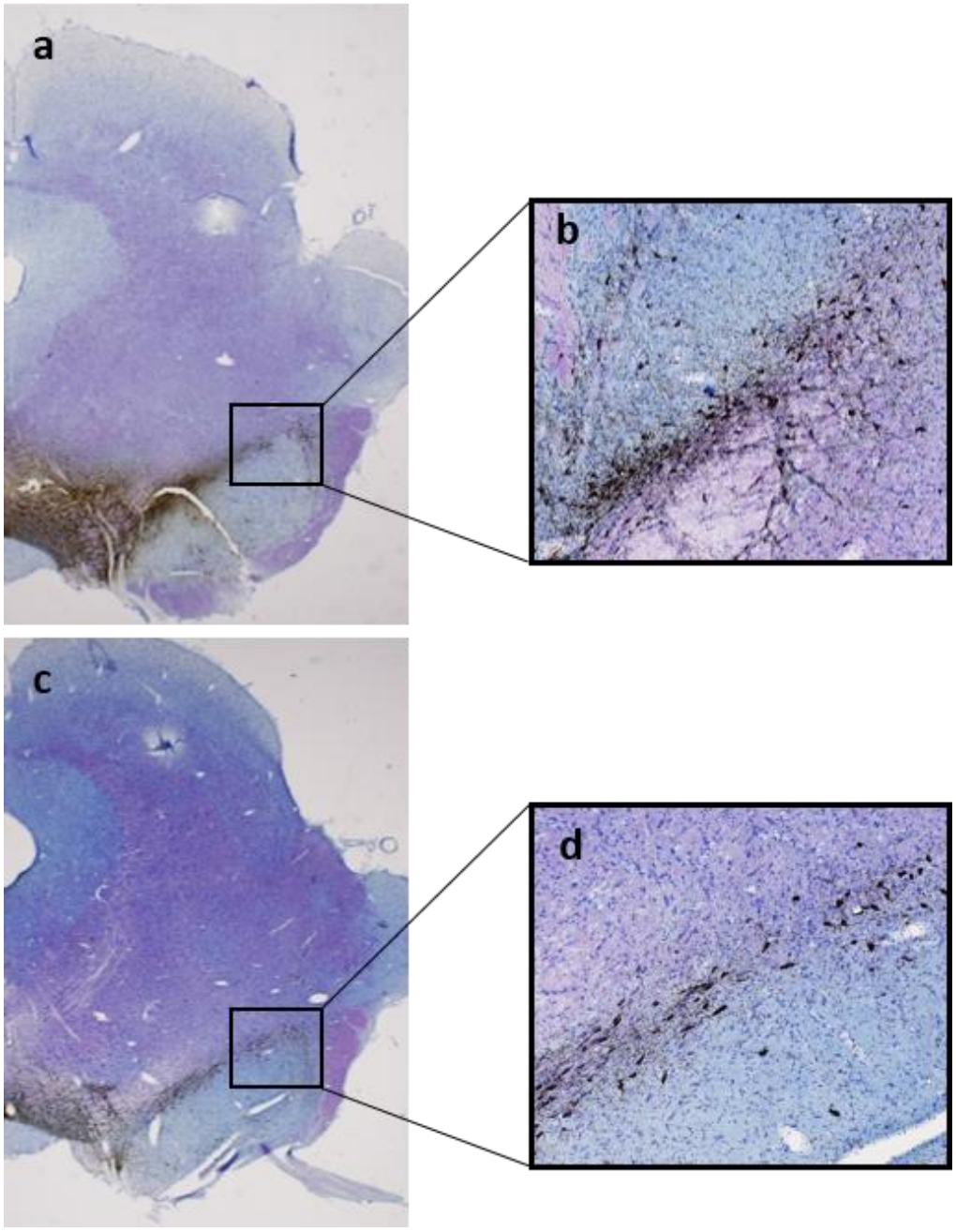
Figure 7: Rotenone induces depression of motor function (Page 93). Representative baseline path maps (**a**) display similar activity in animals from both groups; after eight doses the vehicle-treated rats display a slight reduction in movement, however there is a considerable reduction in the locomotion of rotenone-treated rats. (**b**) Analysis of ambulatory counts revealed significant depression in locomotion from as early as two doses of rotenone. The concurrent reduction in ambulatory counts in the vehicle group, which was sustained from two doses onwards, is likely the result of discomfort due to the daily IP injections and possibly habituation to the apparatus. (**c**) Average ambulatory speed was significantly reduced only at endpoint in the rotenone group compared to baseline and the vehicle-treated group. Data (**b, c**) represents mean ± SEM, n=5-10 rats per group, analysed by one-way ANOVA with repeated measures (**b**) and t-test (**c**). **P<0.01, ***P<0.001.


a
0 Doses
8 Doses
Vehicle
Rotenone
b
Ambulatory Counts
800
600
400
200
0
***
0
2
4
6
8
Treatment Dose
Vehicle
Rotenone
c
Ambulatory Speed (cm/s)
100
80
60
40
20
0
**
Vehicle
Rotenone
Treatment

2.3.3 | Rotenone induces dopaminergic cell loss in the substantia nigra pars compacta

Nigral dopaminergic neurons are the primary cell type affected in PD. To validate this PD model in my hands, I performed stereological cell counting using coronal VMB sections of rats treated with rotenone or the vehicle control. Treatment with rotenone induced a significant reduction in the number of TH-positive neurons in the SNpc. Gross changes in the density of staining are apparent [Figure 8a-d] and unbiased stereological cell counting confirmed a loss of ~45% TH-positive neurons in the rotenone group [Figure 8e; ** $P<0.01$]. The corresponding loss of nissl-positive neurons supports the conclusion that these data indicate a specific loss of dopaminergic nigral neurons, rather than downregulation of the TH phenotype [Figure 8f; *$P<0.05$]. These data support the capacity of the model to induce nigrostriatal damage.

Figure 8: Rotenone induces a loss of dopaminergic neurons in the substantia nigra pars compacta (Page 95). Male Lewis rats, approximately 7 months old, were treated with 2% DMSO in Miglyol 812N (**a, b**) or rotenone (3mg/kg, IP) (**c, d**) daily for eight days. Twenty-four hours after the final dose, rats were euthanised and samples collected for immunostaining, stereological cell counting and analysis. Loss of TH-positive staining in the substantia nigra is evident in rotenone-treated rats (**c**) versus vehicle controls (**a**) at 2x magnification. Reduced density of TH-positive neurons is clear at 10x magnification (**b, d**). (**e**) Stereological counting was utilised to quantify this neuropathological change, demonstrating a loss of ~45% TH neurons in the rotenone-treated group, with corresponding loss of nissl neurons, indictive that it is not a phenotypic loss of TH (**f**). (**a-d**) Representative images. Data (**e**,**f**) represents mean ± SEM, with individual data points, n=3 rats per group, analysed by t-test. *$P<0.05$, **$P<0.01$.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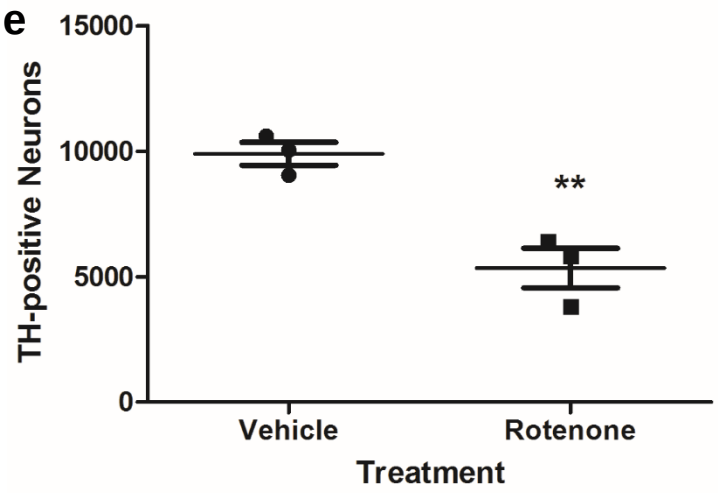
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Rotenone
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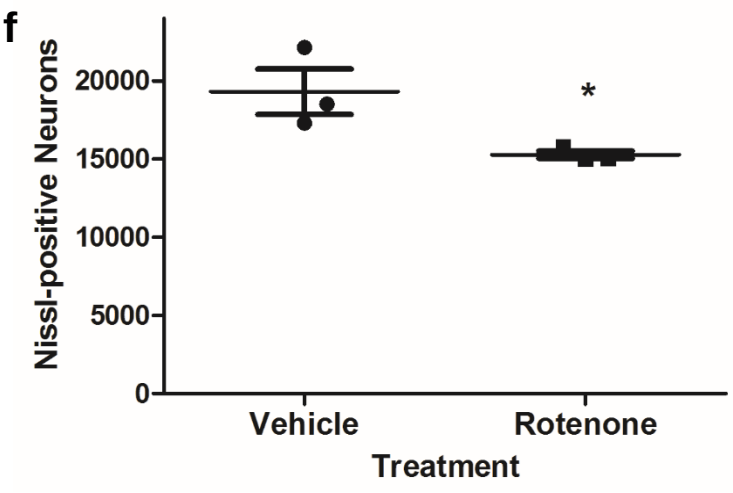
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2.3.4 | Rotenone activates release of the pro-apoptotic factor cytochrome C in the ventral midbrain

To further validate cell death in this rotenone rat model, I assessed cytochrome C release by performing immunoblotting with protein samples from the VMB of rats treated with rotenone or the vehicle control. As seen in Figure 9, rotenone treatment induced a significant increase in cytosolic cytochrome C levels in VMB protein samples [Figure 9, $P<0.05$]. Assessment of protein levels in the cytosolic fraction of micro-dissected VMB tissue revealed a clear increase in the level of this early apoptotic factor compared to vehicle-treated rats when normalised to β-actin levels, indicative that rotenone treatment initiates release of cytochrome C from mitochondria and possible initiation of the apoptotic cascade.

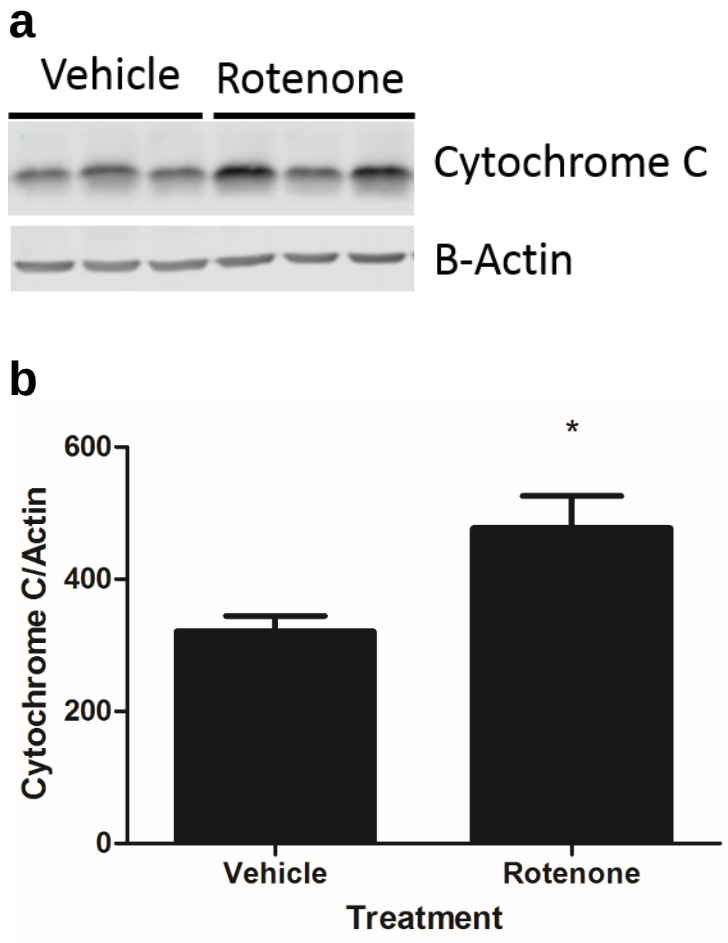


Figure 9: Rotenone induces increased cytosolic cytochrome C levels in the ventral midbrain. Western blot of cytosolic protein samples from the ventral midbrain of vehicle and rotenone treated animals (**a**) revealed that rotenone treatment (8 doses) significantly increased cytochrome C levels, indicative of increased release of this early apoptotic factor from mitochondria (**b**). Data represents mean ± SEM, n=3 rats per group, analysed by t-test, **P*<0.05.

2.4| Discussion

This preliminary work set out to establish the suitability of rotenone to successfully model PD in rodents and as a potential model for the investigation of therapeutic modulation of Drp1 function. Following the methods of Cannon et al (2009), I demonstrated that systemic rotenone administration induced locomotor dysfunction and dopaminergic nigral cell loss, potentially through induction of apoptotic cell death; however, I also discovered that this treatment paradigm induced severe acute systemic side effects, including locomotor depression, reduced feeding behaviour and weight loss. The variability in the severity of systemic side effects in my study does not support the 'high reproducibility' of the model, as it raises concerns in future study design for how to account for the potential loss of animals during treatment. Overall this rotenone treatment protocol induced systemic side effects, including rapid weight loss, hunching and piloerection, necessitating the early sacrifice of a high percentage (50%) of rats in the rotenone-treated group; the severity of these adverse effects and their occurrence in a large proportion of the treatment group is suggestive that the dose may be too high and the locomotor recordings support the conclusion that some of the observed locomotor depression in animals at earlier dose-points may result from acute systemic effects, rather than neuropathological changes. Analysis of behavioural data indicated significantly reduced ambulatory counts in rotenone-treated animals sacrificed prior to endpoint, in which gross loss of TH-positive neurons in the SNpc was not apparent; thus some locomotor parameters must be considered cautiously as they may be attributed to non-neuropathological effects of rotenone in those animals.

Peripheral administration of a high dose of this potent complex I inhibitor increases the likelihood of inducing unanticipated systemic side effects, which may

confound the detection of neuropathology-induced locomotor changes; Table 4 summarises a variety of the rotenone models published to highlight the variability of methods, animals and doses used. From the nine different rotenone administration methods, five resulted in weight loss and/or early mortality (Betarbet *et al.*, 2000; Cannon *et al.*, 2009; Fernández *et al.*, 2011; Fernández *et al.*, 2012; Fleming *et al.*, 2004; Gokul and Muralidhara, 2014; Höglinger *et al.*, 2003; Huang *et al.*, 2006; Lapointe *et al.*, 2004; Liu *et al.*, 2015; Mulcahy *et al.*, 2011; Mulcahy *et al.*, 2013; Ravenstijn *et al.*, 2008; Sharma and Nehru, 2013; Sherer *et al.*, 2003; Sonia Angeline *et al.*, 2013; Tapias, Cannon and Greenamyre, 2014; Tapias, McCoy and Greenamyre, 2019), despite the wide range of rotenone doses, variety of diluents and different administration periods.

In some studies utilising rotenone to model PD, lower doses of rotenone have been administered for longer treatment periods to better model the low-dose chronic exposure to which human PD patients may be exposed, increasing their risk of developing PD. Gokul et al (2014) utilised 1mg/kg daily IP in mice for a period of 21 days, and detected a modest reduction in striatal dopamine release, whilst Bai et al (2016) used rats with a treatment regime of 1mg/kg IP every 48 hours for 24 weeks, successfully recapitulating PD-associated dopaminergic cell loss and locomotor dysfunction. Whilst these models are indicative that low-dose chronic rotenone treatment produces a representative model of PD pathology, their reproducibility has not been demonstrated by repeat publication of the same regime.

Intranigral and intrastriatal stereotactic delivery of rotenone has also been investigated, which removes the risk of inducing life-limiting systemic effects as a result of rotenone treatment, however this route of delivery is less illustrative of environmental rotenone exposure. Previous work utilising direct intrastriatal or

intranigral delivery of rotenone has demonstrated variability in the success of recapitulating some key pathological PD factors, including striatal dopamine depletion and α-synuclein pathology (Carriere, Kang and Niles, 2016; Sindhu, Saravanan and Mohanakumar, 2005; Saravanan, Sindhu and Mohanakumar, 2005; Weetman *et al.*, 2013; Xiong *et al.*, 2009b).

In 2015, Liu et al published an environment contact model utilising dermal and inhalation rotenone exposure in C57BL/6 mice; this model was designed to better reproduce the likely routes by which humans are exposed to rotenone, producing dose-dependent weight loss and early mortality, with variable loss of dopaminergic nigral neurons, changes in striatal dopamine, α-synuclein pathology and locomotor changes. Whilst this model seems promising, it is unclear whether it could be utilised to investigate therapeutic strategies and the nature of exposure may induce variable results due to the difficulty in predicting the true exposure dose of each animal.

Many papers have utilised intraperitoneal injection strategies, with a variety of doses, diluents and administration protocols published. Many of these report weight loss or early mortality as a side effect of the treatment, indicative that a number of animals and breeds display susceptibility to the systemic toxicity of rotenone (Cannon *et al.*, 2009; Fernández *et al.*, 2012; Fernández *et al.*, 2011; Gokul and Muralidhara, 2014; Sonia Angeline *et al.*, 2013; Tapias, McCoy and Greenamyre, 2019; Tapias, Cannon and Greenamyre, 2014). There is also variability between the research's success in demonstrating nigrostriatal degeneration and α-synuclein pathology.

Locomotor changes are widely reported, however as we discovered in our own model some changes may result from the systemic toxicity rather than directly resulting from rotenone-induced neuropathological changes.

Table 4: Summary of the various *in vivo* rotenone models published between 2000-2019, from over 35 papers

Administration Route	Dose Range	Animal Breeds	Animal Age	Diluents	Treatment Duration	Weight loss / Mortality	Nigral Degeneration	Striatal TH / DA Loss	α-synuclein	Motor Changes	References
Environmental Contact	5 - 100 mg/kg/day	C57BL/6 Mice	8-9 wks	Olive oil	2-6 wks	Dose-dependent	25-40% loss	Yes	Yes	Yes	(Liu et al., 2015)
Intragastric	0.0625 mg/g/day	C57BL/6 Mice	1 year	CMC + Chloroform	1.5-3 mths	/	Yes	No	Yes	Variable	(Jia et al., 2014; Pan-Montojo et al., 2010)
Intranasal	2.5 mg/kg/day	C57BL/6 Mice	8 wks	Saline	30 days	/	No	No	/	No	(Rojo et al., 2007);
Intraperitoneal Injection	1-3 mg/kg/day	CST-Swift Mice Lewis rats Sprague-Dawley rats Wistar rats E46K synuclein rats	7 wks to 12 mths	Sunflower oil Miglyol 812N + DMSO DMF + PEG DMSO + PEG	8 days to 6 wks	Yes	Variable	Variable	Variable	Variable	(Alam and Schmidt, 2002; Bai et al., 2016; Cannon et al., 2009; Drolet et al., 2009; Fernández et al., 2011; Fernández et al., 2012; Gokul and Muralidhara, 2014; He et al., 2003; Sonia Angeline et al., 2013; Tapias, Cannon and Greenamyre, 2014; Tapias, McCoy and Greenamyre, 2019b; Zhang et al., 2017)
Oral	0.25-30 mg/kg/day	C57BL/6 Mice C57BL/6xC3H Mice	8 wks to 13 mths	CMC	4-5 wks	No	Yes	Yes	Yes	Yes	(George et al., 2010; Inden et al., 2011; Liu et al., 2017)
Stereotactic Delivery (SNpc)	3-12µg/1µl delivery	Sprague Dawley rats	7 wks≤	DMSO alone DMSO + PEG	Single dose	/	Yes	Variable	Variable	Yes	(Saravanan, Sindhu and Mohanakumar, 2005; Sindhu, Saravanan and Mohanakumar, 2005; Xiong et al., 2009b; Anusha, Sumathi and Joseph, 2017)
Subcutaneous Injection	2-3 mg/kg/day	Sprague-Dawley rats Wistar rats	7 wks≤	Sunflower oil	4-5 wks	Yes	/	/	/	Yes	(Ferris et al., 2013; Fleming et al., 2004; Kaur, Chauhan and Sandhir, 2011; Sharma and Nehru, 2013; Zhu et al., 2004)
Subcutaneous Microspheres	~90 mg/kg	Long-Evans rats Sprague-Dawley rats	7 wks to 9 mths	/	30 days to 12 wks	Variable	Yes	Variable	Variable	Variable	(Huang et al., 2006; Marella et al., 2008)
Subcutaneous Osmotic Minipumps	2-50 mg/kg/day	C57BL/6 mice Lewis rats Sprague-Dawley rats	7 wks≤	DMSO + PEG	1-8 wks	Variable	Variable	Variable	Variable	Variable	(Betarbet et al., 2000; Greene, Norrian and Srinivasan, 2009; Lapointe et al., 2004; Mulcahy et al., 2013; Murakami et al., 2014; Panov et al., 2005; Ravenstijn et al., 2008; Sherer et al., 2003a; Sherer et al., 2003b)

Some models have relatively consistent treatment regimens and findings whilst others display greater variability, illustrating the inconsistencies found in the use of rotenone to model Parkinson's disease. CMC = carboxymethylcellulose, DMF = dimethyl fumarate, DMSO = dimethyl sulfoxide, PEG = polyethylene glycol, / = untested/unreported in administration method.

Ultimately, the high incidence of early mortality induced by the Cannon et al (2009) treatment regime, along with systemically induced locomotor changes and variable dopaminergic neuron loss, contributed to my decision to move away from the model for the ongoing work. To address the aims of the thesis one of the key experimental aims was to address how inhibition of Drp1 via pharmacological and genetic methods would impact the rotenone-induced neuropathology. The severe systemic toxicity and high incidence of early mortality would have required increased animal numbers for experiments which, when the genetic manipulation of Drp1 relied on precise delivery of Drp1-DN rAAV to the SNpc of rats, was deemed risky as the survival outcomes of the work were unpredictable and could have resulted in small final sample size per group, which could result in low statistical power during analysis and difficulty interpreting results. The generalised toxicity induced by rotenone treatment also impacted rotenone behaviour, which can confound interpretation of locomotor depression, not necessarily resulting from rotenone-induced neuropathology.

Future work utilising rotenone to model PD would benefit from more accurate recapitulation of the progressive nature of the disease, to enable the introduction of potential therapeutic agents at different stages of progression; it would be valuable to demonstrate the threshold at which therapeutic interventions are most effective, and whether they remain effective at later stages, as much of the published work has focused on neuroprotective investigation rather than the introduction of therapeutic agents after the disease state has been established. Greater focus on human-relevance in terms of the exposure route, administration dosage and the treatment duration would also be valuable. Many of the current models rely on rotenone treatment which, once initiated, persists until death or sacrifice of the animal; in human

populations this is unlikely to be the case, as occupational or proximity exposure to rotenone would not be a constant steady exposure, rather the increased disease risk is more likely to be the result of sporadic, low dose exposure incidents which, in combination with other risk factors, increase the likelihood of future PD development. Any future model which can demonstrate the persistent and progressive effects of rotenone in periods absent of direct exposure would greatly further our understanding of how this pesticide contributes to the human disease. Recent, unpublished work by the Greenamyre group has included the development of a rotenone model which better represents the progressive, gradual degeneration of the nigrostriatal pathway, with concurrent onset of motor symptoms and the demonstration of neuropathology post-mortem. This model is still being refined; however, I believe it holds promise for the future despite the continued reliance on systemic, non-representative, rotenone administration.

To move forward with my research, for which optimisation and development of the rotenone model would have been costly and time consuming, I opted to focus on utilising the other pesticide model proposed for my project: paraquat. As a result, the remainder of this thesis will detail the *in vivo* and *in vitro* work undertaken to investigate the potential of targeting Drp1 function in the paraquat model and to explore the mechanisms by which this may confer benefit.

Chapter 3| Investigating the potential of paraquat as a neurotoxic mouse model of Parkinson's disease and attenuation of neurotoxicity via Drp1 inhibition with small molecule and transgenic methods

3.1| Introduction

As introduced in Chapter 1.4.1, PQ has been used agriculturally for decades and has been identified as a prominent herbicidal risk factor for PD. The pathogenic mechanism of PQ is distinct from that of rotenone, as despite the structural similarities to MPTP, PQ does not directly inhibit electron transport chain complex function, instead utilising oxidative stress, which may employ electrons from electron transport chain complex I and III to produce H_2O_2 (Castello, Drechsel and Patel, 2007; Cochemé and Murphy, 2008). Multiple experimental models for PQ have been published, demonstrating the ability of PQ-treatment to recapitulate key aspects of PD, including loss of the dopaminergic nigrostriatal pathway, reduced striatal dopamine levels, increased α-synuclein levels and reduced locomotor activity (Manning-Bog *et al.*, 2002; Peng *et al.*, 2004; Prasad *et al.*, 2009; Rappold *et al.*, 2011). For this work I decided to continue using a treatment regime which has previously proved effective in our group. Earlier work demonstrated that organic cation transporter 3 null ($Oct3^{-/-}$) mice display enhanced striatal degeneration following PQ treatment (Rappold *et al.*, 2011), however our group verified that specific dopaminergic nigral degeneration is still induced in C57BL/6 mice with this treatment regime, and therefore it should be suitable for use in $Oct3^{+/+}$ mice also (Rappold *et al.*, 2011).

In this study I utilised two methods of Drp1 inhibition; in a small molecule model of Drp1 inhibition, I used $Oct3^{-/-}$ mice to facilitate concurrent investigation of both nigral and striatal degeneration. For the genetic model of Drp1 inhibition, I utilised global heterozygous Drp1 knockout mice ($Drp1^{+/+}$) mice and their WT ($Drp1^{+/-}$) littermates as controls.

Mdivi-1 is a small-molecule inhibitor of Drp1, characterised by Cassidy-Stone et al (2008) following screening of several chemical libraries using a yeast two-hybrid

system. Mdivi-1 has been shown to inhibit Drp1 function by allosteric inhibition of the fission protein, preventing its assembly into oligomeric ring structures and its GTPase activity (Xie *et al.*, 2013). Mdivi-1 has been applied to multiple disease studies, and data suggests efficacy in several models of neurodegeneration; in 2010 our lab demonstrated that abnormal mitochondrial morphology and function induced by mutant PINK1^{L347P} in N27 cells can be prevented by mdivi-1 treatment (Cui *et al.*, 2010). Further work *in vivo* has supported the protective effects of mdivi-1 against aberrant mitochondrial changes in models of PD (Bido *et al.*, 2017; Rappold *et al.*, 2014), Alzheimer's disease (AD) (Oliver and Reddy, 2019; Reddy *et al.*, 2012), ischemic brain injury (Zhao *et al.*, 2014) and epilepsy (Qiu *et al.*, 2013; Xie *et al.*, 2013), distinguishing its promise as a potential therapy for multiple disorders.

As small-molecule pharmaceutical interventions always present a risk of off-target effects, and a recent paper challenged the accepted mechanics of mdivi-1's method of Drp1 inhibition (Bordt *et al.*, 2017), I also worked to confirm the effects of Drp1 inhibition on PQ-induced neuropathology through use of a transgenic mouse model. These mice, of C57BL/6 background, present with global heterozygous Drp1 knockout. Previous characterisation of these mice was performed to investigate the effect of global heterozygous Drp1 knockout, in comparison to the embryonically lethal global homozygous Drp1 knockout, however they have not been utilised in neurotoxic models of PD until now (Wakabayashi *et al.*, 2009). Characterising the mouse model and confirming the successful breeding of Drp1 global heterozygous mice was also a crucial step before we utilised these animals in the study of potential protection from neurotoxic PQ treatment.

3.2| Materials and Methods

3.2.1| Animal care

$Oct3^{-/-}$ mice (15-30g; 4-6 months old), described by the lab previously (Cui *et al.*, 2009; Rappold *et al.*, 2011), Drp1 heterozygous global knockout ($Drp1^{+/-}$) mice and littermate WT $Drp1^{+/+}$ mice (3-4 months old) (acquired from Institut Clinique de la Souris (ICS), Mouse Clinical Institute, France) were utilised for *in vivo* paraquat experiments. The $Oct3^{-/-}$ mice were utilised in the first portion of this study as our lab previously described a mechanism of PQ uptake into cells, which implicated Oct3 and DAT (Rappold *et al.*, 2011). In its native divalent state, PQ^{2+} is not a substrate for DAT, however conversion to a monovalent cation by cellular diaphorases, such as nitric oxide synthase and NADPH oxidase, produces a monovalent PQ^{+} species which is a DAT substrate. This facilitates its transport and accumulation in dopaminergic neurons, where it induces cytotoxicity and oxidative stress (Day *et al.*, 1999; Rappold *et al.*, 2011). Reports that mutant mice with hypomorphic DAT are resistant to PQ neurotoxicity indicate that functional DAT is necessary for PQ to elicit its neurodegenerative effects (Rappold *et al.*, 2011). PQ^{+} is also a substrate for Oct3 (*Slc22a3*), an abundantly-expressed, poly-specific, bidirectional cation transporter found in dopaminergic and non-dopaminergic cells of the nigrostriatal regions (Tieu, 2016). In mice with Oct3 deficiency ($Oct3^{-/-}$), enhanced striatal damage was detected after PQ treatment. The increased sensitivity likely results from reduced buffering capacity by non-dopaminergic cells, leading to increased availability of PQ^{+} for uptake into dopaminergic neurons by DAT (Rappold *et al.*, 2011), thus Oct3 knockout mice are useful for modelling PQ-induced PD risk (Tieu, 2016).

All mouse care and experimental procedures were approved and conducted in accordance with Home Office Animals for Scientific Procedures Act (ASPA) 1986

guidelines (PPL 30/3088; PIL IC7E2094D) and the Florida International University Institutional Animal Care and Use Committee (IACUC) under protocol number 16-069-AM04. All animals were maintained on a 12-hour light/dark cycle, with access to food and water *ad libitum*.

3.2.2| Confirmation of Tm1b mouse model

Genotyping

At post-natal day 2-3, tail and toe clip samples were taken from mouse pups to enable genotyping and identification of individual animals for ongoing growth rate characterisation experiments. Biological samples were collected in DNase-free Eppendorf tubes on ice. DNA was extracted for PCR using a simple protocol; 75µl alkaline solution (25mM NaOH and 0.2mM EDTA in PCR water) was added to each sample and then incubated in a heat block at 96^{o}C for 45 minutes. After cooling slightly, 75µl 40mM Tris-HCL (ph 7.6) was added to each sample on ice. Residual tissue was pelleted via centrifugation at 15,000 x *g* for 30 seconds at 4^{o}C. 1µl of DNA solution was added to each reaction. PCR products were run on 1% agarose-TBE gels with GelRed™ (Biotium; 41003). Gene Ruler Mix Ladder (ThermoScientific; FERSM0311PM) was used to validate fragment sizes and each sample was mixed with 6x DNA Loading Dye (ThermoScientific; FERR0611PM) prior to loading. The electrophoresis gel was run at 120V/200mA for 45 – 120 minutes and visualised under UV.

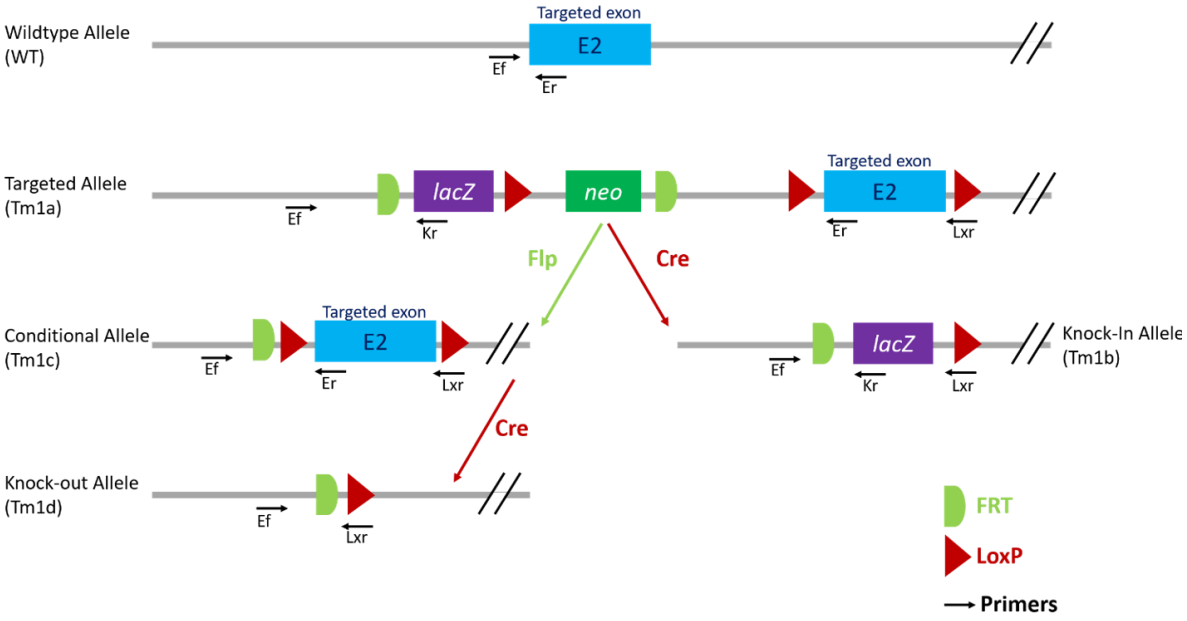


Figure 10: Genotyping strategy for Drp1$^{+/-}$ Tm1b mice. Using the genotyping strategy above we established an effective protocol to distinguish heterozygous Drp1 knockout mice (Tm1b in the above image) from their wild-type Drp1$^{+/+}$ littermates.

Table 5: Primer sequences for genotyping of the Tm1b global heterozygous Drp1 knockout mice

Primer Name	Primer Sequence
Ef	5' GATTGGGCAGGAGCAAGATTCTC 3'
Er	5' GTGAGCTGACTGATGTAGGTGCTG 3'
Kr	5' GGGCAAGAACATAAAGTGACCCTCC 3'

Table 6: PCR conditions for genotyping the Tm1b global heterozygous Drp1 knockout mice

	Stage 1		Stage 2		Stage 3	
Repeats	x 1	x 35			x 1	
Temperature	95 °C	94 °C	61 °C	72 °C	72 °C	20 °C
Time	4 minutes	30 seconds	30 seconds	1 minute	7 minutes	∞
Step	Taq activation	Denature	Anneal	Extend	Final Extension	Hold

qPCR

RT-PCR with TaqMan® probes was utilised to quantify the expression of Drp1 in the heterozygous global Drp1 knockout ($Drp1^{+/-}$) mouse model prior to their use for further experiments. Following sacrifice of $Drp1^{+/-}$ mice and WT litter mate controls, the brain was dissected, and one hemisphere collected for qPCR. The method primarily followed the advised protocol. RNA was isolated using the TRIZOL method (Fisher; 15-596-026); Briefly, one hemisphere of brain tissue was homogenised in TRIZOL reagent with a glass dounce homogenizer, samples were then centrifuged at 12,000 x *g* for 5 minutes at 4^{o}C and the supernatant transferred to a clean Eppendorf tube. Following 5 minute incubation, chloroform was added and the sample incubated for a further 3 minutes. Samples were centrifuged at 12,000 x *g* for 15 minutes at 4^{o}C. The RNA-rich aqueous layer was then transferred to a new tube. The sample was isolated with ice cold isopropanol for 10 minutes prior to centrifugation at 12,000 x *g* for 10 minutes at 4^{o}C. The supernatant was discarded, and the RNA pellet resuspended in 75% ethanol and vortexed briefly. Following centrifugation at 7500 x *g* for 5 minutes at 4^{o}C, the supernatant was again discarded, and the RNA pellet dried for 5 minutes before solubilisation in RNase-free water. Following RNA isolation, the RNA content of each sample was quantified using a NanoDrop™ One^{C} and reverse transcription performed using the Superscript™ IV VILO™ Kit (Fisher; 11756050). 10ng of cDNA was loaded into each well of the 96 well plate and RT-PCR ran in a QuantStudio 6 Flex as specified below. GAPDH was used as the endogenous control and results were analysed using the pfaffl method.

Table 7: Real-time qPCR conditions for characterisation of Drp1 expression in global heterozygous Drp1 knockout mice and age-matched wild type controls

			PCR (40 Cycles)	
	UNG Incubation	**Polymerase Activation**	**Denature**	**Anneal/Extend**
Temperature	50ºC	95ºC	95ºC	60ºC
Time	2 minutes	2 minutes	1 second	20 seconds

Western blotting

To complement the qPCR data, the remaining brain hemisphere from each mouse was taken and processed for protein extraction. The tissue was weighed and RIPA buffer (150mM NaCl, 5mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS and 25mM Tris-HCL ph 7.6) added at a 1:10 (w/v) ratio. Samples were homogenised in tube using plastic homogenisers (10-12 strokes) and then transferred to a syringe. Passage of the sample through a 26G needle 6 times was completed prior to centrifugation of the sample at 12,000 rpm for 25 minutes at 4ºC. The supernatant was collected, and a BCA assay (See Chapter 2.2.8) (Pierce™, Thermo Scientific) used to determine what volume of sample to prepare for each animal. Samples were run on a 12% resolving gel and a 4% stacking gel. Proteins were then transferred to EMD Millipore™ Immobilon™ PVDF (PSQ) using a Biorad wet transfer system and blocked with 5% BSA in TBS-T for one hour before overnight incubation with primary antibodies [Table 8]. Following washing, incubation with IRDye® secondary antibodies and final washes, the blot was imaged using a Licor Odessey® CLx. A protein ladder (Odessey® Protein Molecular Weight Marker, 10-150kDa) was utilised to verify molecular weights of the detected bands, which were then quantified using LI-COR Image Studio™. Data was exported to Microsoft Excel where Drp1 levels were normalised to β-Actin.

Table 8: **Primary antibodies utilised for immunoblotting during characterisation of the Tm1b global heterozygous Drp1 knockout mice**

Target	Host	Supplier	Catalogue #	Dilution
Anti-β-Actin	Mouse monoclonal	Sigma	A5441	1:20,000
Anti-Drp1	Mouse monoclonal	BD Biosciences	611113	1:2000

Investigation of developmental differences

As Drp1 is believed to play a role in development, with embryonically lethal homozygous knockouts reported to be developmentally delayed compared to their WT counterparts (Wakabayashi *et al.*, 2009), it was considered that perhaps global heterozygous Drp1 knockout mice may also demonstrate some developmental differences compared to their WT littermates. In order to assess the rate of growth of these mice, rather than merely the potential weight or size differences at any given age, mice were toe clipped at PND 2 (for identification) and measurements taken every week. All measurements were recorded with the animal's ID number without reference to their genotype, until later grouping of the data. At each measurement, the weight of the mice was recorded along with the length of each animal. A novel method was developed to enable standardised estimation of mouse length; in brief, the mouse was placed on laminated graph paper (5mm x 5mm grid size) and when in a relaxed position a photo was taken directly from above. The images were collated and labelled with mouse ID prior to quantification of the mouse length. Using Fiji, a free image analysis software, the scale was set for each image using the 5mm grid. The use of this grid allowed accurate scale determination regardless of variance in the height from which the image was taken. A midline was then drawn along the centre of the mouse [Figure 11] and the length measured. All data was exported to Excel. The monitoring

of mice throughout their development allowed for comparison of these growth parameters through various stages of development.

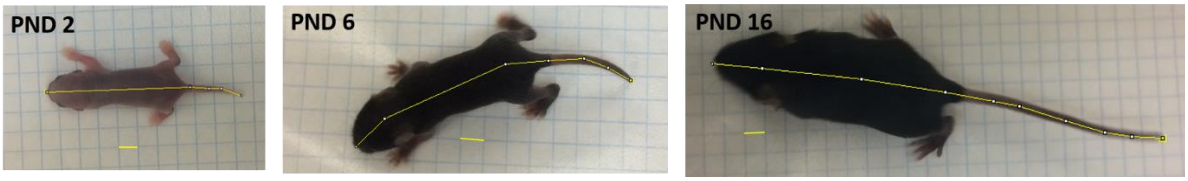


Figure 11: Quantification of mouse lengths. These examples display the scale bar from which the unit was set and the delineation of the midline of each mouse for estimation of their length at three different stages of monitoring

3.2.3| Chemical preparation and animal treatment

Paraquat (Sigma; 856177) was dissolved in sterile saline to create a 1mg/mL working stock, which was freshly prepared prior to each set of injections. Preceding chemical administration, the weight of each mouse was recorded in a tracking chart to enable weight monitoring throughout the duration of treatment. For each injection, the dose was calculated from the weight of the mouse immediately prior to administration. PQ was injected intraperitoneally at a dose of 10mg/kg every 48 hours for a total of ten doses, using a 26G needle and a 1ml syringe. Mice were sacrificed 7 days after the final injection.

Mdivi-1 (3-(2,4-dichloro-5-methoxyphenyl)-2-sulfanyl-4(3H)-quinazolinone; Enzo; BML-CM127) was prepared to a stock concentration of 100mg/mL in DMSO, which was then diluted to a working solution in sterile saline for injections (20mg/kg), with sonication of each dose to assist emulsification due to the poor aqueous solubility of mdivi-1. Mdivi-1 was injected twice daily (IP; 26G needle) for 3 days pre-treatment prior to the initiation of PQ injections, alongside the 20-day PQ injection regime and throughout the 7 day post-treatment lesion stabilisation period to investigate the neuroprotective potential of mdivi-1 in this model.

Mice were monitored for health and weight changes daily. If the weight loss or side effects of PQ administration became debilitating, limiting mobility, grooming, feeding, or leading to excessive weight loss (≤20%), the mouse was euthanised prior to endpoint and samples collected.

3.2.4| Sample collection and preparation

For this study, brain samples were collected and prepared ready for immunohistochemistry following conclusion of the PQ treatment regimen. Animals were anaesthetised with pentobarbitol (Euthatal®) and transcardially perfused with cold 0.1M PBS followed by 4% (w/v) paraformaldehyde (PFA; Sigma) in 0.1M PBS (pH 7.4). The brains were removed, post-fixed in 4% PFA overnight and subsequently cryoprotected in sequential 15% and 30% sucrose phosphate buffer solutions for 2-3 days at 4^{o}C. The brains were subsequently frozen in chilled methylbutane (approximately -50^{o}C) and stored at -80^{o}C until sectioning. Serial coronal sections (30µM) spanning the entire midbrain and striatum were collected free-floating in 0.1M TBS (Leica CM3050S cryostat) and stored at 4^{o}C.

3.2.5| Immunohistochemistry and fluorescent staining

Immunohistochemistry using both fluorophore- and biotin-conjugated secondary antibodies was performed to investigate different aspects of cellular change within the study. For assessment of dopaminergic cell loss in the substantia nigra, every 4th midbrain section was used for stereology (as in Chapters 2.2.5 and 2.2.6), and every 6th striatal section spanning caudal to rostral was stained using the same method for assessment of striatal terminal loss. Following dehydration, striatal

sections were scanned (Epson Perfection V800 Photoviewer) and optical density assessed using Fiji.

For immunofluorescent detection of changes in sub-cellular structures and processes a similar staining method was utilised. Sections were washed in 0.1M TBS, followed by blocking in 4% NGS for 1 hour and overnight incubation with primary antibody solutions [Table 9] in 2% NGS, 0.1% Triton X 0.1M TBS, typically at 4^{o}C. Sections were washed in 0.1M TBS and incubated for 1 hour with the appropriate Alexa Fluor® secondary antibody (1:1000 Alexa fluor 488/568/633/647). Following 10-minute DAPI [2µg/mL] incubation and subsequent final washes in 0.1M TBS, sections were mounted onto Superfrost™ Plus microscope slides and cover-slipped with Prolong® Gold Antifade Mountant. Immunofluorescent staining was imaged using an Olympus Fluoview FV10i confocal microscope (60X objective with 3x optical zoom) or Nikon Eclipse TE2000-U epifluorescence microscope (60X Objective). Co-staining of all sections with TH enabled identification of TH-positive neurons for specific analysis of changes within this vulnerable cell population.

Table 9: Primary antibodies utilised in immunohistochemical assessment of paraquat-induced changes in mouse brain sections

Target	Host	Supplier	Catalogue #	Dilution
Anti-Drp1-616	Rabbit polyclonal	Cell Signalling	D9A1	1:200
Anti-Drp1	Rabbit polyclonal	Santa Cruz	611113	1:200
Anti-HNE	Rabbit polyclonal	Alpha Diagnostic	HNE11-S	1:200
Anti-TOM20	Rabbit polyclonal	Santa Cruz	11415	1:1000
Anti- TH	Chicken polyclonal	Abcam	Ab76442	1:2000

3.2.6| Confocal imaging and image analysis

Stained sections were primarily imaged with an Olympus Fluoview FV10i automated laser-scanning confocal microscope. For each experiment, a minimum of ten fields of view were imaged for each specimen to provide a wide representative sample for analysis. All staining in tissue was accompanied by TH co-staining to enable accurate identification of the dopaminergic neurons in the region of interest which facilitated TH-positive neuron-specific analysis. All imaging and image analysis were performed blind using Fiji. All methods of analysis of *in vivo* staining start with definition of the TH-positive neuron ROIs and segregation of the image into individual channels.

For analysis of Drp1-616 staining, images were segregated into multiple channels and the ROI defined for TH-positive neurons within the image. The Drp1-616 channel was then converted to 8-bit greyscale, a convolve filter applied, a median 3x3 (“despeckle”) filter applied to reduce noise and threshold applied to remove background, providing a binary image for analysis. ‘Analyse particles’ was utilised to provide unbiased counts of the number and size of Drp1 puncta within each image. This process was performed for multiple TH-positive neurons in each image for a minimum of 10 images per animal per experiment. Care had to be taken to avoid underestimating puncta which were in close proximity to each other by ensuring they were clearly differentiated by the thresholding process without inadvertently excluding smaller independent puncta.

Analysis of 4-hydroxynonenal (HNE) adduct formation as an indication of reactive oxygen species generation and evaluation of mitochondrial content in cells utilised corrected total cell fluorescence (CTCF) to determine the signal intensity with consideration of background fluorescence. TH-positive neuron ROIs were defined for

each image. The area, integrated density and mean grey value for each neuron within the ROIs, along with three samples of background signal (background taken to be areas outside of the ROIs) was then measured. Equation 4 was then used to calculate the CTCF for each cell, which was averaged for each animal and then across groups.

Equation 4:

$$CTCF = Integrated\ density\ of\ ROI - (Area\ of\ ROI\ x\ Mean\ grey\ area\ of\ 3\ background\ measurements)$$

In this study, samples for the investigation of changes in mitochondrial morphology were imaged using a Nikon Eclipse TE2000-U epifluorescence microscope (60X Objective). *In vivo* mitochondrial morphology analysis utilised a previously published method from Chen et al (2015). The process for each image is described in brief as follows: The image was converted to grayscale, threshold applied to exclude background signal, a median 3x3 filter ("despeckle") applied to reduce noise and then inverted to provide a black on white image of the mitochondria. The skeletonize plug-in was then applied to provide a single-pixel-width representation of the mitochondrial network, which was analysed within each specific ROI to provide data regarding the morphology of the network. This final analysis step was repeated within each TH-positive ROI in each image, with a minimum of ten images (~30-50 TH-positive neurons) per animal. The skeletal visualisation of the mitochondrial networks was saved for future reference to verify accurate representation of the structures in comparison with the original images.

3.2.7| Stereology

For the assessment of dopaminergic terminal loss in the striatum, serial coronal sections (30µM) were collected throughout the striatum and stained for TH as described in Chapter 2.2.5. Fiji.

Stereology was performed using the same stereological set up and methods as detailed in Chapter 2.2.6 with the following specific modifications for mice: A sampling percentage of 20% of the ROI was counted, using a counting frame area of 2500µm (50µm x 50µm frame) for mice, as determined by pilot studies. A guard zone of 1µm was specified at the upper and lower limits of the dissector. The CE values were <0.1 for all animals.

3.2.8| Statistical analysis

All values are expressed as mean ± SEM or absolute total values. Differences between means were analysed using students T-test, one-way ANOVA, or two-way ANOVA. All analyses were performed in GraphPad Prism 5 (GraphPad Software Inc. California). The null hypothesis was rejected when *P*-value <0.05.

3.3| Results

3.3.1| Mdivi-1 protects against paraquat-induced nigrostriatal dopaminergic cell loss

As PQ-induced cell loss in Oct3$^{-/-}$ mice has been previously shown by our lab (Rappold *et al.*, 2011) and nigrostriatal dopaminergic degeneration is a characteristic of PD, I decided to test whether pharmacological inhibition of Drp1 with a small molecule, mdivi-1, would confer protection against PQ-induced neuropathology. In mice pre-treated and co-treated with mdivi-1 for the duration of PQ treatment, the small molecule effectively protected against dopaminergic cell loss in the SNpc and against striatal terminal loss [Figure 12; *P<0.05; ***P<0.001]. Whilst the neuroprotection was incomplete, the threshold effect of dopamine loss in PD suggests that partial protection or restoration of dopaminergic neurons may be sufficient to attenuate PD pathology and motor function.

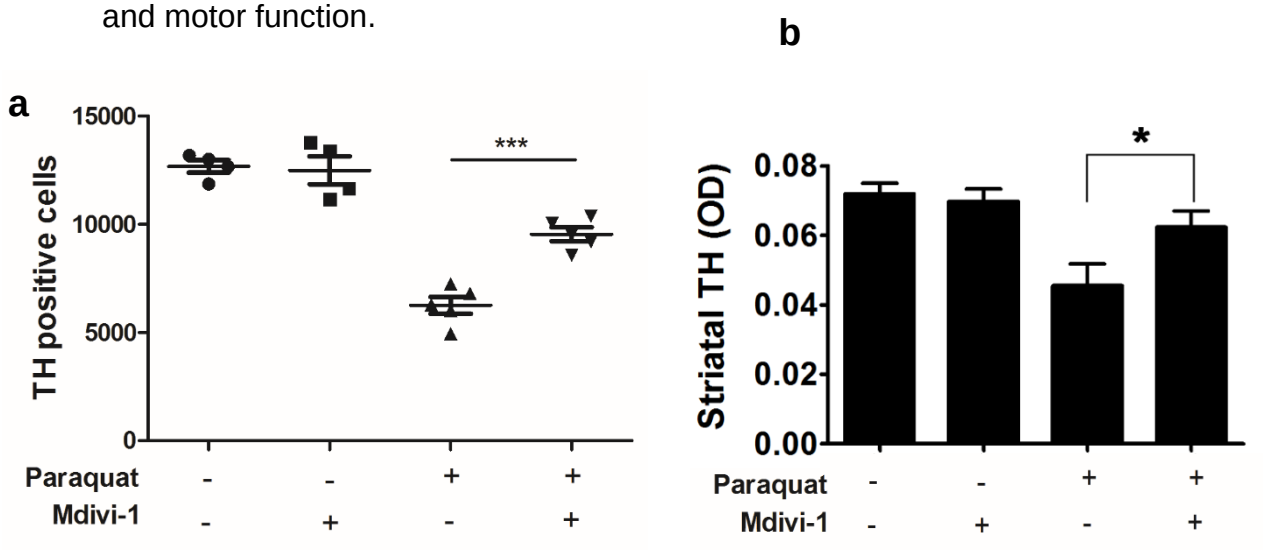


Figure 12: Mdivi-1 protects against paraquat-induced loss of TH-positive nigrostriatal neurons and terminals in Oct3$^{-/-}$ mice. Oct3$^{-/-}$ mice were pre-treated with mdivi-1 (20mg/kg, IP) or DMSO for three days. Mdivi-1 / DMSO treatment continued throughout the experiment. Paraquat (10mg/kg, IP) or saline was administered every 48 hours for 10 injections. Seven days after the last injection mice were sacrificed and assessed for nigrostriatal integrity by performing stereological cell counts of nigral dopaminergic neurons (**a**) and analysis of striatal dopaminergic terminal density (**b**). Data represents mean ± SEM, n=4-5 per group, analysed by one-way ANOVA followed by Newman-Keuls post-hoc test. *P<0.05, ***P<0.001. Stereological assessment of TH-positive neurons in the SNpc of PQ-treated *Oct3*$^{-/-}$ mice demonstrated ~45% loss in the SNpc and optical density analysis demonstrated ~35% loss of dopaminergic striatal terminals. Pre-treatment and concurrent treatment with small molecule Drp1 inhibitor mdivi-1 successfully attenuated this loss. Stereology kindly performed by Dr Mei Cui at Fudan University, China.

3.3.2| Paraquat-induces non-significant increase in phosphorylated Drp1-616 levels

Drp1 post-translational modifications can have differing impacts on the protein function depending on their nature. Drp1 phosphorylation at serine-616 by cyclin-dependent kinases (Cdk1, Cdk5), protein kinase C (PKCδ) or extracellular-signal regulated kinases (ERKs) regulates Drp1 activity and mitochondrial fission during mitosis (Cho *et al.*, 2014; Lee and Yoon, 2014; Tang *et al.*, 2016). Phosphorylation of Drp1 at serine-616 by PKCδ was of particular interest as this post-translational modification is known to induce translocation of Drp1 from the cytosol to mitochondria, where it interacts with other fission factors, such as MFF, Fis1, MiD49 and MiD51 and oligomerises to facilitate mitochondrial fission processes. The specific detection of serine-616 phosphorylated Drp1 provides evidence for its likely, if incomplete, translocation to mitochondria as an early indication of increased mitochondrial fission induction, which is visible in IHC images as distinct puncta. Whilst the variability between the groups prevents the data reaching statistical significance, due to large SEM values and small sample number (3 mice/group), there is a trend towards increased Drp1-616 puncta in TH-positive neurons which may be ablated in the PQ + mdivi-1 group [Figure 13]. Further work is necessary to confirm this effect.

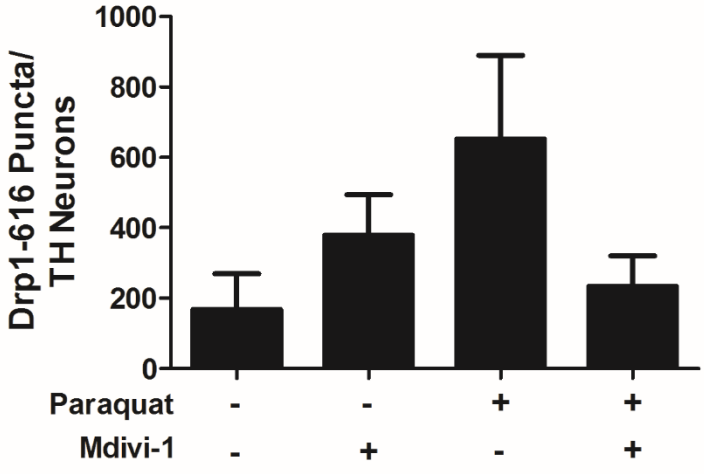
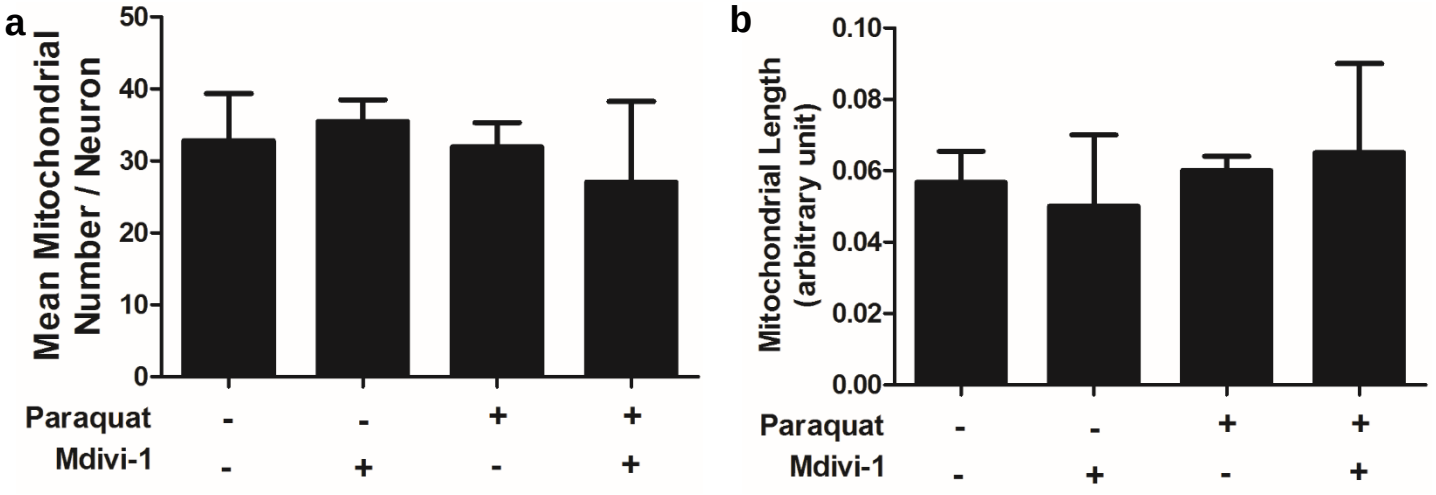


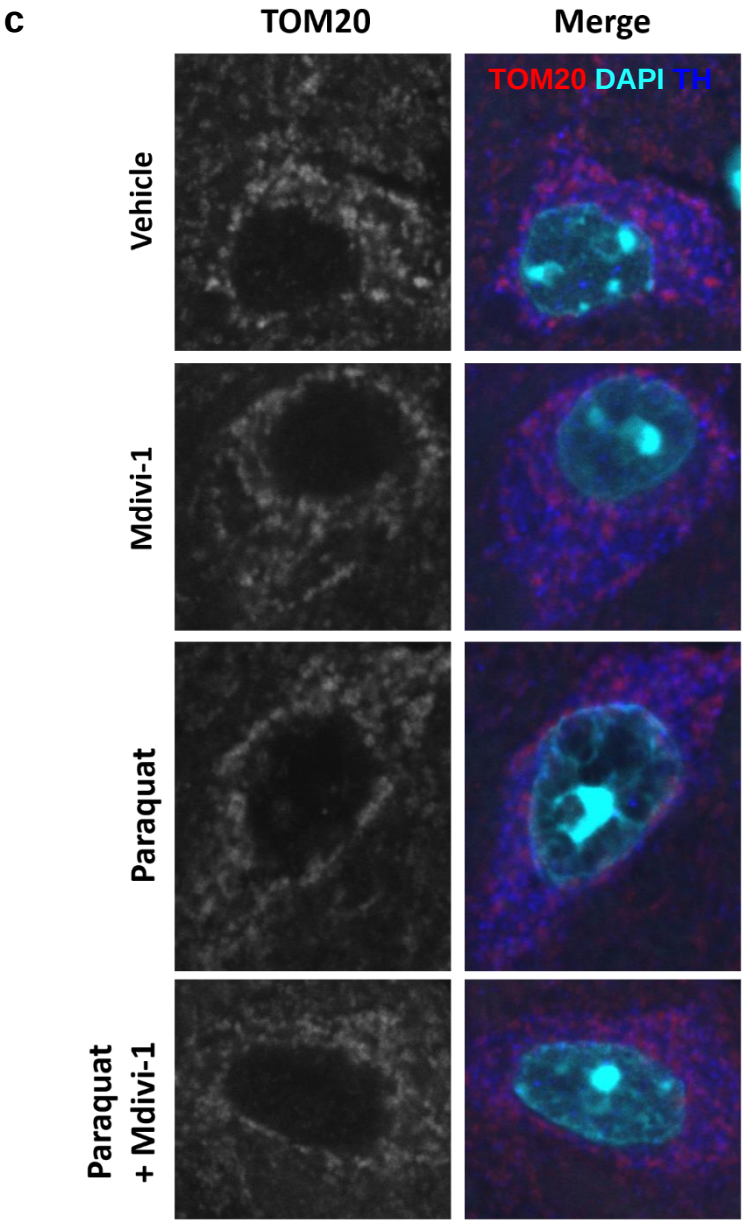
Figure 13: Paraquat- and mdivi-1-treated Oct3$^{-/-}$ mouse SNpc neurons did not display significantly increased Drp1-616 puncta. No significant changes in Drp1-616 puncta were detectable in TH-positive neurons when brain samples were collected one week after the cessation of paraquat treatment, although a trend in increasing puncta was apparent in the paraquat group. Data represents mean ± SEM, n=3-4 per group, analysed by one-way ANOVA followed by Tukey's post-hoc test.

3.3.3| Paraquat did not induce mitochondrial fragmentation in Oct3$^{-/-}$ mice

Mitochondrial fragmentation has been demonstrated in SH-SY5Y cells, primary rat cortical neurons and Mfn2 transgenic mice treated with PQ (Zhao *et al.*, 2017), so I investigated whether this morphological change was replicated in TH neurons of our *in vivo* model. Analysis of mitochondrial number and length demonstrated no significant change in mitochondrial morphology in the brain samples collected seven-days post-cessation of PQ treatment [Figure 14]. This may be due to the dynamic nature of mitochondrial morphology, which may not have sustained any PQ-induced changes beyond the termination of treatment. An increase in the length of mitochondria was not evident in the mdivi-1 treatment group, which is unexpected given that mdivi-1 inhibits Drp1 and would therefore be expected to reduce mitochondrial fission in treated cells. The reliance on immunofluorescent histochemistry for this analysis may also be a limiting factor in the detection of mitochondrial changes, as studies often utilise electron microscopy for analysis of mitochondrial morphology at higher resolution.

Figure 14: Mitochondrial number and length were unchanged in paraquat- and mdivi-1-treated Oct3$^{-/-}$ mouse SNpc neurons (Page 121). No significant changes in mitochondrial number (**a**) or length (**b**) were detectable in TH-positive neurons when brain samples were collected one week after the cessation of paraquat treatment. (**c**) Representative images from each treatment group; TOM20 staining in greyscale (red in merge). Blue TH immunoreactivity. Data represents mean ± SEM, n=3-4 per group, analysed by one-way ANOVA followed by Tukeys post-hoc test.


a
Mean Mitochondrial Number / Neuron
50
40
30
20
10
0
Paraquat - - + +
Mdivi-1 - + - +
b
Mitochondrial Length (arbitrary unit)
0.10
0.08
0.06
0.04
0.02
0.00
Paraquat - - + +
Mdivi-1 - + - +


c
TOM20
Merge
TOM20 DAPI TH
Vehicle
Mdivi-1
Paraquat
Paraquat + Mdivi-1

3.3.4| Characterisation of the Tm1b global heterozygous Drp1 knockout mouse model

Prior to the commencement of experiments which explored the potential protective effects of reduced Drp1 expression in the Drp1 heterozygous global knockout ($Drp1^{+/-}$) mice, I sought to confirm the reduction of Drp1 expression in the animals and to investigate whether Drp1 reduction resulted in any growth disparity, as the embryonically lethal homozygous Drp1 knockout mice displayed a significant reduction in embryonic size (Ishihara *et al.*, 2009; Wakabayashi *et al.*, 2009). Monitoring of mouse growth, as determined by changes in weight and nose-to-tail midline length, from PND 0 to PND 70 demonstrated no effect of the heterozygous Drp1 knockout [Figure 15]. Male mice of both genotypes displayed higher weight compared to females from ~PND 25, however there was no difference between the genotypes [Figure 15c].

Sampling of brain tissue confirmed the reduction of Drp1 expression at the mRNA and protein level, which was further confirmed by immunohistochemistry [Figure 16]. Verification of the suggested PCR parameters for genotyping to distinguish $Drp1^{+/-}$ mice from their WT litter mates was performed, and qPCR provided robust confirmation of reduced Drp1 expression in the brain regions of interest [Figure 16a]. The genotyping protocol was crucial for the determination of mouse genotype prior to allocation to experimental groups in the further work.

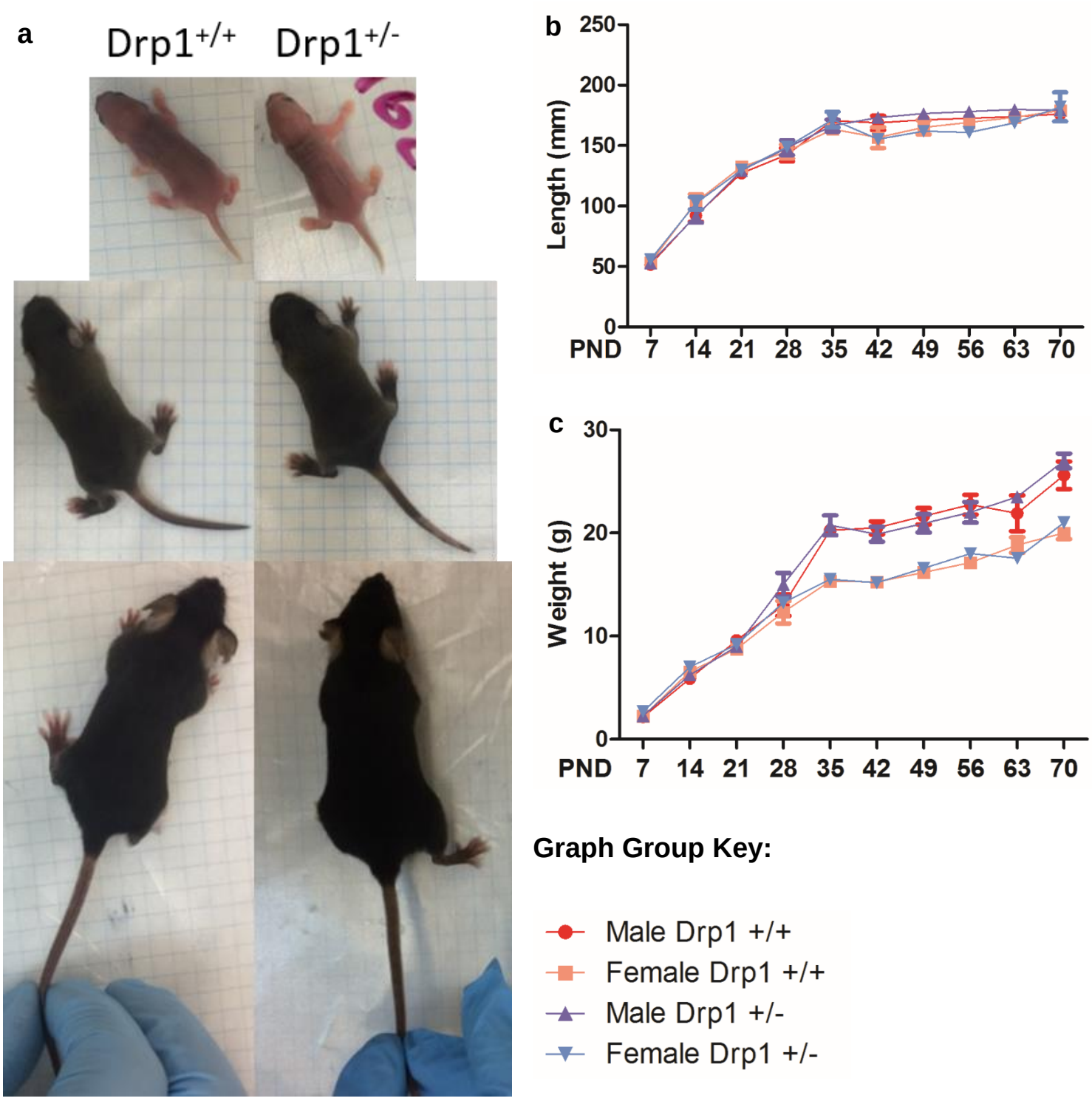


Figure 15: Investigation of growth disparity between Drp1$^{+/+}$ and Drp1$^{+/-}$ mice determined that reduced Drp1 expression does not cause growth disparity or developmental delay. (**a**) Representative images of mice of each genotype at different ages. Examination of the mouse growth rate via digital measurement of midline length (**b**) and weight monitoring (**c**) throughout the first 10 weeks of life confirmed that global heterozygous Drp1 knockout (Drp1$^{+/-}$) mice displayed no growth disparity compared to their Drp1 wild type (Drp1$^{+/+}$) littermates.

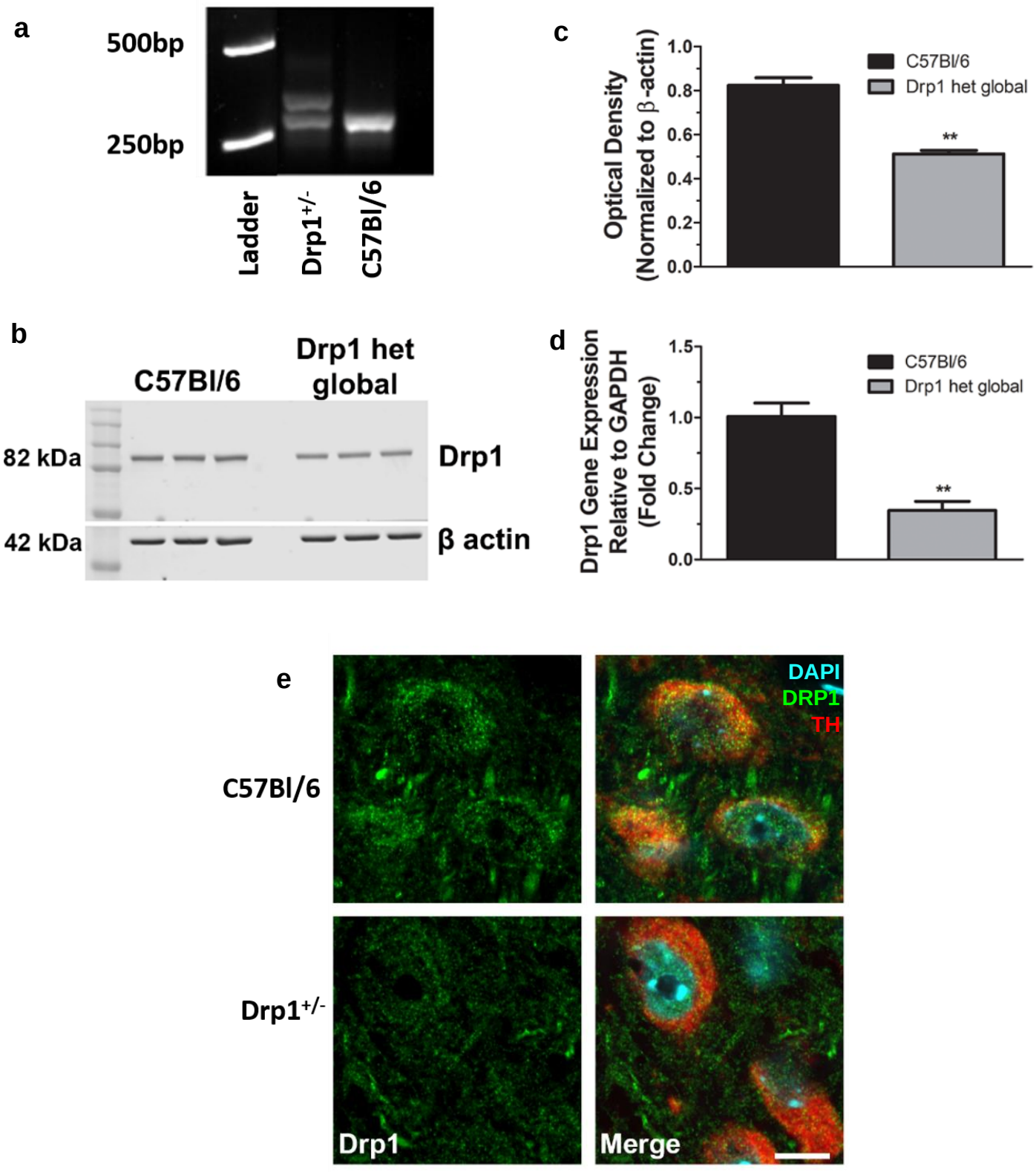


Figure 16: Characterisation of Drp1 expression in global heterozygous Drp1 knockout mice. (**a**) Representative genotyping result to distinguish between Drp1$^{+/+}$ and C57Bl/6 controls. (**b**) Immunoblotting displayed the reduction in Drp1 levels in the global heterozygous knockouts; (**c**) quantification of the optical density of each protein band confirmed this when normalised to β-Actin. (**d**) qPCR validation of the Drp1 reduction at the mRNA level. (**e**) Immunohistochemical confirmation of Drp1 (green) reduction in dopaminergic neurons (red, TH immunoreactivity) of the SNpc in heterozygous knockouts compared with C57Bl/6 controls. Data represents mean ± SEM, analysed by t-test, $^{**}P<0.01$. Immunoblot and qPCR data kindly provided by Dr Martin Helley.

3.3.5| Paraquat failed to induce significant neuropathology in $Drp1^{+/-}$ mice or $Drp1^{+/+}$ littermate controls

To evaluate the protective effects of genetic Drp1 inhibition *in vivo*, $Drp1^{+/-}$ mice and their WT littermates were injected with 10mg/kg PQ IP every 48 hours for 20 days. Combined stereology data from two independent experiments demonstrated that the PQ injection paradigm, with a week rest post-injection for lesion stabilisation, failed to produce a significant loss of TH-positive neurons in the SNpc of these mice [Figure 17]. Segregation of the data by gender or experiment had no effect on the results. It is unclear why the paradigm, which as displayed in Figure 12 and in previous publication by our lab (Rappold *et al.*, 2011) is capable of inducing ~45% reduction in TH-positive SNpc neurons, failed to demonstrate the anticipated cell loss. Following stereological assessment of the first experiment all practical parameters were verified to ensure that no variation in the chemical preparation, storage, administration, or sample collection could account for the unexpected results. Repetition of the experiment with a larger cohort that allowed gender-based segregation of the data still failed to establish PQ-induced TH-positive neuron loss in the SNpc [Figure 17].

Immunohistochemical investigation of intracellular mechanisms which contribute to pathology failed to demonstrate a significant increase in HNE [Figure 18] or Drp1-616 [Figure 19] between vehicle and PQ treated $Drp1^{+/+}$ groups. Drp1-616 is post-translationally modified Drp1 with phosphorylation of Serine 616 residue; this has been previously demonstrated to stimulate mitochondrial fission by promoting Drp1 translocation to mitochondria (Alaimo *et al.*, 2014; Taguchi *et al.*, 2007). The significant reduction in Drp1-616 levels between $Drp1^{+/+}$ and $Drp1^{+/-}$ mice is an expected result of the reduced Drp1 expression between the genotypes. Although the trend suggests PQ-induced increased Drp1-616 puncta, the variability between animals and the low

sample number failed to demonstrate a significant change. Assessment of the induction of oxidative stress by quantification of HNE signal demonstrated no significant changes between groups; although a trend is apparent between vehicle and PQ treatments, there is no significant increase in HNE signal following PQ treatment. If PQ failed to induce significant increase in ROS this could explain its failure to robustly induce Drp1-616 changes or loss of TH-positive neurons, and may be suggestive of impaired PQ uptake into the brain.

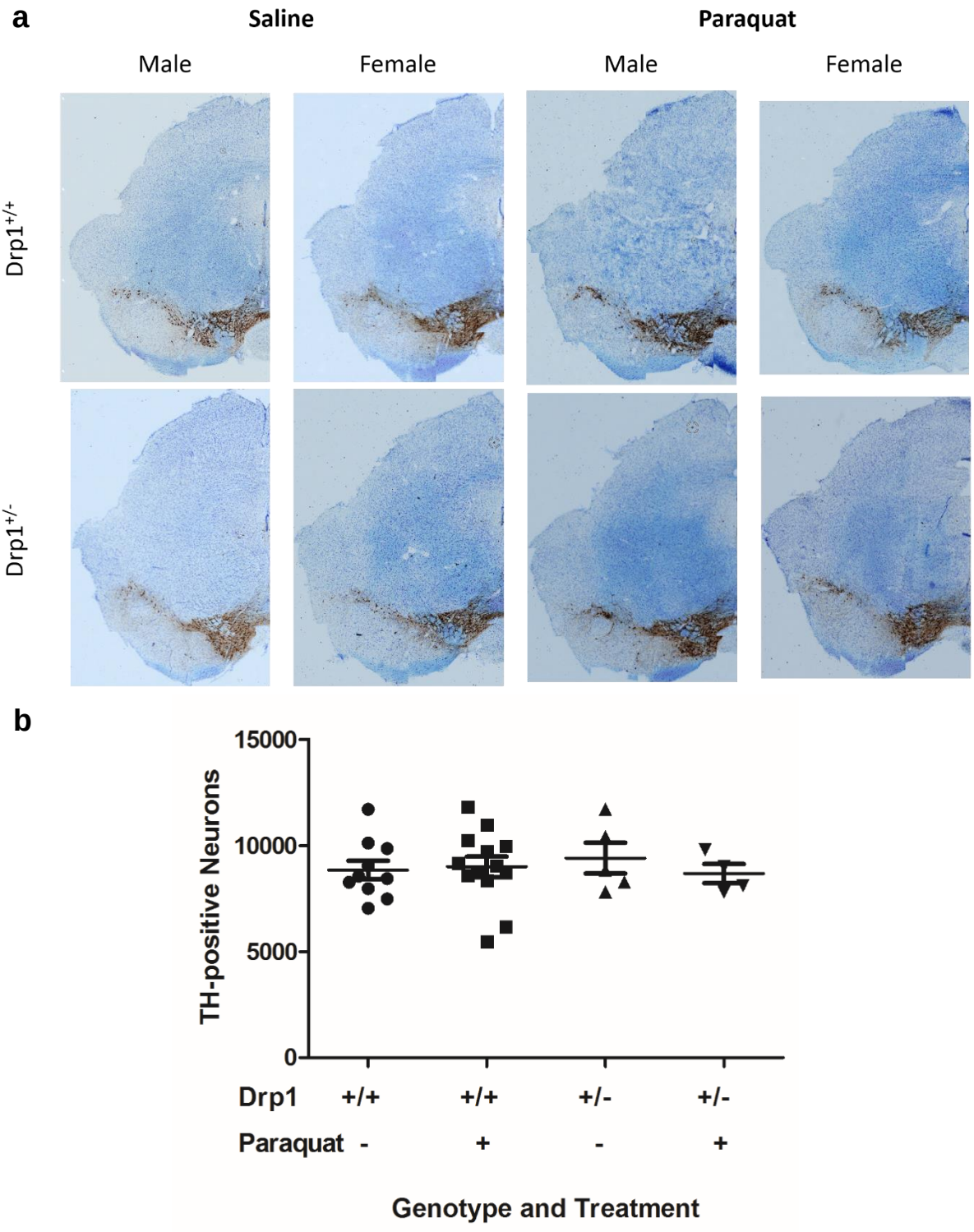


Figure 17: Paraquat failed to induce the anticipated loss of nigral dopaminergic neurons in Drp1$^{+/-}$ mice or the littermate control Drp1$^{+/+}$ mice. 10 doses of vehicle or paraquat (10mg/kg) over a period of 20 days, with sacrifice of animals 7 days following the final injection, failed to produce the anticipated loss of TH-positive neurons in the SNpc, despite previous success with this paradigm in the lab. (**a**) Representative images of midbrain sections counted for stereology. (**b**) Combined stereology data from two independent experiments, with grouping of males and females after confirmation that gender did not affect detection of PQ-induced neurotoxicity. Data represents mean ± SEM, n=3-10 mice per group per experiment, totalling n=5-13 per group when experimental data is combined, analysed by two-way ANOVA.

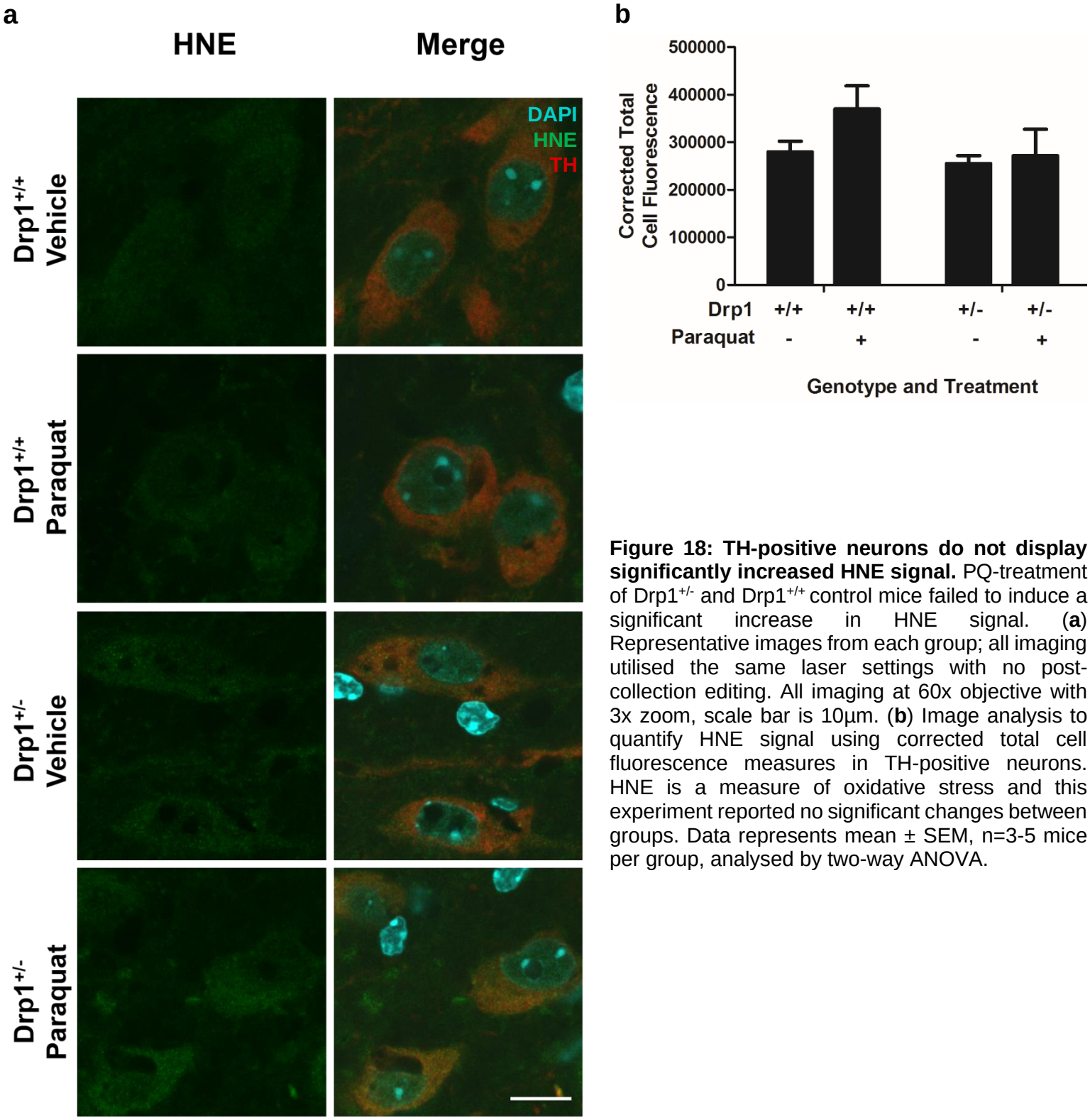
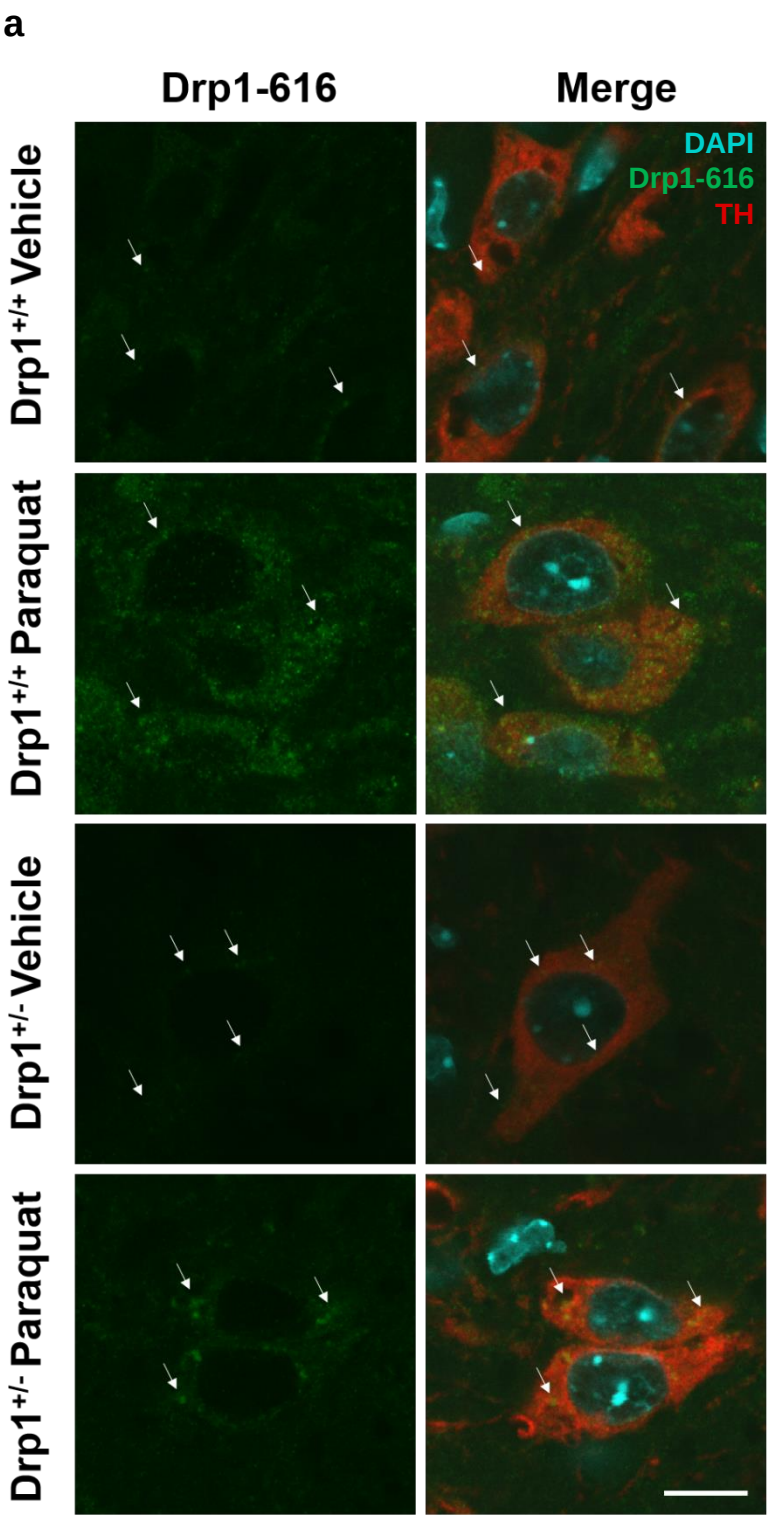


Figure 18: TH-positive neurons do not display significantly increased HNE signal. PQ-treatment of Drp1$^{+/-}$ and Drp1$^{+/+}$ control mice failed to induce a significant increase in HNE signal. (**a**) Representative images from each group; all imaging utilised the same laser settings with no post-collection editing. All imaging at 60x objective with 3x zoom, scale bar is 10µm. (**b**) Image analysis to quantify HNE signal using corrected total cell fluorescence measures in TH-positive neurons. HNE is a measure of oxidative stress and this experiment reported no significant changes between groups. Data represents mean ± SEM, n=3-5 mice per group, analysed by two-way ANOVA.



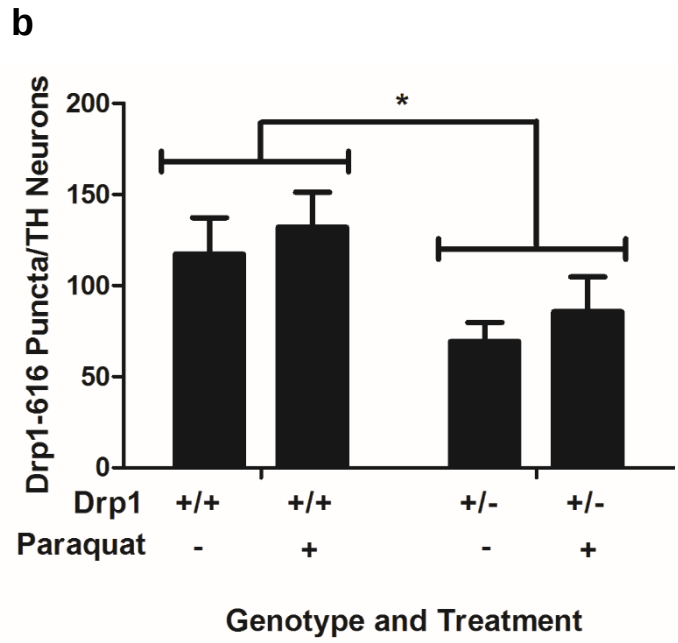


Figure 19: TH-positive neurons display genotype-dependent differences in Drp1-616 puncta. Drp1$^{+/-}$ mice display reduced background signal, likely due to reduced Drp1 expression. (**a**) Representative images from each group; all imaging utilised the same laser settings with no post-collection editing. All imaging at 60x objective with 3x zoom, scale bar is 10µm. Arrows indicate puncta. Paraquat-treated mice do not display significantly increased Drp1 levels; (**b**) Analysis of Drp1 specifically phosphorylated at Serine-616 demonstrated genotype- but not treatment-dependent significant changes. Data represents mean ± SEM, n=3-5 mice per group, analysed by two-way ANOVA. *P<0.05.

3.3.6| Evidence of gender disparity in the presentation of paraquat-induced systemic side effects

As mouse weights were monitored and recorded throughout treatment, to calculate the appropriate PQ dose and to track weight changes as an indicator of systemic PQ toxicity, it became apparent that male mice displayed weight loss whilst female mice demonstrated stable or increasing weight. The weight loss apparent in male mice was rapid, with up to 5% of start weight lost within 24 hours of treatment. During both independent PQ experiments, clear distinction in the reaction of mice to the chemical based on data was detectable from the weight tracking data [Figure 20]. Male mice displayed reduced movement, increased piloerection, and hunching posture, compared with female mice which displayed no behavioural changes as a result of PQ treatment. This data suggests that gender differences may affect the susceptibility of the animals to PQ.

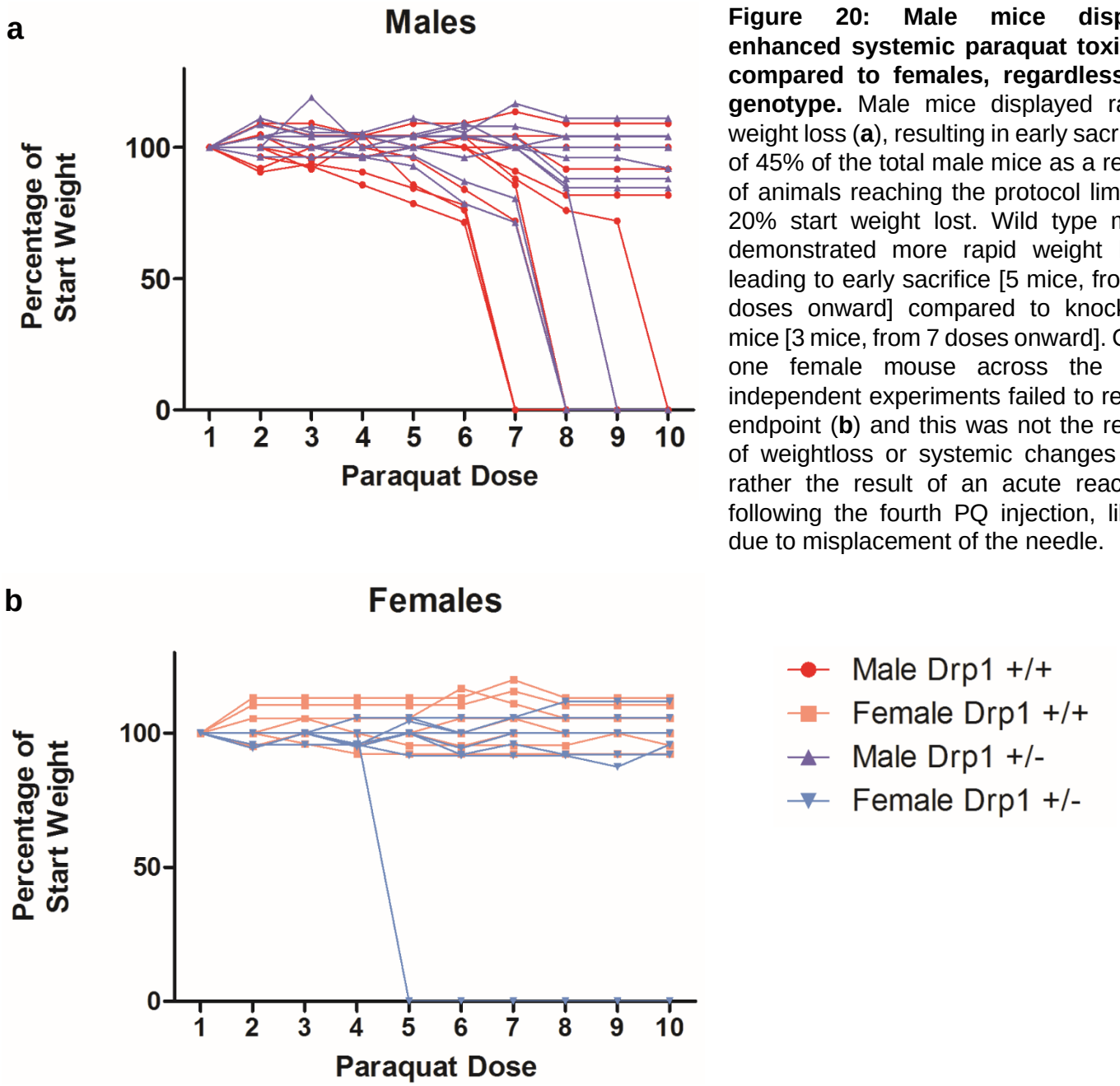


Figure 20: Male mice display enhanced systemic paraquat toxicity compared to females, regardless of genotype. Male mice displayed rapid weight loss (**a**), resulting in early sacrifice of 45% of the total male mice as a result of animals reaching the protocol limit of 20% start weight lost. Wild type mice demonstrated more rapid weight loss leading to early sacrifice [5 mice, from 6 doses onward] compared to knockout mice [3 mice, from 7 doses onward]. Only one female mouse across the two independent experiments failed to reach endpoint (**b**) and this was not the result of weightloss or systemic changes but rather the result of an acute reaction following the fourth PQ injection, likely due to misplacement of the needle.

3.4| Discussion

PQ was demonstrated to successfully induce neuropathological changes in dopaminergic neurons of Oct3$^{-/-}$ mice, supporting previous work by our lab using this treatment regimen (Rappold *et al.*, 2011). Further to this previous work, inhibition of Drp1 by the small pharmacological Drp1 inhibitor mdivi-1 demonstrated neuroprotective effects; the PQ-induced reduction of TH-positive neurons in the SNpc and striatal terminals was partially attenuated by mdivi-1 co-treatment. This is a significant finding as previous work has demonstrated the efficacy of mdivi-1 in other PD models (Bido *et al.*, 2017; Fan *et al.*, 2019; Rappold *et al.*, 2014), however no other papers had investigated the potential application of mdivi-1 in environmental toxin models of PD. The exact mechanism by which mdivi-1 treatment confers protection is not apparent from this data, as changes in Drp1-616 levels did not reach significance. Based on previous research, we expected mdivi-1 to reduce mitochondrial fission in both saline and PQ treated animals, as the literature demonstrates that mdivi-1 impacts the expression of proteins involved in mitochondrial dynamics, increasing mitochondrial fusion protein expression and reducing the expression of fission proteins (Manczak *et al.*, 2019). In this experiment, PQ treatment failed to induce mitochondrial fragmentation, which could lead to an increase in mitochondrial number if the cells waste clearance mechanisms become saturated. It is also possible, however, that the transient nature of mitochondrial dynamics led to rapid changes in morphology which had resolved to an undetectable level after administration of the toxin ceased. As our experimental paradigm specified a week interval between the cessation of treatment and the perfusion of animals, acute changes in mitochondrial morphology may have resolved. As Drp1-616 puncta formation was sustained through this post-treatment interlude, it may be that this post-translational modification contributes to other Drp1

functions which are not yet understood. Co-staining of a mitochondrial marker with the Drp1-616 immunohistochemistry to confirm that these puncta are located at the presumed organelle would further assist in understanding whether this is the case. Drp1 phosphorylation at Serine 616 is mediated by various kinases, dependent upon the stimuli; CDK1/Cyclin B has been implicated in phosphorylation in mitosis (Taguchi *et al.*, 2007), MAPK1 in tumour growth, cancer and a Huntington's disease model (Kashatus *et al.*, 2015; Roe and Qi, 2018) and PKCδ in oxidative stress conditions (Qi *et al.*, 2011). Evidence to date suggests that Drp1 phosphorylation at Serine-616 does not directly alter GTPase activity, so the increased Drp1-dependent mitochondrial fission induced by this phosphorylation is likely mediated by altered Drp1 interactions with other proteins involved in the fission process. As PKCδ is implicated in phosphorylating Drp1 at this residue in oxidative stress conditions, it is the most likely kinase to have been activated by PQ-induced ROS to produce the enhanced phosphorylation of Drp1 detected in this model.

Investigation of the bioaccumulation and persistence of PQ in the literature reports tissue-specific variation, with slower elimination from the brain than from other tissues, such as the liver (Moretto and Colosio, 2011); whilst a half-life of ~28 days was reported for PQ in the brain, and evidence suggests that it accumulates following repeated doses (Prasad *et al.*, 2007), previous work has shown that some PQ-induced changes are short-lived and transient. One paper demonstrated that PQ-induced increase in α-synuclein was only detectable in animals sacrificed two-days post-cessation of treatment, whereas the animals sacrificed with a seven-day interval from treatment cessation revealed the protein levels quickly returned to baseline. This supports the possibility that PQ-induced changes in mitochondrial morphology were not detectable subsequent to this sacrifice interval, and further investigation of the

impact of PQ *in vivo* should consider investigating both short-term and longer-term effects of this agrochemical toxin.

As mdivi-1 is a pharmacological agent, it is possible that it may confer non-specific or off-target effects, aside from its proposed interference with Drp1. A 2017 paper challenged the specificity and mechanism of mdivi-1 inhibition of Drp1 function (Bordt *et al.*, 2017), leading to caution surrounding its use to investigate Drp1 inhibition whilst research was undertaken to further explore and confirm its functional mechanism. For this reason, I determined that the protective effects of Drp1 inhibition should be confirmed in a genetic model of Drp1 depletion to avoid concern regarding off-target effects or misunderstood pharmacological interactions.

Characterisation of the $Drp^{+/-}$ mice, by Dr Martin Helley, robustly demonstrated a reduction in Drp1, which was consistent across mRNA and protein expression investigations, confirming reduction from the transcriptional level due to heterozygous knockout of the gene. Analysis of mouse growth measurements confirmed that global heterozygous Drp1 knockout did not induce any detrimental impact upon the growth rate or final size of the mice, when compared against their $Drp1^{+/+}$ littermates. Previous characterisation of $Drp1^{+/-}$ mice by Manczak *et al* (2012) confirmed that Drp1 reduction had no significant impact upon synaptic, dendritic and mitochondrial proteins compared with WT mice. Mitochondrial function and GTPase activity were also confirmed to be unaffected (Manczak *et al.*, 2012). As $Drp^{-/-}$ is not only embryonically lethal, but knockout mice also demonstrated a developmental delay from embryonic day 8, it seemed that Drp1 may be an important factor in growth (Wakabayashi *et al.*, 2009). My work confirms that reduction of Drp1 (~45%) resulting from heterozygous knockout is not sufficient to interfere with mouse development and growth, as measured by weight and growth tracking.

Table 10: Summary of various paraquat mouse models published from 2002- 2020

Administration Route	Dose Range	Mouse Breed	Animal Age Range	Animal Gender	Diluents	Treatment Frequency
Intraperitoneal Injection	0.3mg/kg* + 10mg/kg*	SWR/J	PND 5-19 + 12 wks	U/S	Saline	Once daily (PND) + twice weekly
	10mg/kg*	*CR3*$^{-/-}$ + C57BL/6J	3 mths	Male	Saline	Twice weekly
	10mg/kg*	DAT-KD + C57BL/6	PND 3-4, 5 wks, 18 mths	U/S	Saline	Single dose
	10mg/kg	hMfn2 OE Tg + WT	12 wks	Male	Saline	Twice weekly
	10mg/kg or 20mg/kg	C57BL/6	9-16 wks	Male	Saline	Once or twice weekly
	10mg/kg	C57BL/6J	8-9 wks	Male	Saline	Twice weekly
	5mg/kg	A30P Syn Tg	12 mths	U/S	Saline	Twice weekly
	1-35mg/kg	C57BL/6	9-10 wks	Male	Saline	1-3 doses, 1 wk apart
	10mg/kg	C57BL/6 + SWR/J	U/S	Male + Female	Saline	Twice weekly
	10mg/kg*	C57BL/6 x 129S	2-3 mths	Male	Saline	Twice weekly
	10mg/kg	*Oct3*$^{-/-}$ + C57BL/6J	10-12 wk	U/S	Saline	Every second day
	10mg/kg or 15mg/kg	CBA/2J or C57BL/6	U/S	U/S	Saline	Single dose
	50mg/kg or75mg/kg	*Prop-1*$^{+/-}$, *Prop-1*$^{-/-}$ + C57BL/6	4-6 mths or 14-20 mths	Male + Female	Saline	Daily
	10mg/kg	C57BL/6	8-12 wks	Male	Saline	Twice or thrice weekly
	0.3mg/kg + 5mg/kg	C57BL/6J	Prenatal+ 10-12 wks	Male + Female	Saline	Prenatal – once + daily (adult)
	10mg/kg	C57BL/6	8 wks	Male	Saline	Once weekly
Dietary	10ppm or 50ppm	C57BL/6J	10 wks	Male + Female	Rodent LabDiet® 5002	Daily in diet
	50µg/ml	M83 Tg + C57BL/6	8 wks	Male + Female	Drinking water	Daily in water

ENS, enteric nervous system; * indicates co-treatment with 30mg/kg maneb; U/S, unspecified

Table 10 continued: Summary of various paraquat mouse models published from 2002- 2020

Treatment Duration	Sacrifice Interval	Weight loss / Mortality	Nigral Degeneration	Striatal TH / DA Depletion	Synuclein Pathology	References
2 wks (PND) + 6 wks	U/S	U/S	Yes	/	/	(Colle *et al.*, 2020)
6 wks	U/S	U/S	Yes	/	/	(Hou *et al.*, 2017; 2018)
N/A	1 wk	U/S	Yes	/	No	(Richter *et al.*, 2017)
4 wks	1 wk	U/S	Yes (~20%)	Yes (~30%)	/	(Zhao *et al.*, 2017)
3 wks	1-7 days	Weight loss	No	No	/	(Smeyne *et al.*, 2016)
6 wks	2 wks	U/S	No	Yes	/	(Allen *et al.*, 2014)
6 wks	U/S	U/S	No	No	Yes	(Nuber *et al.*, 2014)
1-3 wks	1 wk	Yes, weight loss and mortality	No	No	/	(Breckenridge *et al.*, 2013)
3 wks	1 wk	U/S	Breed-specific	/	/	(Jiao *et al.*, 2012)
6 wks	U/S	Co-treatment	/	/	Yes	(Wills *et al.*, 2012)
20 days	1 wk	U/S	Yes	*Oct3*$^{-/-}$ specific	/	(Rappold *et al.*, 2011)
N/A	U/S	U/S	Breed-specific	/	/	(Yin *et al.*, 2011)
1-6 days	Death as endpoint	Breed-specific mortality	N/A	/	/	(Bokov *et al.*, 2009)
8-12 wks	1 wk	Yes, weight loss and mortality	Yes	/	/	(Prasad *et al.*, 2009)
8 days	1wk	U/S	Gender-specific	Gender-specific	/	(Barlow *et al.*, 2003)
3 wks	2 or 7 days	U/S	/	/	Yes, 2-day only	(Manning-Bog *et al.*, 2002)
13 wks	0 days	No	No	No	/	(Minnema *et al.*, 2014)
6-8 wks	0 days	No	/	/	Yes, ENS	(Naudet *et al.*, 2017)

; /, un-investigated in the model.

Although previous studies utilising C57Bl/6 mice without the Oct3 knockout have successfully demonstrated loss of TH-positive neurons in the mouse SNpc [Table 10] after PQ treatment, I failed to recapitulate this key phenotypic indicator of PQ-induced PD-related neuropathology in two independent experiments. The initial investigation into whether global heterozygous *Drp1* knockout may confer protection against PQ-induced neuropathology utilised a total cohort of 24 mice, three per treatment (vehicle or PQ) per gender (male or female) per genotype (*Drp1*$^{+/+}$ or *Drp1*$^{+/-}$); we followed our previously established PQ injection regime (Rappold *et al.*, 2011) of ten doses at 10mg/kg/dose over 20 days, with a seven day interval following the final injection before collection of tissue samples for analysis. Blinded stereological quantification of the SNpc demonstrated no significant reduction in either TH-positive neurons or Nissl stained neurons, indicative that PQ failed to induce the anticipated reduction in dopaminergic neurons.

Following meticulous verification of all procedures, including modification of the injection procedure to include the production of a fresh 10mg/ml PQ stock for each injection dose rather than making and freezing (-20^{o}C) a single stock and defrosting aliquots for each injection, the experiment was repeated. This second study used a larger cohort [34 mice total] with a minimum of 6 animals per PQ-group per gender and genotype, to account for the weight loss and mortality evidenced in the male PQ groups from the first study. This increase in group size in the experimental design allowed for the segregation of data based on gender, in case combining the data was masking gender-specific effects of PQ in these mice. The same injection paradigm was followed, including the 7-day interval between cessation of treatment and collection of tissue samples. Blinded stereological assessment of these samples again failed to demonstrate a significant difference in the number of TH-positive neurons in

the SNpc of these mice. Extensive review of the literature to explore other experimental regimes for PQ-induced PD neuropathology in rodent models failed to elucidate a clear explanation for these results, although variation in the results of other PQ studies, which used a variety of rodent strains and injection regimes, suggests that some mouse strains and ages may be more susceptible to neuropathological changes following PQ exposure [Table 10].

Although the gross neuropathology of TH-positive neuron loss was absent in these mice, I was curious to investigate whether other pathological changes may be present at the intracellular level. Immunohistochemical investigation of HNE production, a marker of oxidative stress, demonstrated no significant PQ-induced change in $Drp1^{+/+}$ mice. Examination of Drp1-616 puncta formation in these samples further confirmed the genotype-specific reduction of Drp1 signal ($p=0.0156$), supporting the pre-experimental characterisation of the $Drp^{+/-}$ mice. Although there is a trend towards PQ-induced increase in Drp1-616 puncta, in a similar manner to that seen in the earlier PQ-mdivi-1 $Oct3^{-/-}$ experiment, this increase was not statistically significant. As with the earlier work it seems that the Drp1-616 data could confer greater value to understanding the impact of PQ treatment in TH-positive neurons if I had co-stained with a mitochondrial marker to enable investigation of co-localisation to confirm whether these puncta form as a result of enhanced Drp1 translocation to mitochondria.

As there was an absence of significant change in the oxidative stress in these experiments, which is well known to be induced by PQ treatment *in vivo* (Breckenridge *et al.*, 2013; Colle *et al.*, 2020; Hou *et al.*, 2017; Hou *et al.*, 2018; Ossowska *et al.*, 2006; Prasad *et al.*, 2009; Zhao *et al.*, 2017), the data suggest that insufficient PQ entered the brain, which may explain the lack of neuronal death observed in these

animals. It remains unclear as to why inadequate PQ entered the brain to induce neuropathology, as previous PQ studies with less frequent dosing protocols have reported nigral dopaminergic neuron loss (Colle *et al.*, 2020; Hou *et al.*, 2017; Hou *et al.*, 2018; Prasad *et al.*, 2009; Richter *et al.*, 2017; Zhao *et al.*, 2017). Whilst many other injection regimes also included this one-week post-injection interval prior to sample collection, few have investigated mitochondrial morphology, so it is difficult to determine whether we are alone in failing to demonstrate sustained morphological changes or not. The lack of neuropathological changes could relate to PQ entry to the brain, which is mediated by the neutral amino acid transporter (Shimizu *et al.*, 2001); further work to investigate PQ uptake into the brain of this mouse strain, at different doses and time points, could improve our understanding of PQ transmission into the brain which will contribute to its role as a PD risk factor.

The age of the mice utilised in the *Drp1*$^{+/-}$ study was in line with other mouse studies which have utilised less aggressive PQ-treatment regimens and still successfully demonstrated degeneration of the nigrostriatal system [Table 10]. Some studies however, have demonstrated mouse breed-specific and gender-specific variation in the susceptibility of mice to PQ toxicity, so it is possible that the genetic background of these mice may have conferred some resistance, as the *Drp1*$^{+/+}$ mice did not demonstrate loss of TH-positive neurons. Overall, whilst there are similarities in the dosing paradigms and working concentrations of PQ used to model PD *in vivo*, there is a lot of variation in the resultant pathology. Further work to better characterise the impact of mouse strain, gender and age in neurotoxic models of PD could further knowledge of how genetic variation, gender and age impact the risk conferred by different environmental factors; this could better inform regulations around working

with such chemicals, including the introduction of considerations for additional PPE and safety procedures for those in higher risk groups.

Despite the inability of PQ to replicate the neuronal loss anticipated in this study, monitoring of the mice throughout the treatment period of the two independent experiments clearly demonstrated a distinction in weight loss and systemic effects between the genders. The rapid weight loss and enhanced systemic toxicity, which resulted in early sacrifice of males in the PQ group of both WT and $Drp1^{+/-}$ heterozygous knockout mice, suggests that males are more susceptible to PQ toxicity. Disease monitoring in humans demonstrates that men are at greater risk of developing PD than women, with a 1.5 – 2 fold risk increase for men (Cerri, Mus and Blandini, 2019). The sex disparity in systemic reactions to PQ in this study confers relevance to the sex disparity seen in disease presentation as it suggests that males may be more sensitive to some environmental risk factors, which may then confer enhanced risk which culminates in the increased disease incidence in males. Research suggests that biological sex impacts disease risk factors and potentially influences molecular mechanisms involved in PD pathogenesis. Clinical data demonstrates that men and women display distinct motor and non-motor symptoms as their PD progresses; Motor symptoms often appear later in women, who also display elevated risk of L-DOPA-related motor complications (Haaxma *et al.*, 2007). Distinct clinical presentation and variable contribution of different risk factors support the theory that PD development may involve specific pathogenic mechanisms, or the same mechanism to different degrees, in men and women (Cerri, Mus and Blandini, 2019). Oestrogens have been highlighted as potentially significant; they may confer disease protection as demonstrated by the similar PD incidence in men and post-menopausal women, potentially linked to the rapid progression as women age and become menopausal,

losing this protective effect. As oestrogens have anti-inflammatory and anti-oxidant effects, the defensive effects may be linked to suppression of neuroinflammation, which has been identified as a pathogenic mechanism in multiple models of PD (Bourque, Dluzen and Di Paolo, 2009). Oestrogen has been reported to confer protection to gonadectomised rodents in MPTP and 6-OHDA mouse models, further supporting the proposed protective role of this hormone in models of PD (Dluzen and Horstink, 2003; Miller *et al.*, 1998). Tamoxifen, a selective oestrogen receptor modulator, abolished oestrogens protective effects upon MPTP-induced nigrostriatal neurotoxicity (Dluzen, McDermott and Anderson, 2001) and there are increased PD diagnosis rates in female breast cancer patients receiving adjunct hormone therapy (Latourelle *et al.*, 2010).

Specific investigation of the impact of PQ on female mice by Litteljohn *et al* (2011) is one of few studies to date which specifically focused on the effects of this neurotoxin on female mice; there are few publications concerning sexual dimorphism in response to environmental PD risk factors and the majority of studies have been undertaken solely in male mice [Table 10]. This study in C57BL/6 females revealed that they were largely resistant to the PQ-induced nigrostriatal changes and the locomotor changes reported in males. Similarly, in contrast to PQ-induced reduction in BDNF reported in male mice, female mice displayed a PQ-related increase in the growth factor. Results suggest that female mice may be less susceptible to nigrostriatal dopaminergic degeneration and motor effects induced by some environmental toxins, consistent with reports of greater PD incidence in males. Previous studies have demonstrated that female rodents are less susceptible to MPTP and 6-ODHA, two laboratory-based PD toxin models (Dluzen and Horstink, 2003; Dluzen, McDermott and Anderson, 2001; Gillies *et al.*, 2014; Miller *et al.*, 1998).

Mangano *et al* (2012) revealed that female C57BL/6 mice were resistant to many of the nigrostriatal dopaminergic changes and accompanying motor impairment normally provoked by PQ in males, despite utilising an identical treatment regime (10mg/kg IP, 3x per week for 3 weeks). A reduction in DOPAC levels suggests a small effect on the dopaminergic system in these female mice, however no loss of nigrostriatal dopaminergic neurons or terminals was detected. Gillies *et al* (2014) found that 6-OHDA administration to the medial forebrain bundle caused significantly greater depletion of striatal dopaminergic levels and loss of SNpc dopaminergic neurons in male rats compared with females, with the disparity consistent over a five week period post-treatment; this was considered as confirmation of a true sex difference rather than merely a difference in the rate of neurodegeneration. Overall, these studies suggest that females may have a higher threshold than males for PQ-induced neuropathology, suggestive that a higher cumulative dose or an altered dosing regimen could be used to elicit more profound changes in future studies. Endogenous hormone levels in females could contribute to their enhanced resilience to dopamine deficiency. As our understanding of the sexual dimorphism in human responsiveness to PD risk factors improves, it may suggest a need for personalised, gender-considerate PD therapies, as well as additional consideration of the effect of gender when designing *in vivo* PD studies. The significant degree of neuroprotection against the early stages of clinical or experimental PD, as seen in human and rodent females compared with males, necessitates a better understanding of sexual dimorphisms in the intact and injured nigrostriatal dopaminergic system to elucidate mechanisms which have the potential to delay or even halt the progression of PD. Sex-specific hormone-based therapeutic agents hold promise for developing treatments if further research confirms how oestrogens may confer protection.

As a result of the difficulties confirming the protective effects of Drp1 inhibition against PQ-induced neuropathology *in vivo*, and due to the limited time available for completion of my lab work, I made the decision to move to *in vitro* models. Work by my colleagues has demonstrated the protective influence Drp1 inhibition in both a genetic and a heavy metal based cell model of PD, so I was interested to see whether we could replicate similar effects with PQ and to investigate the potential cellular mechanisms which may be involved.

Chapter 4| Paraquat-induced autophagy blockade is attenuated by Drp1 inhibition *in vitro*

4.1| Introduction

Due to difficulties reproducing PQ-induced neuropathology in the *Drp1*$^{+/-}$ mouse model using our previously established PQ treatment regime, and limited time remaining to complete lab work which prevented the further investigation of this discrepancy in the model, I decided to move investigation of PQs mechanism of cell death and the potential impact of Drp1 modulation to *in vitro* models. Whilst a variety of previous investigations of PQs effects *in vitro* have been published, none have researched how influencing Drp1 may alter the pathology and many utilise concentrations of PQ which detrimentally impact cell viability, with some doses inducing loss of up to 50% of cells within the treatment period (de Oliveira *et al.*, 2017; Delic *et al.*, 2017; Hirayama *et al.*, 2018; Zhao *et al.*, 2017).

Previous research into the impact of PQ *in vitro* has predominantly focused on the impact of toxic doses in SH-SY5Y neuroblastoma cells (Hirayama *et al.*, 2018; Hou *et al.*, 2017; Huang *et al.*, 2019; de Oliveira *et al.*, 2017; Li *et al.*, 2017; Yang and Tiffany-Castiglioni, 2005; Zhao *et al.*, 2017), with the investigation of numerous cellular processes including apoptosis, ferroptosis, mitochondrial fragmentation and dysfunction, cellular uptake mechanisms, α-synuclein aggregation and mitochondrial fission/fusion proteins. To date there has been no investigation of the impact of modulating Drp1 in PQ models, although Zhao *et al* (2017) demonstrated that PQ treatment induced increased Drp1 levels for four hours post-treatment, indicative that the impact of PQ in cells likely includes variation of Drp1s function.

Earlier studies have explored how α-synuclein is affected by PQ treatment, with examination of potential protective effects conferred by expression conflicting with evidence from models which suggest that α-synuclein increases cellular sensitivity to this toxic insult. Musgrove *et al* (2011) suggest that α-synuclein may be upregulated

in response to chronic low level oxidative stress as a protective mechanism against apoptosis, which conflicts with more recent work suggesting that α-synuclein increases cellular sensitivity to PQ (Anandhan *et al.*, 2017; Kumar *et al.*, 2016; Kumar, Ganini and Mason, 2016; Musgrove *et al.*, 2019; Naudet *et al.*, 2017). Study of the impact of PQ in α-synuclein over-expressing M17 cells demonstrated that over expression of the protein increased cellular sensitivity to PQ (Delic *et al.*, 2017); as with many other *in vitro* PQ studies, the dose used was high [500μM]. Induction of cytochrome C release to the cytosol by PQ, alone or in conjunction with maneb, has also been implicated in the formation of α-synuclein protein radicals, which are believed to contribute to aggregation formation (de Oliveira *et al.*, 2017; Kumar, Ganini and Mason, 2016) .

Whilst there is some previous investigation of the influence of PQ on autophagic processes, earlier studies were limited by their focus solely on changes in LC3 levels, which we now understand to be an inaccurate indicator of autophagy flux when considered independently of other changes (Gottlieb *et al.*, 2015; Mizushima, Yoshimori and Levine, 2010). Targeting autophagy has previously been suggested as a potential strategy for the treatment of neurodegenerative diseases (Menzies, Ravikumar and Rubinsztein, 2006; Menzies, Fleming and Rubensztein, 2015), with impaired autophagic-lysosomal pathways implicated in the development of PD (Pan *et al.*, 2008). The increase in α-synuclein aggregates induced by PQ treatment suggests that autophagy may be involved in the toxic mechanisms of PQ pathology, as do the changes in some other protein levels. Hirayama *et al* (2018) demonstrated that high dose treatment of SH-SY5Y cells [600μm for 24 hours], which led to 30% loss of neuronal viability, resulted in increased LC3-II levels without a reduction of P62; this is indicative of blocked autophagy flux as LC3-II is a membrane-bound autophagy

protein which may accumulate if autolysosomal maturation is interrupted, preventing degradation of the substrate. The concurrent maintenance of P62 levels suggests that there is no enhancement of P62 degradation; the same accumulation of this protein may not be detected as it can be degraded by other non-lysosomal pathways.

The aim of this final portion of work in my project was to investigate the impact of PQ *in vitro*, with specific consideration given to whether it impacted autophagy flux and whether targeting Drp1 may attenuate such changes. This work involved treatment with PQ alone and also in conjunction with α-synuclein expression, as the interplay of genetic and environmental risk factors in PD is important to further our understanding of how toxic insults interact to induce disease pathology, and to properly assess how effective purported therapeutics may be.

4.2| Materials and Methods

4.2.1 | Cell culture

Two cell types were utilised to study the effects of PQ on mitochondria and autophagy *in vitro*, individually and in conjunction with α-synuclein. The use of two cell models enabled investigation of PQs effects in a model designed for assessment of autophagy (mRFP-GFP-LC3 HeLa cells, tfLC3), which was followed by confirmatory work to determine whether this effect was conserved in a more specific neuronal model (N27 cells). Potential protective methods were investigated in both. Cell populations were maintained in T25 flasks, cultured in 6-well plates for western blotting samples, 96-well plates for cell viability assessment and on uncoated (tfLC3) or poly-D-lysine-coated (N27) glass coverslips (Fisher, CB-40210) in 24-well plates for immunocytochemistry.

mRFP-GFP-Hela cells

HeLa cells stably expressing tandem fluorescent mRFP-GFP-LC3 (tfLC3, kindly provided by Dr Shouqing Luo, ptfLC3 plasmid, Addgene #21074) were developed by Kimura et al (2007) to specifically study autophagy flux. tfLC3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco; 12-430-062) supplemented with 10% Foetal Bovine Serum (FBS; Gibco; 10-082-147), 100 U/mL penicillin/ 100µg/mL streptomycin (Fisher; SV3001) and 100µg/mL G418 (Gibco; 10-131-035) at 37ºC in 5% CO_2.

N27 cells

Rat dopaminergic neuronal 1RB3AN27 (N27) cells (original cells provided by Dr Anumantha Kanthasamy) with inducible expression of WT α-synuclein were generated by our lab, using The Complete Control® Inducible Mammalian Expression

System (Stratagene, La Jolla, CA) as previously described (Cui *et al.*, 2010; Fan *et al.*, 2019) [Figure 21]. N27 cells were cultured in Roswell Park Memorial Institute 1640 Medium (RMPI; Gibco; 11-875-119) supplemented with 10% FBS, 500µg/mL G418 and 200µg/mL Hygromycin B (Gibco; 10-687-010) at 37°C in 5% CO_2. WT α-synuclein expression was induced by the addition of 20µM Ponasterone A (PonA; ENZO; ALX-370-014-M005) to the media for 24 hours; for experiments to investigate the combined impact of PQ and α-synuclein, protein expression was induced by PonA 24 hours prior to treatment with PQ.

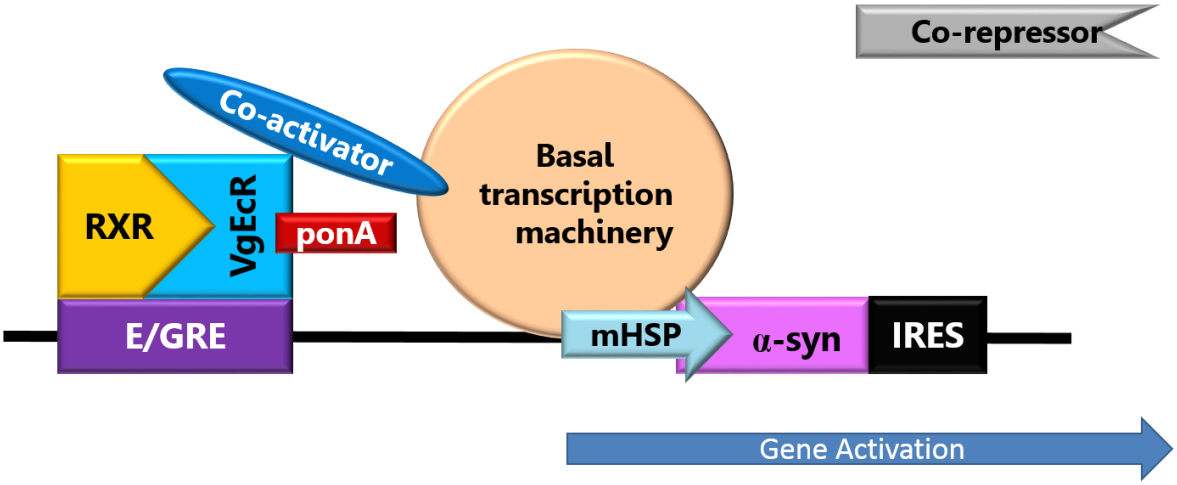


Figure 21: Inducible overexpression of α-synuclein in rat dopaminergic N27 cells. Stable cells containing a vector that constitutively expressed the ecdysone receptor (VgEcR) and the retinoid X receptor (RXR) were created. The cells were then stably transfected with a second vector that contained the ecdysone-responsive element (E/GRE) and a multiple cloning site for the insertion of human wild type α-synuclein. The VgEcR-retinoid X receptor heterodimer binds to the E/GRE. In the presence of Ponasterone A, an ecdysone analogue inducer, the co-repressors are released from VgEcR, and co-activators are recruited, resulting in gene activation via the minimal heat shock promotor (mHSP). IRES = internal ribosomal entry site.

4.2.2 | Transfections

To study the effects of PQ in conjunction with WT α-synuclein in tfLC3 cells, and to study the effect of modulating Drp1 expression with siRNA or $Drp1^{K38A}$ dominant

negative plasmids in tfLC3 and N27 cells, they were transfected 24 hours prior to treatment with PonA or PQ.

All transfections were performed in plate according to the Lipofectamine™ 3000 (Invitrogen; L3000008) protocol on plated cells at ~75% confluency. N27 cells were transfected with DNA plasmid LC3-Cherry (0.3µg/well, mCherry-hLC3B-pcDNA3.1 plasmid) to enhance LC3 for immunofluorescent detection. tfLC3 cells and N27 cells were transfected with either $Drp1^{K38A}$ dominant-negative (0.3µg/well, Drp1-K38A-pcDNA3 plasmid, from Dr Craig Blackstone) for dominant-negative modulation of Drp1 function, or 10nM siRNA Drp1 (siGenome smartpool DNM1L Dharmacon, against both rat and human Drp1) or a scramble siRNA (siGenome smartpool siRNA scramble) control for siRNA Drp1 inhibition experiments.

In brief, the Lipofectamine™ 3000 reagent was diluted with Opti-MEM™ Medium (Gibco, 31-985-070). A master mix of DNA and/or siRNA was prepared by diluting the nucleic acid products in Opti-MEM™ Medium. In experiments which only included DNA plasmid transfection, P3000™ reagent was added to the master mix. The DNA master mixes were added to the diluted Lipofectamine™ 3000-Opti-MEM™ solutions [1:1 ratio] and incubated for 10-15 minutes at room temperature. Cells were prepared for transfection by removal of normal culture media and gentle washing with sterile 0.1M PBS immediately prior to addition of the transfection reagents. After 8-10 hours in the presence of the transfection reagents, the Opti-MEM™ was removed and replaced with normal culture media. Cells were treated with compounds at the indicated concentrations 24 hours post-transfection and incubated for a further 24 hours before fixation or protein preparation.

4.2.3 | Chemical preparation

Paraquat (*N, N*-dimethyl-4,4'-bipyridinium dichloride; Sigma; 856177) was dissolved in 0.1M sterile PBS to create a 50mM stock, which was then diluted to a working dilution of 10µM in the appropriate growth media, depending upon the cell type. Chloroquine (Sigma; C6628) was dissolved in PBS to a 1mM stock concentration, then diluted 50x to a 20µM stock in cell culture media; this was then diluted to the working concentration (10µM) in culture media for cell treatment. PonA was dissolved in 100% ethanol to a stock solution of 2mM/mL; for use the stock was diluted 100x in cell culture media to 20µM, with a corresponding dilution of 100% ethanol in media as a negative control for the vehicle groups.

4.2.4 | Calcein cell viability assay

Cells were plated at variable densities depending on the cell line and treatment period. N27 cells were plated at $1x10^4$ cells/well (for 24-hour treatment) and $8x10^3$ cells/well (for 48-hour treatment). tfLC3 cells were plated at $6x10^4$ cells/well (for 24-hour treatment) and $4x10^3$ cells/well (for 48-hour treatment). N27 cell viability was assessed in the presence and absence of PonA concurrent with PQ treatment. The Calcein-AM assay (Fisher; C3099) was utilised to assess cell viability following treatment with different concentrations of paraquat. Briefly, cells were gently washed three times with 0.1M PBS prior to 1-hour incubation in 100µL Calcein [2µM in 0.1M PBS] at 37^{o}C in 5% CO_2, protected from light. Six replicate wells were included per condition with four wells of assay media included as blanks. The fluorescent Calcein-AM signal was recorded using a 495nm excitation and 520nm emission filter with a Synergy H1 plate reader (Biotek). For analysis, the mean blank value was subtracted

from all wells and then normalised to control values. Only live cells hydrolyse Calcein AM to produce fluorescent calcein signal.

4.2.5 | Immunocytochemistry

For immunocytochemistry, tfLC3 cells were grown on uncoated circular glass coverslips (9mm) whilst N27 cells were grown on poly-D-lysine-coated circular glass coverslips (9mm). Both cell lines were cultured in 24 well plates, in which the immunocytochemistry protocol was performed and from which the coverslips were collected for mounting on to microscope slides. In brief, cells were seeded and allowed to adhere to coverslips overnight, following which they were transfected, as described in Chapter 4.2.2, and subsequently treated with PQ and/or PonA. At the end of the treatment period (24 hours), cells were fixed in warm 4% paraformaldehyde (Fisher; AA433689M) in media for 20 minutes at 37^{o}C. The fixation solution was then removed, and cells were washed three times with 0.1M PBS. Coverslips were blocked in 5% NGS for 1 hour at room temperature, followed by three PBS washes. Coverslips were incubated in primary antibody solution [Table 11] overnight at 4^{o}C; primary antibody solutions were diluted in 0.1M PBS with 2% NGS and 0.1% Triton-X-100. Following removal of primary antibody solution and subsequent washing with 0.1M PBS, cells were incubated with secondary antibody solution [Table 12] in 0.1M PBS with 2% NGS at room temperature for 1 hour. After the removal of secondary antibody solution and three final washes with 0.1M PBS, coverslips were mounted onto Superfrost™ Plus Microscope Slides using Prolong Gold Antifade Reagent. No DAPI staining was included in these experiments as all four channels were utilised to assess proteins; for investigation of proteinase-K-resistant α-synuclein Prolong Gold Antifade Reagent

with DAPI was applied to mount coverslips as fewer channels were utilised for antibody detection.

Table 11: Primary antibodies utilised in immunocytochemistry

Target	Host	Supplier	Catalogue #	Dilution
Anti-α-Synuclein	Mouse monoclonal	BD Biosciences	610787	1:500
	Rabbit polyclonal		AB5038	
Anti-DLP1	Mouse monoclonal	BD Biosciences	611113	1:500
Anti-LC3	Rabbit polyclonal	Novus	NB100-2220	1:200
Anti-P62	Rabbit polyclonal	Novus	NDP1-48320	1:200
Anti-TOM20	Rabbit polyclonal	Santa Cruz	Sc-11415	1:1000

Table 12: Secondary antibodies utilised in immunocytochemistry

Antibody	Supplier	Catalogue #	Dilution
AlexaFluor 350 goat anti-mouse	Invitrogen	A21049	1:1000
AlexaFluor 350 goat anti-rabbit	Invitrogen	A21068	1:1000
AlexaFluor 488 goat anti-mouse	Invitrogen	A11029	1:1000
AlexaFluor 488 goat anti-rabbit	Invitrogen	A11034	1:1000
AlexaFluor 568 goat anti-mouse	Invitrogen	A11004	1:1000
AlexaFluor 568 goat anti-rabbit	Invitrogen	A11011	1:1000
AlexaFluor 633 goat anti-mouse	Invitrogen	A21052	1:1000
AlexaFluor 633 goat anti-rabbit	Invitrogen	A21070	1:1000

4.2.6 | Fluorescent imaging and image analysis

Cell culture specimens were primarily imaged with an Olympus Fluoview FV10i automated laser-scanning confocal microscope. For each experiment, a minimum of ten fields of view were imaged for each coverslip to provide a wide representative sample for analysis. Immunocytochemistry for mitochondrial morphology was imaged using a Nikon Eclipse TE2000-U epifluorescence microscope (60X Objective). All image analysis was performed using Fiji.

In vitro mitochondrial morphology analysis was performed using a previously validated method from our lab. Following conversion to grayscale images, a convolve filter was applied (normalise Kernel), followed by a 3x3 median filter to minimise noise (“despeckle”). The threshold was then applied, and the mitochondria analysed.

Assessment of autophagy flux was performed in a similar manner to image analysis for Drp1-616 [Chapter 3.2.6], utilising the same protocol for quantification of intracellular puncta. In tfLC3 cells, Equation 5 was used to calculate the number of autolysosomes, whilst the number of autophagosomes corresponds to the number of GFP puncta. In N27 cells transfected with LC3 Cherry and stained for P62, the raw counts of LC3 and P62 puncta per cell were treated as indicators of the levels of these autophagy proteins.

Equation 5: $\boldsymbol{Autolysosomes = RFP\ puncta - GFP\ puncta}$

4.2.7 | Proteinase K assay

To determine whether the PonA-induced α-synuclein expression in N27 cells induced aggregate formation representative of the protein aggregates detected in PD patient brains, inducible N27 cells were transfected with rat Drp1 siRNA and plated onto poly-D-lysine coated glass coverslips. Cells were treated with 20μM PonA, to induce α-synuclein expression, or 100% ethanol vehicle, with or without the addition of 50μm PQ for 24 hours. Chloroquine (CQ) [10μM] was included as a positive control for autophagy blockade, which leads to protein aggregation. Cells were processed for immunocytochemistry as described above [Chapter 4.2.5] however prior to blocking, coverslips were either treated with 0.34 U/mL proteinase K (Sigma, P4850) or vehicle (0.1M PBS) for 15-20 minutes at room temperature, whilst protected from light. Cells were subsequently stained for α-synuclein (AB5038); this antibody was chosen due to its previous success in detecting higher order protein structures. Following imaging, the number of insoluble aggregates was quantified using Fiji. A minimum of 30 cells per condition across triplicate experiments were quantified for statistical analysis.

4.2.8 | Western blotting

To complement the immunocytochemistry data, western blot analysis was used to confirm the siRNA knockdown of Drp1 expression. Cells were cultured in 6 well plates and 24 hours after siRNA transfection, trypsin was utilised to dissociate the cells from the plate. A cell pellet was collected for each well and stored at -20^{o}C until protein preparation. Cell pellets were resuspended in 50µl RIPA buffer (150mM NaCl, 1% IGEPAL, 0.5% Sodium deoxycholate, 0.1% Triton-X, 50mM Tris and 1:100 Thermo Scienftic™ Halt Protease and Phosphatase Inhibitor Cocktail (PI78443)) and then transferred to ice cold dounce homogenisers. After 10-12 strokes to homogenise the sample, the solution was transferred to 1.5ml eppendorfs and centrifuged at 13,000 rpm for 15 minutes at 4^{o}C. The supernatant was then collected, protein concentration assessed using the BCA assay [method detailed in Chapter 2.2.8] and loading volumes were prepared by boiling with 2x Laemmli buffer for 10 minutes.

The samples were run using a 4% stacking gel and a 12% resolving gel, with ten wells per gel. The protein ladder (PageRuler™ Plus Prestained Protein Ladder, Fisher, 26619) was run in the first well of each gel, followed by the samples. The gel was run at 95V for 30 minutes, then the voltage was increased, and the gel was run at 130V for 90 minutes. Samples were transferred to EMD Millipore™ Immobilon™ PVDF (PSQ) at 100V for 90 minutes. Following probing with the appropriate primary antibodies [Table 13) and incubation with IRDye® secondary antibodies, the blot was imaged using a LI-COR Odessey® CLx. Quantification of the protein bands was performed in the LI-COR Image Studio software (Ver 5.2), with normalisation to β-actin in Microsoft Excel.

Table 13: Primary antibodies utilised for immunoblotting confirmation of protein reduction following knockdown with Drp1 siRNA

Target	Host	Supplier	Catalogue #	Dilution
Anti-β-Actin	Mouse monoclonal	Sigma	A5441	1:20,000
Anti-DLP1	Mouse monoclonal	BD Biosciences	611113	1:1000

4.2.9 | Statistical analysis

All values are expressed as mean ± SEM. Differences between means were analysed using a one-way analysis of variance followed by post-hoc testing for pairwise comparison. All analyses were performed in GraphPad Prism 5 (GraphPad Software Inc. California). The null hypothesis was rejected when *P*-value was <0.05.

4.3| Results

4.3.1| tfLC3 HeLa cells and N27 cells display time- and dose-dependent sensitivity to paraquat treatment

Cell viability was assessed prior to PQ use for *in vitro* experiments to establish sub-lethal doses for use in the investigation of cellular changes and toxicity. Calcein AM assay assessment of cell viability following 24-hour or 48-hour treatment of tfLC3 HeLa cells and N27 cells, with and without induction of α-synuclein expression by PonA, demonstrated time- and dose-dependent reductions in cell viability. Calculation of LD_{50} values demonstrates that tfLC3 HeLa cells are the most sensitive to PQ treatment, followed by PonA-induced N27 cells and untreated N27 cells. The LD_{50} value for untreated N27 cells could not be calculated from these data for 24 hours treatment as the response to even the highest dose did not reach 50% loss of cells. PQ treatment was tested at 10µM, 25µM, 50µM, 100µM, 200µM, 400µM, 800µM and 1000µM; these data provided a clear range of sub-toxic PQ doses which were utilised for further experiments.

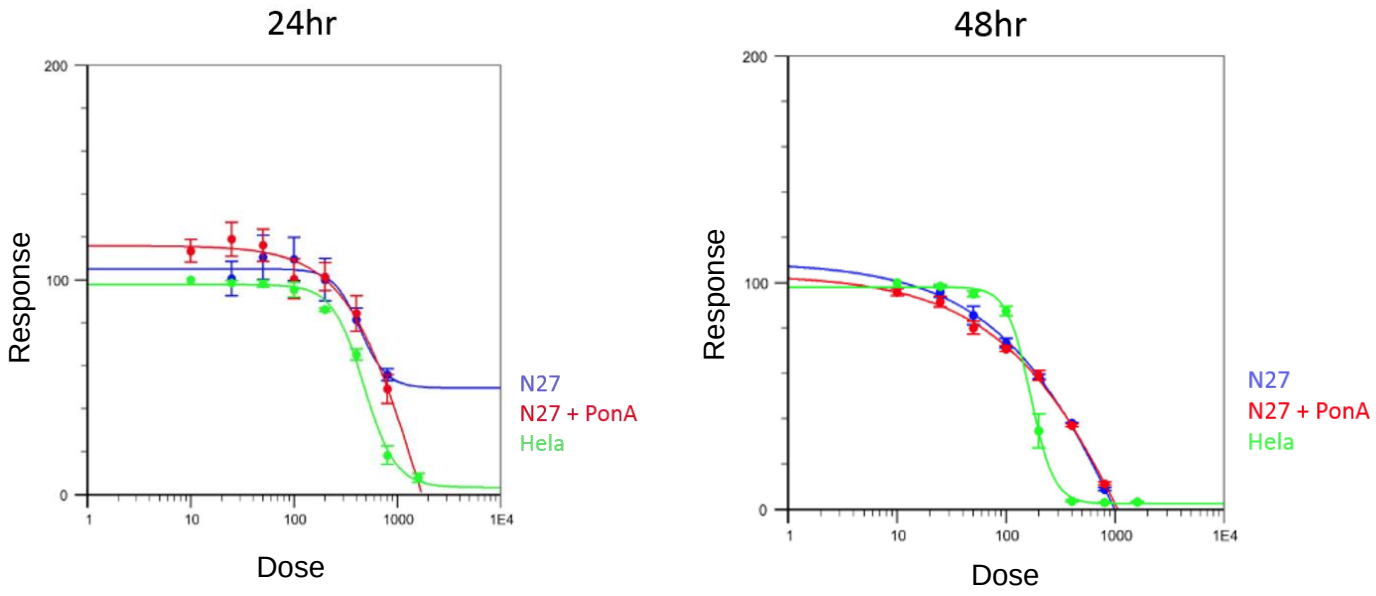


Figure 22: Dose-response and time-course studies of paraquat in N27 and tfLC3 HeLa cells. Clear differences in the toxicity of cell lines to PQ at 24 and 48 hours, through the dose range investigated. Calcein AM Assay performed by Carolina Sportelli, with raw data provided for calculation of LD_{50} values.

Table 14: LD_{50} of paraquat in N27 and tfCL3 HeLa cells. Results indicate that PonA induction of α-synuclein expression increased N27 cell sensitivity to paraquat treatment.

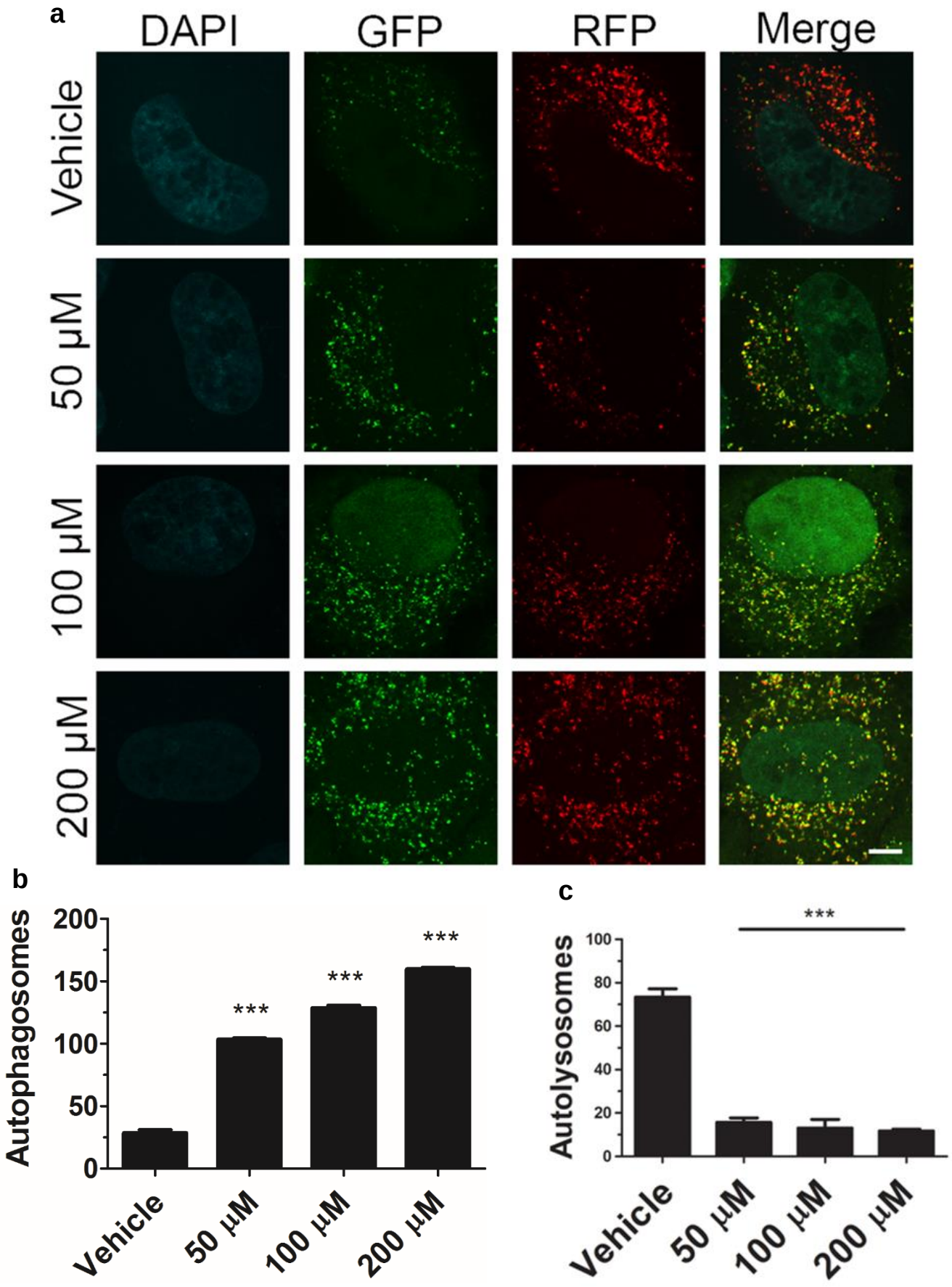
	LD_{50} (μM)	
Cell Lines	**24hr**	**48hr**
N27	>1000	268.16
N27 + PonA	798.16	261.03
Hela Cells	481.85	168.71

4.3.2| Paraquat induces dose-dependent blockade of autophagy flux in tfLC3 HeLa cells

TfLC3 HeLa cells were developed by Kimura et al (2007) for use in the investigation of changes in autophagy flux. TfLC3 HeLa cells utilise a tandem-fluorescent LC3 tagged protein, which enables easy determination of the impact of treatments upon autophagy flux; in the cytosol and autophagosomes, tfLC3 appear as yellow puncta due to the co-localisation of RFP and GFP signal. After fusion of the lysosome to autophagosome, to form the autolysosome, GFP signal is quenched by the acidic environment within the autolysosome so only RFP puncta present. Increased yellow signal is indicative of reduced progression of autophagy through to autolysosome formation. Treatment of tfLC3 HeLa cells on glass coverslips with different doses of PQ [50µM, 100µM, 200µM] for 24 hours facilitated assessment of PQ-induced dose-dependent differences in autophagy flux.

Quantification and analysis of the RFP and GFP puncta demonstrated dose-dependent increases in autophagy blockade [Figure 23]. The dose-dependent significant increase in autophagosomes [Figure 23b] is indicative of an enhanced rate of accumulation of undegraded cellular waste as a result of PQ treatment, suggestive that PQ can impact the rate of autophagosome formation. The stable reduction in autolysosome levels [Figure 23c] confirms that even the lowest PQ dose is sufficient to interrupt autophagosome fusion to the lysosome.

Figure 23: Paraquat induces dose-dependent autophagy blockade in tfLC3 HeLa cells (Page 160). (**a**) Enhanced yellow signal, which results from the co-localisation of RFP and GFP signal on LC3 due to the tandem fluorescent construct indicates blockade of autophagy flux, as GFP is usually quenched by the acidic environment inside autolysosomes. (**b**) Quantification of the signals to determine autophagosome (**b**) and autolysosome (**c**) numbers per cell confirms the PQ-induced autophagy blockade. Data represents mean ± SEM, n=3, analysed by one-way ANOVA followed by Tukeys post-hoc test, ***P<0.001. Scale bar 20µM. Data produced by Carolina Sportelli.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100 μM
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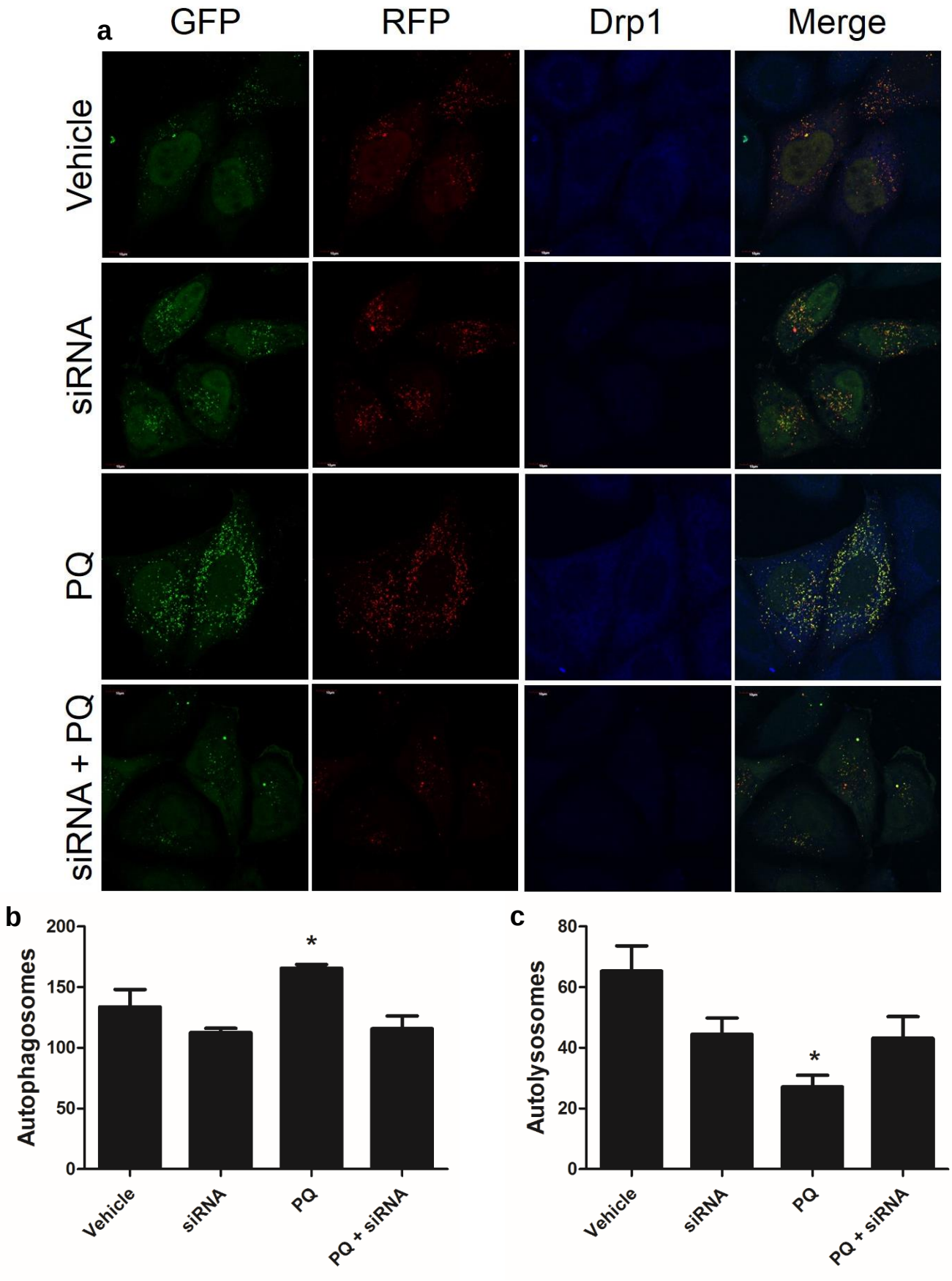
4.3.3| Inhibition of Drp1 function by siRNA knockdown protects against paraquat-induced autophagy blockade in tfLC3 HeLa cells

Subsequent to the discovery that treatment of tfLC3 cells with PQ [50µM] for 24 hours was sufficient to induce blockade of autophagy flux, I investigated whether modulation of Drp1 via siRNA inhibition would alter the impact of PQ treatment. TfLC3 HeLa cells were transfected with Drp1 siRNA 24 hours prior to treatment with 50µM PQ for 24 hours. Following fixation and immunocytochemistry, assessment of the impact of Drp1 knockdown on PQ-induced disruption of autophagy flux demonstrated the protective impact of this protein modulation. Knockdown of Drp1 with siRNA was demonstrated to be effective in tfLC3 cells by assessment of Drp1 staining intensity. Staining intensity also suggests that PQ treatment may increase Drp1 levels, which suggests that PQ may induce other Drp1-related cellular changes. Quantification of autophagosome and autolysosome signal confirmed PQ-treatment significantly inhibits autophagy flux, which was prevented by Drp1 knockdown prior to PQ treatment [Figure 24].

Figure 24: siRNA Drp1 reduces paraquat-induced autophagy blockade in tfLC3 HeLa cells (Page 162). Treatment of tfLC3 Hela cells with 50uM PQ for 24 hours induced autophagy blockade as demonstrated in Figure 23. Transfection with Drp1 siRNA 24 hours prior to PQ treatment attenuated the inhibition of autophagy flux. (**a**) Representative images from the treatment groups demonstrate the induction of autophagy blockade by PQ treatment, with the protective effects evident in the siRNA + PQ group. Quantification of autophagosome (**b**) and autolysosome (**c**) signal support the initial conclusions from imaging. Data represents mean ± SEM, n=3, analysed by one-way ANOVA with Newman-Keuls post-hoc test, *P<0.05. PQ group significantly different to Vehicle, Drp1 siRNA alone and PQ plus Drp1 siRNA groups (**b**, **c**). Scale bar 10µM.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4.3.4| Expression of α-synuclein induces autophagy blockade in tfLC3 HeLa cells

α-synuclein is a genetic risk factor in PD development and a hallmark of disease, with intracellular inclusions apparent in the brains of both familial and sporadic PD patients. As PQ is an environmental risk factor, which has been associated with increased α-synuclein in some experimental models of PD, investigating the combined effects of these two risk factors is important to better understand how gene-environment interactions may contribute to disease pathology. Prior to the co-treatment experiments, I established that Drp1 knockdown via siRNA treatment was sufficient to protect against α-synuclein-induced autophagy blockade. Transfection of tfLC3 HeLa cells with WT α-synuclein plasmid demonstrated significantly increased autophagosomes and significantly reduced autolysosomes 24-hours post-transfection, which was abrogated by co-transfection with Drp1 siRNA [Figure 25]. Cells were fixed and immunocytochemistry performed 24 hours post-transfection. Knockdown of Drp1 and expression of α-synuclein was confirmed by staining for the two proteins in all groups; the control groups for Figures 24, 25 and 26 are all the same as the data is from a large experiment which has been subdivided for ease of interpretation.

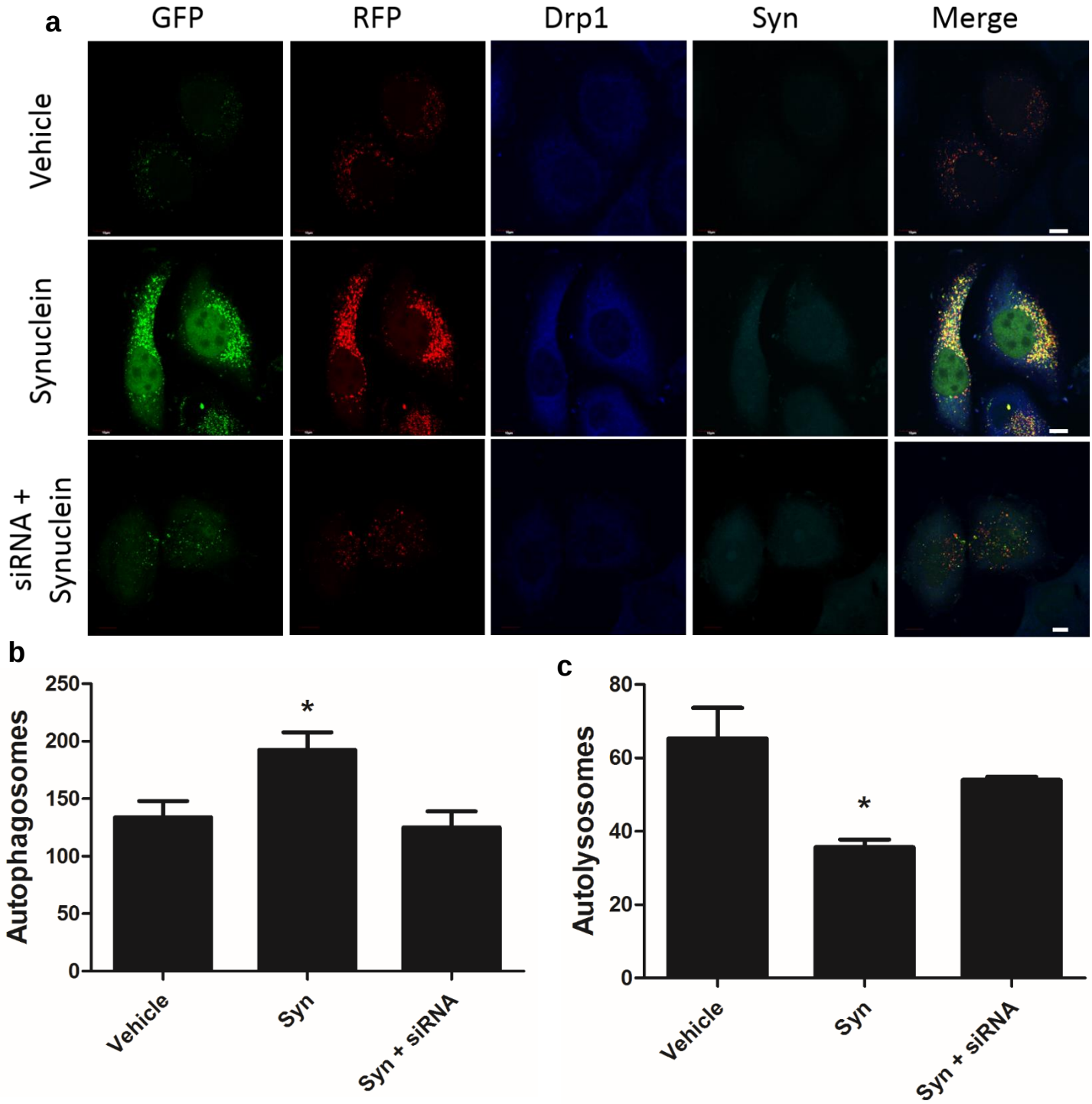
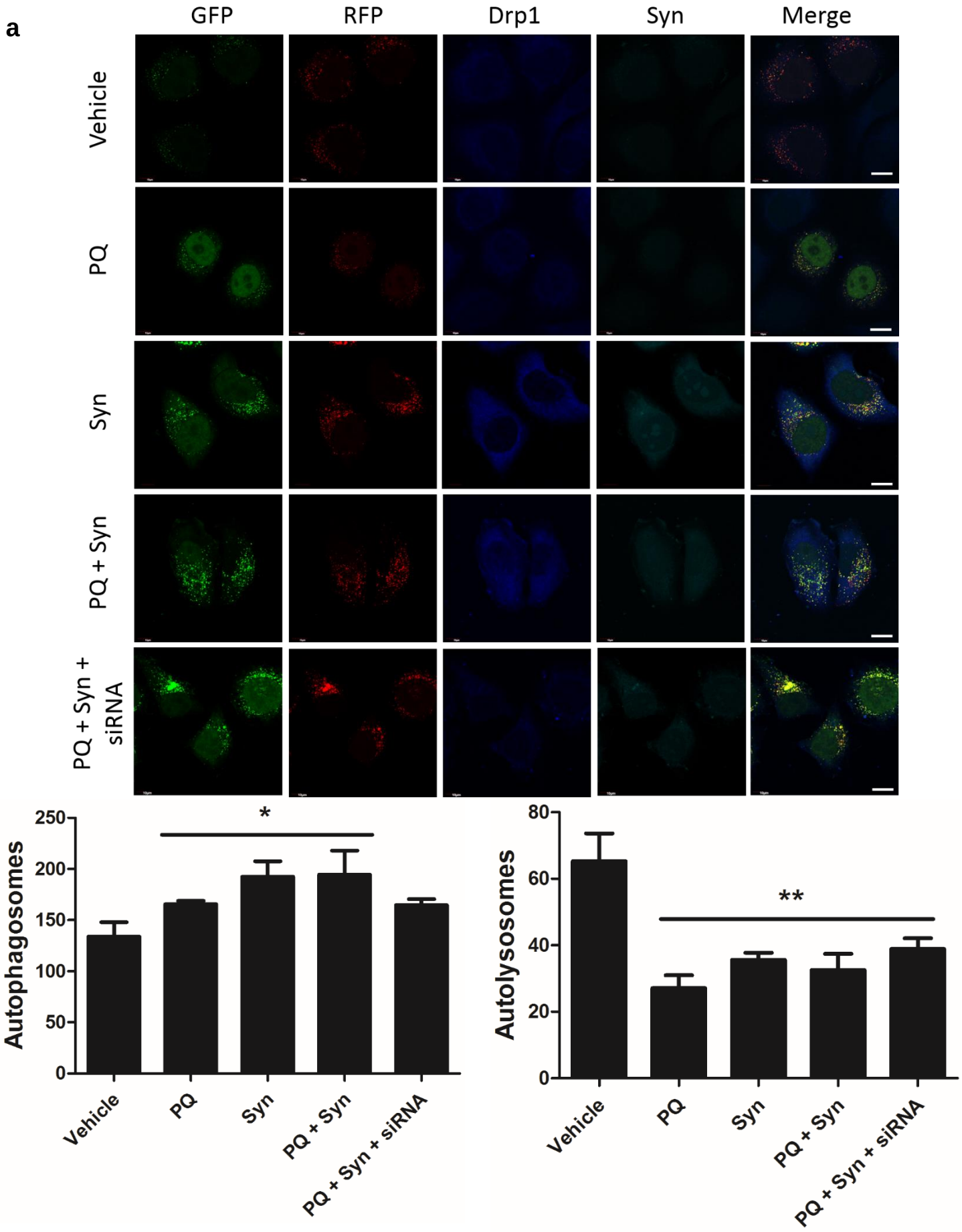


Figure 25: siRNA Drp1 reduces α-synuclein-induced autophagy blockade in tfLC3 HeLa cells. Co-transfection of tfLC3 HeLa cells with Drp1 siRNA and WT α-synuclein plasmid (pcDNA3) for 24 hours attenuated the autophagy blockade induced by WT α-synuclein alone. (**a**) Representative images from the treatment groups demonstrate the induction of autophagy blockade by WT α-synuclein transfection, with the increased α-synuclein expression and reduced Drp1 expression apparent in the relevant treatment groups. Quantification of autophagosome (**b**) and autolysosome (**c**) signal supports the initial conclusions from imaging. Data represents mean ± SEM, n=3, analysed by one-way ANOVA with Newman-Keuls post-hoc test, **P*<0.05. α-synuclein group significantly different to vehicle and α-synuclein + Drp1 siRNA (**b**, **c**). Scale bar 10μM.

4.3.5| Treatment of tfLC3 HeLa cells with paraquat in conjunction with α-synuclein expression induces autophagy blockade which could not be attenuated by Drp1 inhibition with siRNA knockdown

As previous experiments demonstrated efficacy of Drp1 inhibition with siRNA to attenuate PQ induced autophagy blockade, and α-synuclein induced autophagy blockade in tfLC3 HeLa cells, I investigated the combined impact of co-treatment with PQ and α-synuclein. Whilst there was not a significant increase in the degree of autophagy blockade induced by the dual treatment compared to each treatment independently, the trend in data suggests an increased accumulation of autophagosomes in some datasets. The number of autophagosomes in the dual treatment group with siRNA treatment was not significantly reduced from the co-treatment group, indicative that siRNA knockdown of Drp1 failed to significantly protect against PQ and α-synuclein induced autophagy blockade [Figure 26b]. The autolysosomes were significantly reduced in all groups compared to vehicle, confirming an inability of Drp1 siRNA treatment to restore autophagy flux [Figure 26c]. These data suggest that the dual impact of PQ treatment with α-synuclein expression induces more severe cellular effects, as Drp1 siRNA treatment was able to restore autophagy flux in individual treatment groups; whether this is due to autophagy blockade or due to other effects induced by the dual treatment is not discernible from this experiment.

Figure 26: siRNA Drp1 was insufficient to rescue the autophagy blockade induced by combined α-synuclein expression and paraquat treatment in tfLC3 Hela cells (Page 166). Transfection of tfLC3 Hela cells with wild type α-synuclein plasmid (pcDNA3) and/or Drp1 siRNA for 24 hours followed by 24-hour treatment with 50μM paraquat induced significant autophagy blockade. (**a**) Representative images from the treatment groups demonstrate the induction of autophagy blockade by paraquat treatment, wild type α-synuclein transfection and the combination of both treatments. Quantification of autophagosome (**b**) and autolysosome (**c**) signal supports the initial conclusions from imaging. Drp1 siRNA treatment failed to significantly recover autophagy blockade in the dual treatment group. Data represents mean ± SEM, n=3, analysed by one-way ANOVA with Newman-Keuls post-hoc test, **P<0.01, *P<0.05. Paraquat, α-synuclein and dual treatment groups significantly different to vehicle and dual treatment with Drp1 siRNA (**b**, **c**). Scale bar 10μM.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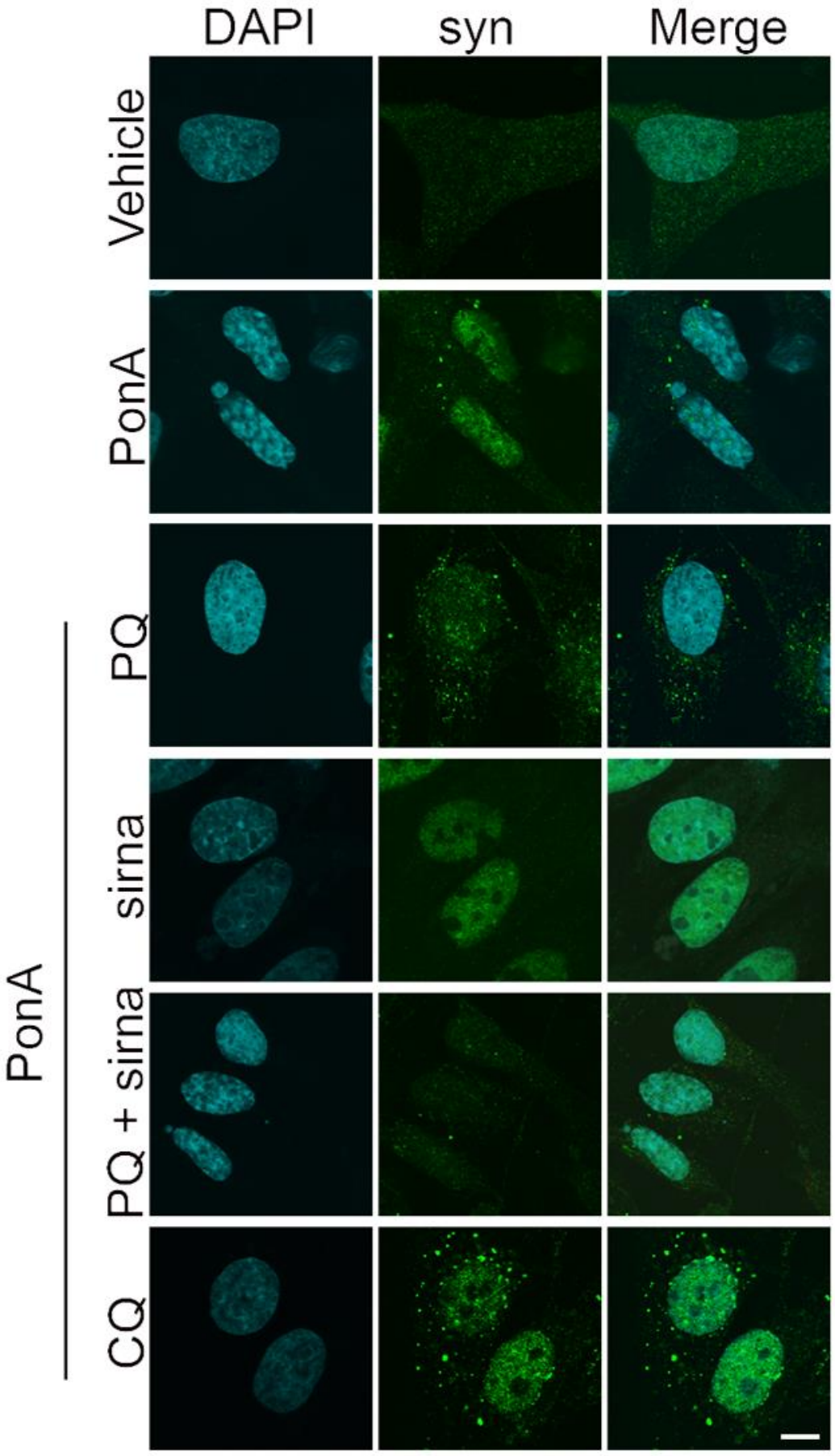
4.3.6| Induction of α-synuclein expression in N27 cells with paraquat treatment causes accumulation of proteinase K-resistant α-synuclein aggregates

To further understand the impact of PQ treatment with α-synuclein expression in relation to PD, investigation of these effects was transitioned into inducible N27 cells. Induction of α-synuclein expression by PonA treatment of N27 cells for 24 hours followed by 24-hour treatment with PQ [50μM] resulted in significant accumulation of proteinase K (PK) resistant α-synuclein aggregates [Figure 27a & b]. These proteinaceous inclusions were prevented by transfection of the cells with Drp1 siRNA prior to PonA induction of α-synuclein expression. This is contrasting with the finding that siRNA inhibition of Drp1 was insufficient to rescue autophagy flux in tfLC3 HeLa cells with α-synuclein expression and PQ treatment. Effective siRNA knockdown of Drp1 was confirmed by western blot analysis of a single well of control and siRNA cells across four independent experiments; protein samples were collected 24 hours after transfection, confirming that efficient Drp1 knockdown would be established prior to PonA induction of protein expression or PQ treatment of N27 cells [Figure 27c]. Quantification revealed a consistent reduction in Drp1 protein levels to 20-45% of Drp1 in the vehicle group [Figure 27d].

a



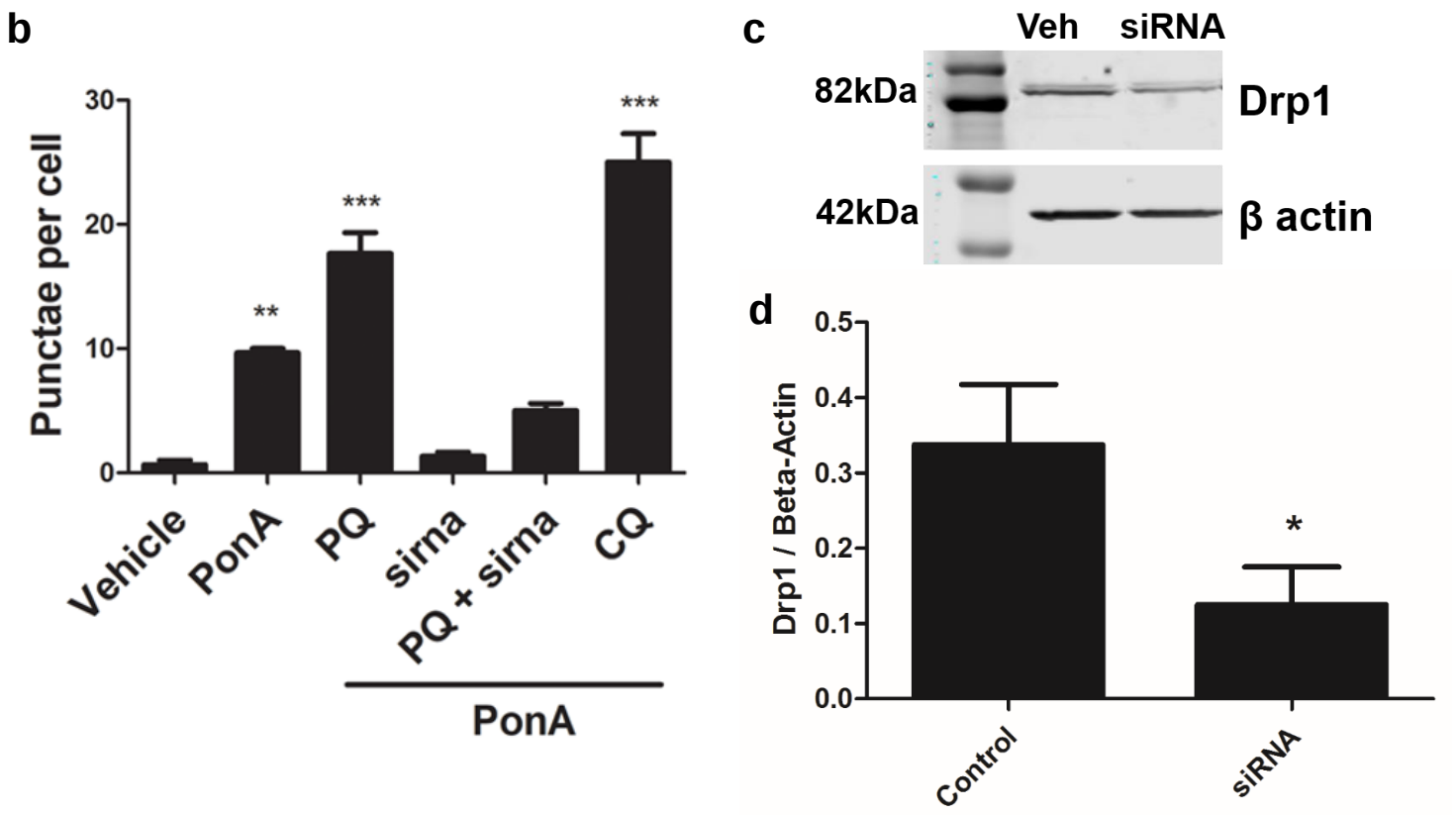
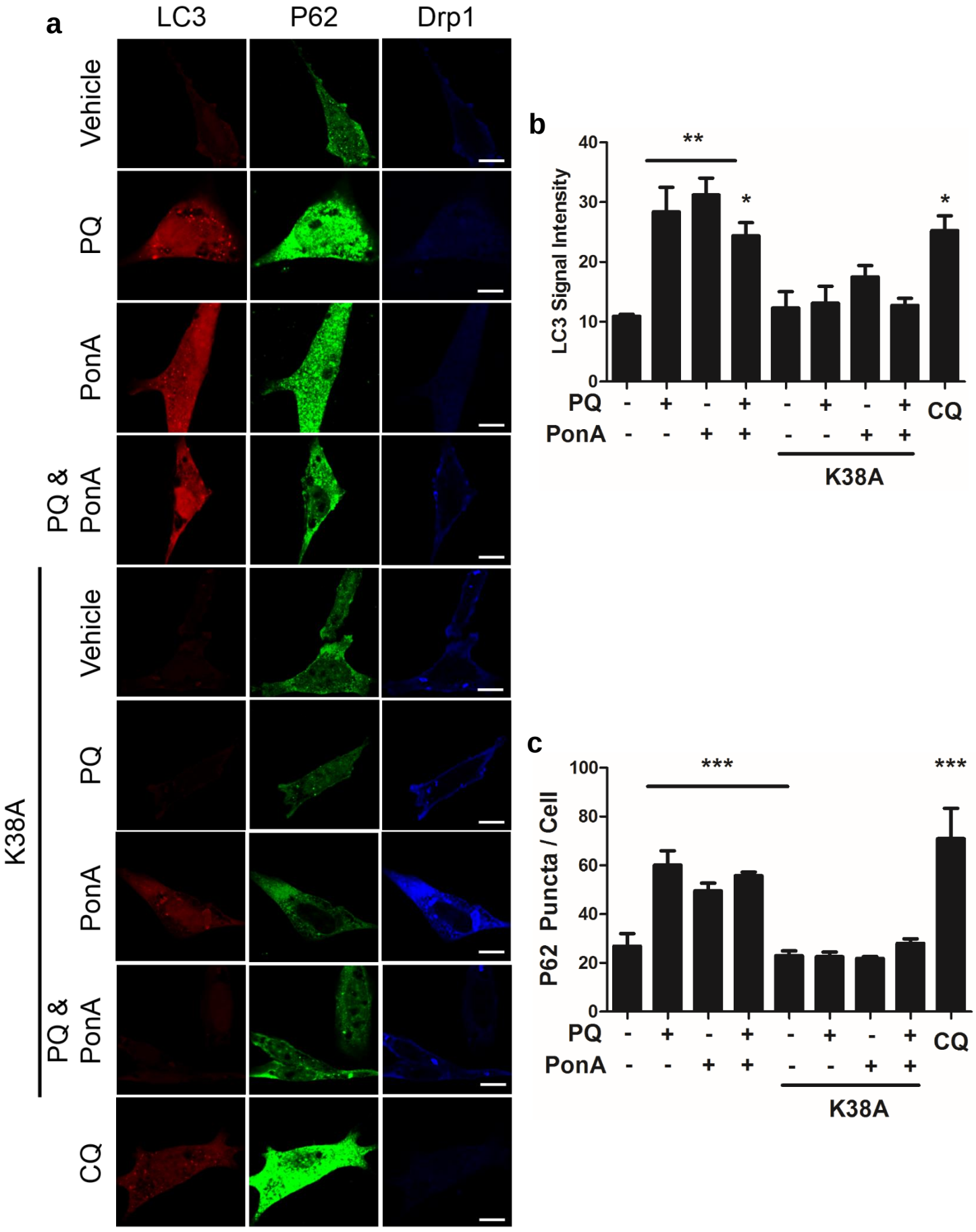


Figure 27: siRNA Drp1 reduces accumulation of proteinase-K-resistant α-synuclein in N27 cells (Page 168-169). 24-hour siRNA knockdown of Drp1, as confirmed by western blotting for protein levels, significantly reduced the accumulation of proteinase K resistant α-synuclein aggregates in N27 cells with PonA induced protein expression, in the absence or presence of PQ treatment. (**a**) Representative images from each group display PK-resistant α-synuclein aggregates with quantification (**b**) revealing significantly increased aggregates in PonA ± paraquat treatment groups attenuated by siRNA Drp1 knockdown. CQ included as a positive control of autophagy inhibition. (**c**) Representative blot from one of four independent experiments to confirm consistent reproducible Drp1 knockdown with siRNA (**d**). Data represents mean ± SEM, n=3-4 independent repeats, analysed by one-way ANOVA with Tukeys post-hoc (**b**) or students t-test (**c**), **P*<0.05, ***P*<0.01, ****P*<0.001, Scale bar 20μM. All significantly different groups (**b**) were different to each other aside from paraquat and chloroquine. Proteinase-K-resistant α-synuclein aggregate data provided by Carolina Sportelli.

4.3.7| Modulation of Drp1 function by expression of Drp1^{K38A} dominant negative protects against autophagy blockade induced by paraquat treatment with or without α-synuclein expression in N27 cells

To investigate whether the GTPase function of Drp1 may be important for its role in modulation of autophagy flux, I used transfection of the dominant-negative mutant Drp1^{K38A}, as siRNA reduces protein expression but does not alter function. Transfection of N27 cells with Drp1 dominant negative K38A, which inhibits Drp1 GTPase activity via modification of a lysine to an alanine in a key functional residue in the GTPase domain, protected N27 cells against PQ- and α-synuclein-induced autophagy blockade [Figure 28]. Cells were treated with PonA 24 hours post-transfection with LC3-cherry with or without Drp1^{K38A}, then fixed 24 hours later or treated with 50μM PQ for 24 hours. Quantification of the P62 puncta and LC3-cherry signal in these cells indicates an accumulation of both proteins following treatment, which was abrogated by K38A transfection. This supports the conclusions from tfLC3 HeLa cells which demonstrated that Drp1 knockdown was protective against PQ-induced autophagy; this extends our understanding of the relationship between Drp1 and autophagy flux, specifying the importance of GTPase activity in its regulatory role.

Figure 28: Drp1^{K38A} expression protects against autophagy blockade induced by paraquat with or without α-synuclein expression in N27 cells (Page 171). Treatment of N27 cells with PonA to induce α-synuclein expression with and without treatment with paraquat for 24 hours demonstrated inhibition of autophagy flux, confirmed with use of chloroquine as a positive control. Inhibition of Drp1 GTPase activity by expression of the K38A dominant negative protein via transfection 24 hours prior to treatment demonstrated protective effects which attenuated the treatment-induced autophagy blockade. Data represents mean ± SEM (**b**, **c**), n=3 independent repeats, analysed by one-way ANOVA with Newman-Keuls post-hoc test, **P*<0.05, ***P*<0.01, ****P*<0.001, Scale bar 20μM.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4.3.8| Treatment of N27 cells with a sub-toxic dose of paraquat for 24 hours did not induce changes in mitochondrial morphology

As Drp1 inhibition is effective at protecting against PQ-induced autophagy dysfunction, I was curious to investigate whether this protective impact could be related to its mitochondrial fission function through maintenance of mitochondrial network morphology. Imaging and morphological analysis of TOM20-stained mitochondria in N27 cells across three independent experiments found that PQ did not induce significant differences in mitochondrial morphology, thus investigation of potential attenuation of changes by siRNA or $Drp1^{K38A}$ was not explored [Figure 29]. The absence of morphological changes after 24-hour treatment with 50μM PQ, which is the same dose demonstrated to induce autophagic dysfunction, suggests that PQs impact on autophagy is independent of any morphological impact on mitochondria; this suggests that the protective impact of Drp1 against PQ-induced autophagy inhibition is independent of its regulation of mitochondrial fission.

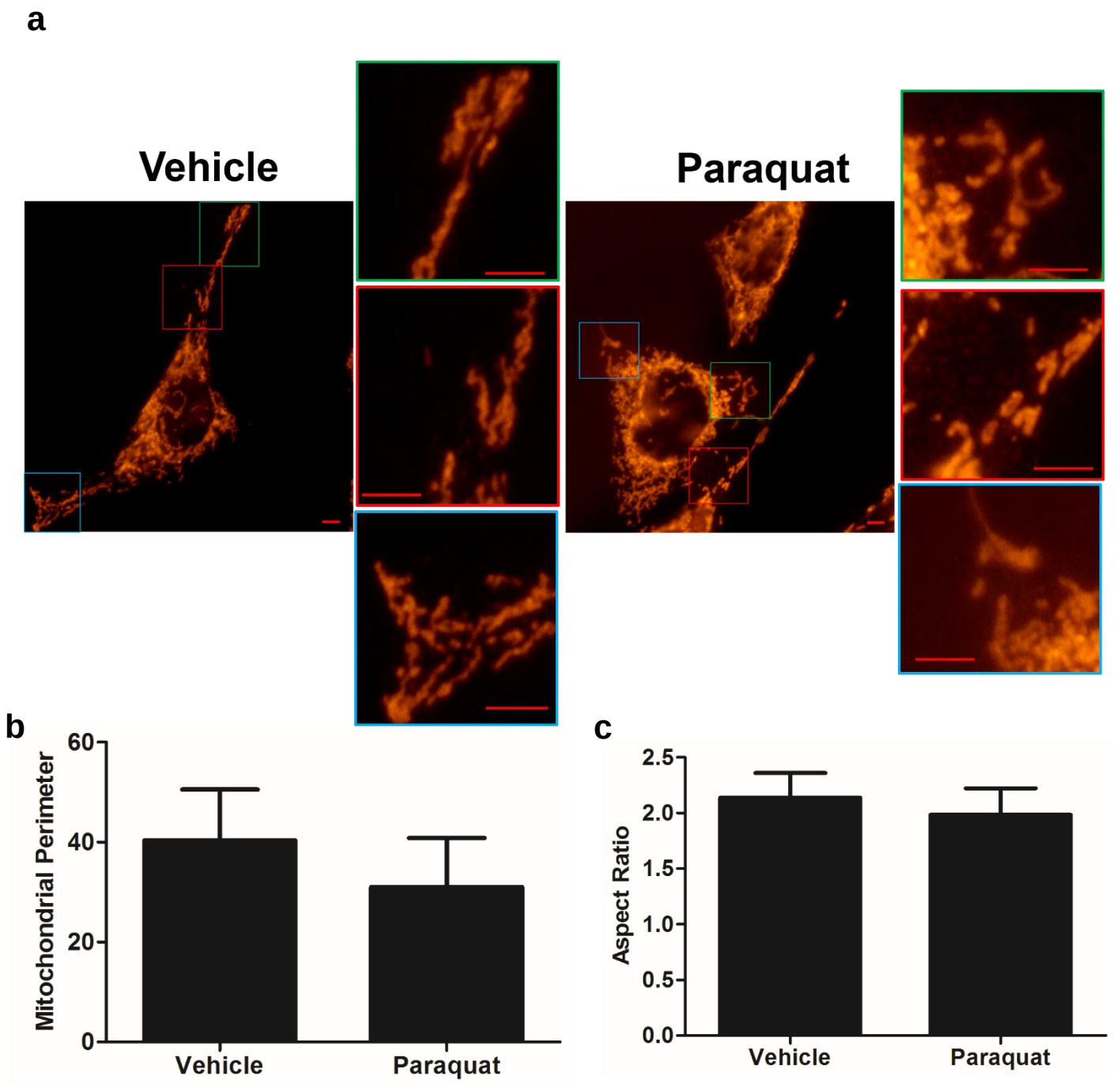


Figure 29: Mitochondrial morphology was unaffected by treatment of N27 cells with a sub-toxic paraquat dose for 24 hours. There was no significant difference detected between the mitochondrial morphology (perimeter of mitochondria (**b**) or aspect ratio (**c**)) in vehicle and paraquat treated N27 cells. Direct comparison of the images, including enlarged areas of view, reveal no obvious difference in the connectivity of the network (**a**). Scale bar 500 pixels.

4.4| Discussion

In this study, I have demonstrated for the first time that PQ-induced autophagy blockade can be abrogated by inhibition of Drp1, by both siRNA knockdown and expression of the Drp1^{K38A} dominant negative protein. Knockdown of Drp1 levels with siRNA was also shown to be effective at reducing proteinase K-resistant α-synuclein aggregates in inducible N27 cells.

The tfLC3 HeLa cells provided an ideal model for the initial assessment of the impact of PQ treatment on dynamic autophagy flux, facilitating confirmation that sub-toxic PQ doses induced blockade of autophagy flux. siRNA knockdown of Drp1 proved effective to prevent the autophagy blockade in cells treated with either PQ or overexpression of α-synuclein, however the combination of α-synuclein overexpression and PQ treatment induced changes which were not prevented by Drp1 reduction. In N27 cells I utilised LC3-cherry plasmid to overexpress LC3, as detection of native levels in this cell type with immunocytochemistry proved difficult, despite confirmation of the antibody in mouse brain sections; this suggested that native LC3 levels are low in this cell type. Inhibition of Drp1 through expression of the K38A dominant negative, which abolishes GTPase activity by changing a critical lysine to an alanine in a key catalytic residue (Smirnova *et al.*, 2001; Ugarte-Uribe *et al.*, 2014), also proved effective at preventing PQ-induced autophagy blockade, both independently and in conjunction with α-synuclein expression: this indicates that sufficient Drp1 GTPase activity is required for PQ-induced autophagy disruption.

Autophagy is a highly dynamic cellular degradation process, which can make it challenging to study as methods often rely on analysis of single snapshots at a pre-determined timepoint. The use of multiple methods to confirm changes in autophagy flux would have been valuable in this study to further support the conclusions; had live

cell imaging been possible, especially in the tfLC3 Hela cell line, it would have been useful to provide a better understanding of the progression rate of changes in autophagy and to explore whether the previously reported increase in autophagy following PQ-treatment preceded the blockade which we detected 24 hours post-PQ-treatment (González-Polo *et al.*, 2007a; 2007b; 2009). Assessment of autophagy protein levels via western blot could also have provided a more quantitative method for the assessment of these PQ-induced changes.

Previous research which has investigated the impact of PQ on autophagy using *in vitro* models has published conflicting results, which may be due to the methods utilised to investigate this dynamic process and challenges interpreting the data; Gonzalez-Polo et al (2007a; 2007b) found that in SH-SY5Y neuroblastoma cells, low dose [<100µM] PQ treatment induced several characteristics of autophagy, including cytoplasmic accumulation of autophagic vacuoles (AV) and recruitment of GFP-LC3 protein to the AVs. Combined with increased long-lived protein degradation and dephosphorylation of mTOR, these data were interpreted as PQ increasing autophagy flux, which was believed to confer protection against apoptotic cell death as inhibition of autophagy accelerated apoptotic cell death. This study was limited by the lack of investigation of other autophagy factors, such as changes in P62 and LAMP1.

More recent work, however, supports my conclusion that PQ treatment induces blockade of autophagy flux; Zhou et al (2017) found that 24 hour treatment of PC12 cells with 4mM PQ increased levels of p62 and LC3-II proteins, detected by western blot. These results indicate that PQ treatment induced autophagy blockade as a mechanism contributing to cytotoxicity in these cells, as 4mM was the highest dose investigated and the only dose sufficient to induce significantly increased LDH release. Ramirez-Moreno et al (2019) found that induction of autophagy via rapamycin

treatment prior to PQ treatment (500µM) in SH-SY5Y cells prevented PQ-induced autophagy blockade, increased oxidative stress and resultant cell death. PQ-exposed cells presented abnormally enlarged autophagosomes enclosing mitochondria, which correlate to increased P62, an essential mitophagy regulator; rapamycin induction of autophagy prior to PQ treatment resulted in autophagosome size and number similar to control cells. Li et al (2017) found that upon internalisation of PQ to SH-SY5Y cells, it localised to endosomes or lysosomes, through which it could potentially mediate its inhibitory autophagic effects; this was observed following short (10 minute) treatment of the cells, with increased treatment duration leading to PQ localisation to a broader range of subcellular organelles. Hirayama et al (2018) found PQ [600µM] treatment of SH-SY5Y cells for 24 hours, which induced 30% reduction in cell viability, significantly increased LC3-II without concurrently altering P62, suggestive of impaired autophagy flux. The absence of P62 accumulation may be due to the capacity for degradation of this protein by other proteases, so it may still be degraded by lysosome-independent mechanisms during impaired autophagy. The authors also found decreased BNIP3 levels; BNIP3 binds to LC3 and links damaged mitochondria to autophagy (Hanna *et al.*, 2012), which suggests that PQ could reduce autophagy via reduction of BNIP3 as a part of this mechanism. As with previous studies investigating autophagy flux *in vitro*, the above studies all utilised cytotoxic PQ doses for investigation of autophagy and other cellular changes; based on a review of the literature, my study is the first to demonstrate that non-toxic doses of PQ inhibit autophagy in a neuronal cell model.

PQ has also been shown to impact autophagy flux *in vivo;* one study found that co-treatment of mice with PQ and MB increased mTOR levels, indicative that the dual agrochemical treatment inhibited autophagy flux (Wills *et al.*, 2012). This study relied on assessment of the LC3-II:LC3-I ratio via western blotting for protein levels in the

striata, correlating with post-mortem human PD samples which demonstrated a reduced ratio indicative of impaired autophagy flux. Enhanced mTOR expression has also been observed in post-mortem human PD striatum samples. Taken together these data support the finding that autophagy flux may be reduced or impaired in PD patients to contribute to disease pathology (Wills *et al.*, 2012).

The protective impact of Drp1 reduction or GTPase inhibition suggests that Drp1 plays a role in autophagy flux; whether direct or indirect is unclear from this data alone so further work will be necessary to ascertain how it contributes to regulation of this process. A recent publication from our group (Fan *et al.*, 2019), which investigated the protective impact of Drp1 inhibition in α-synuclein PD models, demonstrated that modulation of Drp1 via genetic and pharmacological methods protected against α-synuclein-induced mitochondrial dysfunction and impaired autophagy flux. Inhibition of Drp1 in this model revealed that reducing Drp1 function increased autophagy flux by blocking mTOR activity. My study supports the role of Drp1 in autophagy flux in a PQ model and a dual-risk factor model of PQ with α-synuclein overexpression; whether the protective impact of Drp1 inhibition demonstrated in my work is related to modulation of mTOR activity remains to be established.

Whilst our research demonstrates a clear role for Drp1 reduction in the induction of autophagy flux following toxic insult, research in cardiomyocytes found that downregulation of Drp1 suppresses autophagosome formation and autophagic flux at baseline (Ikeda *et al.*, 2015). Cardiac-specific Drp1 knockout has also been shown to suppress autophagic flux, demonstrating that Drp1 disruption can alter autophagy, which in these cells can lead to cardiac dysfunction. These findings suggest that basal Drp1 activity is necessary for functional autophagy and indicate that Drp1 may play differential roles in autophagy dependent upon the cell type.

Autophagy is considered to be a protective cell response to stress, including nutrient deprivation and oxidative stress, with ROS identified as a mechanism to relay pro-apoptotic signals within cells (Galluzzi, Kepp and Kroemer, 2011); this 2011 study found that mitochondrial elongation was associated with autophagy induction as less Drp1 translocated to the outer mitochondrial membrane, due to hyperphosphorylation at Ser637 resulting from increased cAMP levels and PKA activation. Mitochondrial elongation was demonstrated to efficiently preserve cell viability during the autophagic response to stress, evidenced by defective fusion in OPA1$^{-/-}$ cells in which mitochondria could not elongate. Further implicating autophagy in neurodegenerative disease, Drp1 was recently found to mediate inflammation and microglial activation, which have been associated with neurodegenerative pathogenesis (Chae *et al.*, 2019). LPS-treatment of microglia promoted increased GFP-LC3 puncta, which was repressed by inhibition of mitochondrial fission and ROS production, with subsequent reduction of P62 expression; these results indicated that LPS-stimulation produced increased ROS and increased mitochondrial fission which triggered P62-mediated autophagy in microglia (Chae *et al.*, 2019).

No significant changes in mitochondrial morphology were observed in this study; based on previous research, the absence of morphological mitochondrial changes in this work could be related to the dose of PQ used, the duration of treatment or the method utilised to assess this cellular characteristic. Previous studies which have reported changes in mitochondrial morphology *in vitro* have utilised much higher doses of PQ with a longer treatment duration. Zhao et al (2017) utilised 500µM PQ in SH-SY5Y cells to demonstrate mitochondrial fragmentation after 24-hour treatment, however at a lower dose of 62.5µM PQ for 24-hours, no significant change in mitochondrial morphology was detected. The 500µM PQ dose induced significantly

increased Drp1 levels within 30 minutes of treatment, which continued to increase until they plateaued after four hours, which could explain the increased fragmentation as upregulation of Drp1 expression can mediate increased mitochondrial fission. Yang et al (2005) also used 500μM PQ for 48 hours in SH-SY5Y cells; this paper found PQ increased intracellular ROS, decreased glutathione levels and decreased mitochondrial membrane potential, however they did not investigate mitochondrial morphology. This dose of PQ was shown to induce 50% reduction in cell viability in SH-SY5Y cells.

All current papers which report changes in mitochondrial morphology in PQ models, both *in vitro* or in *C. elegans*, utilised electron microscopy to investigate these changes (Dilberger *et al.*, 2019; Zhao *et al.*, 2017); the use of immunocytochemistry-based images for analysis may not provide the necessary resolution to demarcate subtle changes in the morphology of these organelles after only 24 hours of treatment with a sub-toxic PQ dose in N27 cells in my study. Increased PQ dose, duration of treatment and investigation of other methods for assessment of mitochondrial morphology could have furthered our understanding of whether PQ induced morphological changes alongside the induction of autophagy blockade. However, the major caveat of using a higher PQ dose is determining whether the observed impairment in autophagy flux would directly result from PQ treatment or whether it would result from PQ-induced mitochondrial dysfunction. By utilising a low, non-toxic PQ dose which does not affect mitochondrial morphology, I have demonstrated that the effect of PQ on autophagy is independent of morphological changes; future confirmation of whether this PQ dose impacts mitochondrial function is required.

This *in vitro* work investigated the impact of PQ treatment, and expression of α-synuclein, as PD aetiology involves the complex interplay of multiple environmental

and genetic risk factors. α-synuclein aggregation is a feature in both sporadic and familial PD patient brain samples, indicative that this protein plays a role in disease pathogenesis in idiopathic cases. This study confirmed that autophagy flux blockade is a relevant mechanism in a gene-environment PD model, with dysfunctional autophagy rescued in the two independent models via interference with Drp1. The gene-environment hypothesis in PD models, which suggests that PD pathology results from multiple concurrent or sequential risk exposures, suggests that potential therapeutic approaches tested in singular models alone may not be representative of the interplay in human presentation. My work correlates with a recent publication which investigated the effects of PQ on autophagy in SH-SY5Y cells and the mechanism of abnormal α-synuclein aggregation (Wu *et al.*, 2020). The study demonstrated that PQ-treatment reduced cell survival in a time- and dose-dependent manner, with increased LDH activity and altered levels of autophagy proteins. P62, which tags cellular substrates for degradation, was increased following PQ treatment, whilst other factors, including Beclin-1, VPS34, and the pro-autophagic conversion of LC3-I to LC3-II was reduced. These changes corresponded with increased α-synuclein expression and protein levels. Co-treatment with the pro-autophagic rapamycin induced the opposite effect in the SH-SY5Y cells, with reduced α-synuclein accumulation and increased Beclin-1 and LC3-II levels. This paper supports my conclusion that PQ treatment induces dysfunctional autophagy flux in neuronal cells, including the concurrent accumulation of PD-relevant α-synuclein aggregates. My study, however, extends beyond this publication to investigate the interplay between PQ and α-synuclein when PQ was utilised in α-synuclein expressing cells, along with examination of how Drp1 inhibition may confer protection in the model.

This project is the first to illustrate the inhibitory impact of sub-toxic PQ doses on autophagy flux, and to identify that reduced Drp1 levels or inhibition of Drp1 GTPase activity confers protection against the effect of this PD-related agrochemical. It provides rationale for the future investigation of Drp1 modulation in promoting autophagy in PD, as a potential therapeutic intervention which may alter disease prognosis via modulation of neuropathological changes.

5| General discussion and future perspectives

5.1| Major findings

In this study, I have demonstrated for the first time that PQ-induced autophagy blockade can be abrogated by inhibition of Drp1 by both siRNA knockdown and expression of the $Drp1^{K38A}$ dominant negative protein *in vitro*. I have provided initial evidence that pharmacological inhibition of Drp1 with mdivi-1 in an *in vivo* PQ model can attenuate the induced neuropathology, however due to unanticipated issues recapitulating PQ-induced neuropathology in the global heterozygous Drp1 knockout mice and the littermate controls, I was unable to pursue this further. I also characterised the rotenone Lewis rat model, published by Cannon et al (2009), confirming the value in utilising rotenone to model PD behavioural and neuropathological changes, although the induction of severe systemic toxicity and early mortality stipulates the necessity to further develop the treatment regime to improve the models ability to reproduce the progressive neurodegeneration characteristic in PD.

In relation to the specified project aims [Chapter 1.7], I address all three through this body of work. I established the neurotoxic impact of rotenone and PQ *in vivo*, including dissection of which motor changes specifically related to rotenone-associated neuropathology and which were the result of acute systemic toxicity. In the PQ model, I explored the impact of blocking Drp1 function with pharmacological and genetic methods; Mdivi-1 confirmed the protective impact on the PQ-induced loss of dopaminergic neurons in the nigrostriatal system of *Oct3*$^{-/-}$ mice, however PQ failed to induce neuropathology in the Drp1 heterozygous knockout mice and their controls. The *in vitro* investigation detailed in Chapter 4 demonstrates one mechanism by which targeting Drp1 is effective at conferring protection in the PQ PD model, providing the

first evidence that PQ-induced dysfunction of autophagy flux can be prevented by modulation of Drp1.

Although the project aims were not addressed in both of the animal models as originally planned, this study provides early evidence of the important role which Drp1 plays in PD pathology beyond the previously established function in mitochondrial fission. To build upon the work of this study *in vivo,* further work is essential to explore the impact of Drp1 inhibition in the rotenone model once a strategy is devised to reduce the acute systemic toxicity currently reported, and exploration of the PQ *in vivo* model to determine why the reported nigrostriatal neuropathology was not recapitulated in the Drp1 global heterozygous knockout mice and their WT littermates could further our understanding of human susceptibility to this risk factor.

My colleagues have explored the capability of Drp1 inhibition to confer protection in an α-synuclein model of PD through modulation of autophagy flux (Fan *et al.*, 2019), so the demonstration here that this approach may be an applicable intervention in an environmental model of PD offers hope for its future translation to clinical use. The multifactorial aetiology of PD contributes to the difficulties in developing new treatments, as our experimental models cannot fully reproduce the interplay of factors which contribute to the human condition, so the development of novel therapeutic approaches which are successful across a range of PD models holds promise for their efficacy in patients.

5.2| Interpretation and relevance

Mitochondrial dysfunction and Drp1 imbalance has been identified as a contributing factor in a number of diseases, including Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis, Down's syndrome, multiple sclerosis,

spinobulbar muscular atrophy and spinocerebellar atrophy 1 (Hu, Huang and Li, 2017; Manczak *et al.*, 2010; Manczak, Calkins and Reddy, 2011; Reddy *et al.*, 2011; Silva-Ayala *et al.*, 2013; Wang *et al.*, 2008; Wang *et al.*, 2009b). As Drp1 has been strongly linked to excessive mitochondrial fission and fragmentation, which play an active role in mitochondrial dysfunction, apoptosis, autophagic cell death and necrosis (Barsoum *et al.*, 2006; Estaquier and Arnoult, 2007; Frank *et al.*, 2001; Twig *et al.*, 2008; Wang *et al.*, 2012b), the implications for disease treatment by targeting Drp1 stretch across a range of diseases and disorders.

A few recent publications have reviewed the impact of mitochondrial dynamics in a range of neurodegenerative disorders (Oliver and Reddy, 2019; Pinnell and Tieu, 2017); as Drp1 was first reported in 1997 (Shin *et al.*, 1997), there has been varied investigation into its role in a number of diseases over the past two decades. In AD models, modulation of Drp1 function by pharmacological inhibition has been effective at abrogating toxic protein effects. In APP and presenilin-1 (PS1) double heterozygous transgenic mice, treatment with 10mg/kg or 40mg/kg mdivi-1 per day for forty days demonstrated dose-dependent prevention of APP and PS1 toxicity. APP and PS1 mice without mdivi-1 treatment displayed stunted, rounded mitochondria, increased ROS production, inhibited anterograde mitochondrial transport and reduced mitochondrial biogenesis (Baek *et al.*, 2017); these pathological changes were reduced in mdivi-1-treated mice. Richetin et al (2017) also used the APPxPS1 mouse model to demonstrate that restoration of the defective mitochondrial function in adult-born neurons stimulated dendritic growth and spine formation, supporting the importance of mitochondrial functionality in protecting neurons and restoring neurogenesis in AD. Research using an APP mouse strain (CRND8 mice) found that mdivi-1 rescued mitochondrial fragmentation, function and cellular distribution (Wang

et al., 2017); it also reduced the Aβ-42/Aβ-40 ratio, reduced amyloid deposition and prevented cognitive deficits. Hyperphosphorylated and truncated tau has also been implicated in mitochondrial dysfunction in AD, with reports of abnormalities in mitochondrial structure, function, and oxidative stress levels, compared with neurons expressing full length tau (Quintanilla *et al.*, 2012). Aβ has also been demonstrated to interact with cyclin-dependent kinase 5 (CDK5) which can induce Drp1 phosphorylation at Ser-616, leading to caspase-3 cleavage and execution of indiscriminate apoptosis resulting in neuronal death. In rats, CDK5 phosphorylates Drp1 at the conserved Ser-585 residue (Ser-616 in humans) which upregulates Drp1 function, inhibiting mitochondrial biogenesis, increasing oxidative damage caused by ROS overproduction and increasing glutamate NMDA receptor binding which inhibits Ca^{2+}, necessary for learning and memory (Guo *et al.*, 2018). As deficits in learning and memory are symptoms of AD, disruption of the neuronal pathways necessary to maintain these functions as a result of Drp1 modification highlights its direct link to Alzheimer's pathology. These AD-associated proteins have also been shown to alter the expression of mitochondrial fission and fusion proteins, through investigation in AD patient samples, mouse and cell models (Cho *et al.*, 2009; Reddy *et al.*, 2011; Wang *et al.*, 2008; Wang *et al.*, 2009a; Wang *et al.*, 2009b). Overexpression of APP was shown to reduce Drp1 and OPA1 levels, whilst increasing Fis1 to cause an imbalance of mitochondrial dynamic proteins in M17 cells, corroborating the finding that AD-derived hippocampal tissue showed significant reduction of Drp1, OPA1, Mfn1 and Mfn2 expression, with enhanced Fis1 expression (Manczak, Calkins and Reddy, 2011; Wang *et al.*, 2008; Wang *et al.*, 2009a). Reduction of Drp1 levels through generation of $Drp1^{+/-}$ APP or $Drp1^{+/-}$ Tau P301L transgenic mice reduced the toxic protein levels

and improved mitochondrial function in these AD models (Kandimalla *et al.*, 2016; Manczak *et al.*, 2016).

Mutant Huntingtin protein (mHtt) has been reported to induce excessive mitochondrial fission, resulting in fragmentation and impaired transport of the organelles in neurons (Jin *et al.*, 2013; Kim *et al.*, 2010; Shirendeb *et al.*, 2011; Song *et al.*, 2011). Excessive mitochondrial fission was identified as a direct result of interactions between mHtt and Drp1 causing increased Drp1 GTPase activity (Shirendeb *et al.*, 2011; Song *et al.*, 2011). Altered levels of mitochondrial dynamic proteins have been distinguished in HD mouse models, with Drp1 significantly increased and Mfn1 significantly decreased resulting in a shift of the fission-fusion protein balance which likely contributes to the increased levels of fission and fragmentation in these models (Kim *et al.*, 2010). Combined data from these studies supports the conclusion that mitochondrial fragmentation is an early occurrence, prior to the onset of neurological symptoms, neuronal death and mHtt aggregation to contribute to HD pathogenesis. This verifies the finding that metabolic impairment precedes the characteristic loss of striatal neurons in HD patients (Kuhl *et al.*, 1984). mHtt has also been shown to impair mitochondrial biogenesis, through repression of PGC1-α expression via interference with TAF4/CREB (Cui *et al.*, 2006), which reduces CBP-dependent gene expression (Riley and Orr, 2006), further delineating the role of this toxic protein in disease progression.

Two proteins which have been implicated in PD pathogenesis through their identification as genetic risk factors have been linked with post-translational modification of Drp1; α-synuclein affects mitochondrial dynamics and impacts Drp1 activity both directly and indirectly whilst LRRK2 phosphorylates Drp1 to increase mitophagy, and mutant LRRK2 may induce mitochondrial fission and fragmentation

(Ho *et al.*, 2018; Pozo Devoto and Falzone, 2017). Toxic insults, including with rotenone and PQ, have been shown to interact with α-synuclein and LRRK2 to modulate their post-translational modifications (Pajarillo *et al.*, 2019), which can in turn modulate their interactions with Drp1. Pathogenic modifications of α-synuclein following environmental exposures have also been shown to promote α-synuclein aggregation and abnormally increase LRRK2 kinase activity, which indicates how genetic and environmental risk factors may converge to impact mitochondria through Drp1 modification. Modulation of post-translational modifications in LRRK2 and α-synuclein by toxic insult may also impact the localisation and clearance of these proteins, which can impact their function and may contribute to disease pathology. Rotenone is associated with increasing α-synuclein phosphorylation and aggregation *in vitro* (Yuan et al., 2015), and it has also been shown to modulate α-synuclein nitration (Mirzaei *et al.*, 2006). PQ was shown to increase α-synuclein aggregation and inclusion body formation in α-synuclein overexpressing mice, however no signs of neurodegeneration were associated with these pathogenic hallmarks (Manning-Bog *et al.*, 2002; Manning-Bog *et al.*, 2003), so further work is required to ascertain the significance of the PQ-induced synucleinopathy. Primary dopaminergic neurons treated with 10μM purified α-synuclein, 10nM rotenone or 12.5μM PQ presented mitochondrial morphological alterations and loss of TH-positive neurons (Sashourpour *et al.*, 2017). Treatment with *S. pinnatifida* extracts conferred neuroprotection against PQ and α-synuclein induced toxicity, however treatment was ineffective against rotenone-induced damage, suggestive that these extracts are protective against α-synuclein aggregation and ROS production but not against functional mitochondrial impairment (Sashourpour *et al.*, 2017); this paper didn't investigate mitochondrial changes however so further confirmation of this is required. The selective capacity of

this proposed neuroprotective treatment highlights a limitation of current PD research, in that selection of the model utilised to reproduce PD features can impact the success of the therapeutic interventions; as such I believe that my study, combined with previously published work from our lab and others, robustly supports the neuroprotective effectiveness of Drp1 inhibition, as it has been demonstrated in a range of PD models.

In septic cardiomyopathy, the Drp1-Fis1 interaction has been shown to mediate mitochondrial dysfunction. Balb/c mice treated with LPS displayed reduced cardiac function and abnormal mitochondrial morphology which was abrogated by treatment with P110. P110 is a novel peptide-based Drp1 inhibitor which reduces Drp1 GTPase activity to block the interaction of Drp1 with Fis1 (Qi *et al.*, 2013). LPS-treated cardiomyocytes demonstrated increased oxidative stress, with reduced mitochondrial respiration and membrane potential (Haileselassie *et al.*, 2019); these effects were attenuated by P110 treatment in *in vitro* and *in vivo* LPS models. In a similar study by the same group, the authors investigated the impact of LPS treatment on the blood brain barrier, as Drp1-mediated mitochondrial dysfunction has been shown to propagate impairment of the blood brain barrier in septic encephalopathy (Haileselassie *et al.*, 2020). In primary microvascular epithelial cells, LPS treatment was associated with increased blood brain barrier permeability and loss of tight junctions; P110 abrogated these defects, indicative of a critical role for the Drp1-Fis1 interaction in mediating sepsis-induced brain dysfunction. P110 has also been shown to be protective in MPTP-treated mice (Filichia *et al.*, 2016).

My findings in this study support the implication of environmental risk factors in PD pathogenesis, however further research is necessary to confirm whether this may be related to modulation of Drp1 post-translational modification as Drp1-616 changes

were non-significant in the *in vivo* PQ models of PD. As such, the mechanism by which this post-translational modification may be upregulated following PQ treatment requires further exploration in future studies.

Recent work has explored the tissue-specific expression of Drp1 isoforms, which result from alternative splicing. A 2018 paper identified a novel isoform which is highly enriched in the brain (Itoh *et al.*, 2018) and has been found to localise to mitochondria and lysosomes/late endosomes, suggestive of a Drp1 function beyond mitochondrial fission. $Drp1_{ABCD}$ contains four alternative isoforms, two of which modulate its translocation to interorganelle junctions between mitochondria and lysosomes. Itoh et al (2018) found that assembly of $Drp1_{ABCD}$ onto lysosomes and mitochondria was regulated by its oligomerisation and GTP hydrolysis, which may relate to the importance of the two exons which both present in the GTPase domain. Lysosomal pH, but not protease activity, was also found to contribute to regulation of this association, with deacidification of the lysosomes reducing Drp1 association. Rab7 is another GTPase which has been shown to interact with interorganelle junctions between mitochondria and lysosomes (Wong, Ysselstein and Krainc, 2018); this study found that mitochondria-lysosome contact sites form dynamically in healthy mitochondria and are distinct from the targeting of damaged mitochondria to lysosomes for degradation. The lysosomal GTPase Rab7 promoted contact formation, with untethering of this interaction mediated by mitochondrial recruitment of TBC1D15, a Rab7 GTPase-activating protein. TBC1D15 relies on interaction with Fis1 for recruitment to mitochondria, where it drives Rab7 GTP hydrolysis and the dissociation of the mitochondria-lysosome contact (Wong, Ysselstein and Krainc, 2018). As Drp1 is another GTPase shown to localise to these same contact sites in neurons, it may

be that it plays a similar function in regulating the duration of these interactions, which have been proposed to be an important part of the maturation process for lysosomes. Wong, Ysselstein and Krainc (2018) found that mitochondria-lysosome contact sites marked subsequent sites of mitochondrial fission, demonstrating lysosomal regulation of the mitochondrial network, whilst mitochondrial association with TBC1D15 regulates dissociation of the mitochondria-lysosome contact sites, indicative of bidirectional regulation between the organelles. This crosstalk may explain why defects are often seen in both organelles and their functions in human disease. These contact sites provide a potential cellular mechanism for simultaneous regulation of the organelles and their dynamic connections, with mitochondria and LAMP-1 positive vesicles shown to form stable contacts in HeLa cells (Wong, Ysselstein and Krainc, 2018) and Drp1 localisation to focal points of interorganelle junctions following lentiviral expression in Drp1 KO MEFs (Itoh *et al.*, 2018). Drp1 has been previously implicated in peroxisomal division (Wakabayashi *et al.*, 2009), so localisation to lysosomes/late endosomes could suggest a role in the subdivision of these cellular components; in the Drp1 KO MEF study, Itoh et al (2018) identified Drp1 homo-oligomerisation suggestive that Drp1 oligomers, rather than single Drp1 monomers, colocalised to mitochondria-lysosome contact sites. As Drp1 oligomerisation is required for its mitochondrial fission function, it may be that this oligomerisation is necessary for its currently unknown function at these interorganelle junctions.

Rab7 GTPase-activating protein (TBC1D15) mutants demonstrate increased contact duration without alteration of contact formation, due to interference with the untethering process, and Fis1 mutants display failed mitochondrial recruitment of TBC1D15, confirming dependence on this mitochondrial recruitment for Rab7 GTP hydrolysis and contact site untethering. Inhibition of hydrolysis leads to defective

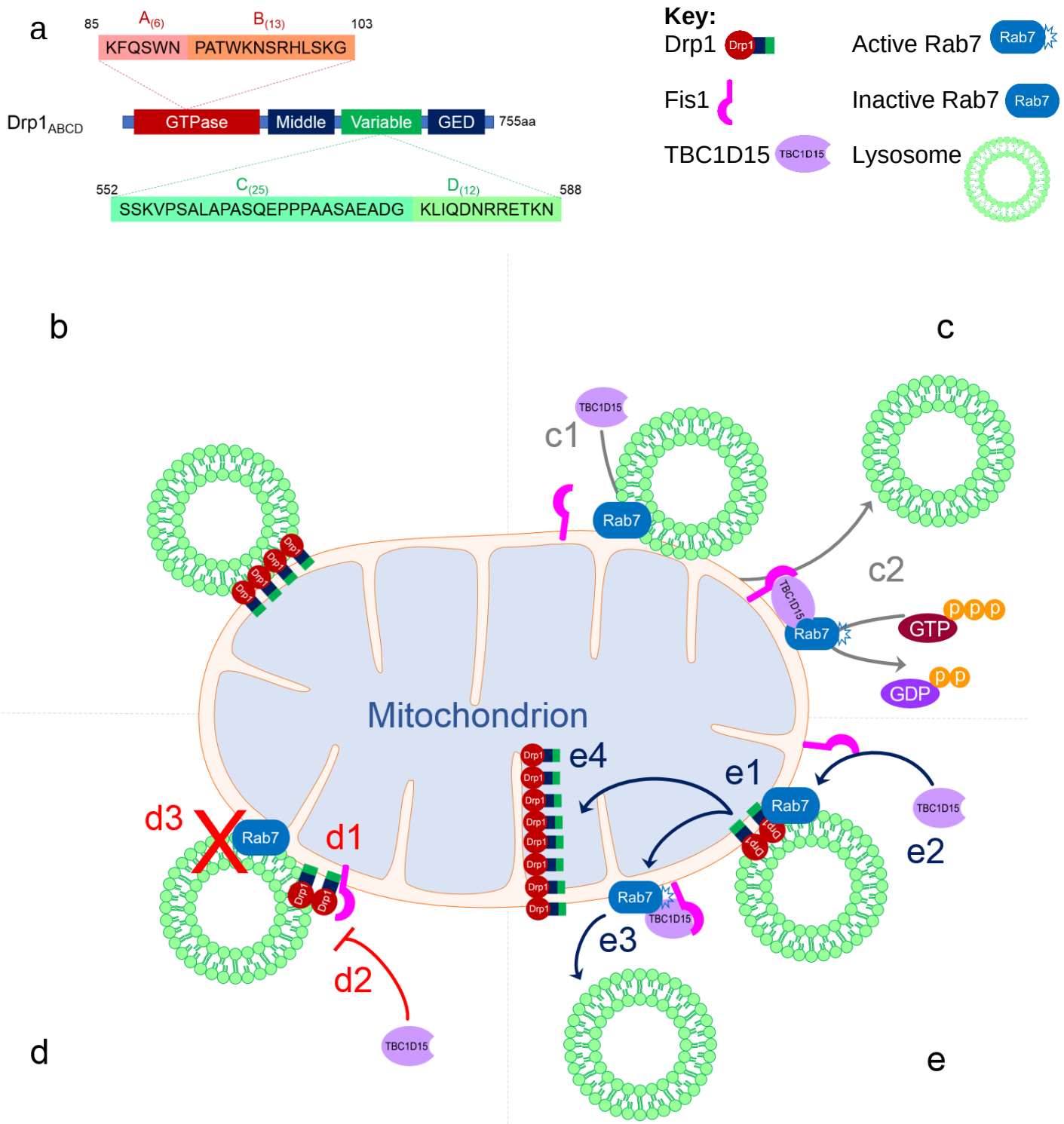
lysosomal morphology and extended persistence of mitochondria-lysosome contact sites (Wong, Ysselstein and Krainc, 2018). As mitochondria-lysosome contact sites seem to mark sites of subsequent mitochondrial fission, implicating Drp1 in this process seems likely as it is a key factor for fission. Increased Rab7 expression reduced the rate of mitochondrial fission (Wong, Ysselstein and Krainc, 2018), suggestive that perhaps Rab7 sequesters Drp1 at these contact sites to regulate free Drp1 to oligomerise for fission. Reduction of TBC1D15 recruitment to mitochondria, both through TBC1D15 and Fis1 mutations, also reduced the rate of fission (Wong, Ysselstein and Krainc, 2018), suggestive that TBC1D15 may play a role in the regulation of this process; perhaps Rab7 releases sequestered Drp1 upon activation by TBC1D15. Inhibition of Rab7 hydrolysis further supports this implication of Drp1 regulation, as it led to an increased proportion of cells displaying abnormal, hypertethered and elongated mitochondrial networks (Wong, Ysselstein and Krainc, 2018), indicative that Rab7 hydrolysis may be an essential step in mitochondrial fission progression [Figure 30].

Investigation of a potential interaction between Rab7 and Drp1, or TBC1D15 and Drp1, could progress understanding of this potential interconnection of the processes, as we know from Itoh et al (2018) that Drp1 is also present at these interorganelle contact sites. Reducing Drp1 levels or oligomerisation, which seem to be essential for recruitment to these contact sites, could promote the untethering of these contacts to restore or increase autophagy flux. Investigation of the impact of α-synuclein and/or PQ on the morphology of these contact sites, and the subsequent changes induced by Drp1 inhibition, could further understanding of how these sites may regulate autophagy and mitochondrial fission in these models; this may provide a mechanistic understanding of how these toxic insults induce autophagy blockade

and why modulation of Drp1 levels confers protection, as evidenced by my *in vitro* study.

It remains to be established whether Rab7 is important in the regulation of mitochondrial-lysosomal contact sites in neurons, and whether constitutive $Drp1_{ABCD}$ localisation to interorganelle junctions is confirmed without regulation of Drp1 expression, before we can further specify the role of these proteins in mitochondrial-lysosomal contacts and potentially in the regulation of autophagy. Work to date suggests that Drp1 may exert a novel function at these mitochondria-lysosome/late endosome contact sites, which could be relevant to the protective effect of reducing Drp1 as demonstrated in the *in vitro* PQ model.

Figure 30: Proposed potential mechanism for Drp1 function in the regulation of autophagy via interaction at mitochondria-lysosome contact sites (Page 194). (**a**) Itoh et al (2018) described a specific Drp1 isoform enriched in the brain, $Drp1_{ABCD}$, which contains four variant exons. Exons A and B in the GTPase domain regulate its localisation to mitochondria-lysosome interorganelle contact sites, which requires oligomerisation and GTPase activity (**b**). Wong, Ysselstein and Krainc (2018) describe regulation of mitochondria-lysosome contact sites via the function of Rab7 GTPase (**c**), which is activated by TBC1D15 following its recruitment to mitochondria via Fis1, an OMM protein (c1). Rab7-mediated GTP-hydrolysis mediates dissociation of the mitochondria-lysosome contact site, releasing the lysosome for autophagic function (c2). As my findings support a functional role of Drp1 in the regulation of autophagy, I propose that Drp1 may interact with these mitochondria-lysosome contact sites, which are purported sites of downstream mitochondrial fission. (**d**) Drp1 may control TBC1D15 recruitment to mitochondria through its known interaction with Fis1 (d1), which could inhibit TBC1D15 recruitment (d2) as shown in TBC1D15 and Fis1 mutants, as interaction with Fis1 appears to be essential for mitochondrial TBC1D15 recruitment. This would then prevent Rab7 GTP hydrolysis leading to continued anchoring of the mitochondria and lysosome at the interorganelle contact site (d3). (**e**) Alternatively, Drp1 may sequester Rab7 interaction at the mitochondria-lysosome contact site (e1), limiting its availability for activation by TBC1D15 (e2). Release of Rab7 from Drp1 could then facilitate Rab7 activation and dissociation of the organelle contact site (e3) to release the lysosome, concurrently freeing Drp1 for oligomerisation and mitochondrial fission (e4). Both of these scenarios would lead to enhanced autophagy in circumstances of Drp1 inhibition or knockdown, as Drp1 would then be unable to extend contact duration which has been shown to produce hyper-tethered, elongated mitochondrial networks and result in dysfunctional or misformed lysosomes for autophagic function.


a
85
A(6)
B(13)
103
KFQSWN
PATWKNSRHLSKG
Drp1ABCD
GTPase
Middle
Variable
GED
755aa
552
C(25)
D(12)
588
SSKVPSALAPASQEPPPAASAEADG
KLIQDNRRETKN
Key:
Drp1
Fis1
TBC1D15
Active Rab7
Inactive Rab7
Lysosome
b
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d
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Mitochondrion
c1
c2
e1
e2
e3
e4
d1
d2
d3
Rab7
TBC1D15
GTP
GDP
Drp1

5.3| Strengths and limitations

As strengths and limitations have been discussed in the relevant data chapters in this thesis, I will focus here on the major factors which strengthen the research conclusions and the limitations which should be considered.

In the rotenone study, inclusion of behavioural motor assessment through use of the open field chamber is a strength of the study, as it facilitated monitoring of rotenone's impact on the rats behaviour and the progressive changes which it induced; this included the delineation of changes attributable to acute toxicity and discomfort caused by the injection regime from the motor dysfunction resultant from the nigrostriatal dopaminergic neuropathology. A limitation of this work was the use of only one functional assessment method: other PD research typically utilises two or more functional tests to dissect the locomotor skills impacted (Anusha, Sumathi and Joseph, 2017; Bai *et al.*, 2016; Cannon *et al.*, 2009; Fleming *et al.*, 2004; Gokul and Muralidhara, 2014; Liu *et al.*, 2015; Mulcahy *et al.*, 2013; Ravenstijn *et al.*, 2008; Sharma and Nehru, 2013; Sonia Angeline *et al.*, 2013; Tapias, Cannon and Greenamyre, 2014; Xiong *et al.*, 2009b; Zhang *et al.*, 2016) and further work which includes a range of behavioural tests could yield greater insight into rotenone-induced changes.

The method utilised to examine apoptosis as a potential cellular pathway contributing to loss of the nigral dopaminergic neurons following rotenone treatment was limited by its design; investigation of apoptosis usually involves appraisal of multiple apoptotic proteins, including Caspase-3. Whilst my work found a clear increase in the cytosolic levels of cytochrome C in the rotenone-treatment group compared to control samples, the conclusions would have been strengthened by the measurement of multiple factors, as apoptosis is a dynamic process which is regulated

by complex pathways of protein interactions. Inclusion of Bax and caspase assays, and potentially imaging analysis to assess the colocalization of cytochrome C with other apoptotic factors, would further bolster the finding that rotenone induces apoptotic cell death. Further to which, testing of other complex I inhibitors for comparison could reveal whether it is a direct effect or a downstream result of complex I inhibition.

A major limiting factor, which prevented all of the research aims being addressed in the rotenone model, was the induction of acute systemic toxicity which led to early deterioration of some motor functions, as determined by their rapid decline and sustained low scores. The necessity to euthanise rats at variable time points introduced an additional level of complexity which was determined to be too great a challenge for exploration of the potential protective impact of Drp1 inhibition. Neuroprotection by Drp1 inhibition was due to be investigated via genetic manipulation of the protein in the rotenone model through stereotactic delivery of rAAV Drp1-dominant negatives (K38A and A395D) to the substantia nigra of the rats; following a six week period to establish expression of the mutant protein, rats would have been subjected to rotenone treatment. Reduced confidence in a high survival rate of rotenone-treated animals negated the potential benefits of this further study, as we would have had to increase the number of rats subjected to surgery without certainty that a sufficient number would survive the rotenone treatment for assessment of the impact of rAAV-Drp1$^{K38A/A395D}$ expression. Overuse of rodent numbers would not adhere to the reduction principle of the Animal Scientific Procedures Act 1986 (ASPA) to ensure survival of sufficient rats for downstream assays. Due to the time-consuming nature of stereotactic surgery and the uncertainty about rodent survival, this line of investigation was not pursued which prevented investigation of the impact of

modulating Drp1 following the establishment of the neuropathological and behavioural effects of rotenone treatment in this treatment regime.

The use of blinded optical fractionator stereological cell counting to estimate TH-positive and Nissl-positive neurons in the SNpc of rat and mouse brain sections in this thesis is a strength of this work; stereology is considered the gold standard because it provides precise, unbiased measurements of three dimensional structures (Altunkaynak *et al.*, 2012; Howard and Reed, 1998; Kipanyula and Sife, 2018). The blinding of all slides prior to stereology removed any potential bias in the selection of neurons to count, as the experimental groups of the samples were only revealed following completion of data collection. All stereology was performed by the same operator to remove the potential for introduction of variation due to inconsistency between counting selection, so this work provided unbiased estimations of neuronal counts.

Whilst the rotenone model was ineffective for the investigation of Drp1 modulation in this study, its promising induction of PD-related neuropathology supports the value of using rotenone to study PD. Further titration of the rotenone dose and adjustment of the treatment regime may provide a more effective model for reproducing PD pathology in future research, which would be a valuable tool for testing therapeutics. A current challenge in neurodegenerative research is the lack of replicable and reproducible animal models, as variability between investigative approaches, models and findings create disparity in the conclusions published. Establishing effective models of neurodegenerative diseases, including PD, is crucial to the development of future therapies, which will enable us to better treat the diseases and provide better care to patients.

The PQ *in vivo* study was strengthened by the use of an established transgenic mouse model of cation knockout mice ($Oct3^{-/-}$), in which PQ had been previously shown to induce PD-related neuropathology by our lab (Rappold *et al.*, 2011). Utilising this reputable model, I demonstrated neuroprotective effects of pharmacological Drp1 inhibitor mdivi-1 against PQ-induced neuropathology, attenuating the loss of dopaminergic neurons in the SNpc and of dopaminergic striatal terminals.

Validation of the Drp1 reduction in the global heterozygous Drp1 knockout ($Drp1^{+/-}$) mice robustly confirmed the reduced expression of the gene and reduced protein levels, whilst verifying the absence of phenotypic differences between $Drp1^{+/-}$ mice and controls. The progression of work utilising the model to investigate neuroprotective effects of reduced Drp1 levels however was limited by difficulties recapitulating the PQ-induced neuropathology in *$Drp1^{+/-}$* mice and their *$Drp1^{+/+}$* littermate controls. The absence of PQ-induced neuropathology prevented the investigation of any potential neuroprotective effects conferred by the reduction in Drp1 levels. The PQ *in vivo* model was then limited by my failure to identify the factor behind this differential reaction to an established PQ treatment paradigm, which could be related to PQ uptake into the brain. This inability to reproduce the previously established PQ-induced neuropathology could not be remedied for future work in this mouse model within the time constraints of the study.

Another limitation of this work was the reliance on immunohistochemical methods for assessment of cellular changes, which in this work prevented the identification of significant changes in Drp1-616 puncta and HNE signal. The sole use of immunohistochemistry meant that quantitative data was produced via image analysis; this is limiting as the threshold established may exclude or include irrelevant signal, which can lead to increased variation within experimental groups. Both Drp1-

616 and HNE data suggest trends of change in the PQ groups, however due to large SEM the variation failed to reach statistical significance which limits the conclusions that can be drawn from the data.

The apparent gender disparity within the model must be considered in future work utilising PQ to recapitulate PD features, as lack of consideration of gender differences could obscure significant gender-specific effects of the agrochemical. Gender-disparity is also apparent in the clinical manifestation of PD, with men displaying an increased risk of developing the disease (Cerri, Mus and Blandini, 2019), so the finding that this variance was reproduced in systemic changes of the mice may be important to understand the apparent protection in females.

The PQ *in vivo* study provided valuable understanding of the neuroprotective impact of pharmacological inhibition of mitochondrial fission in a PQ PD model, however the precise mechanisms by which mdivi-1 confers protection against this neuropathology was not further studied. Due to the difficulties with the global heterozygous Drp1 knockout mice, the effect of genetically manipulating mitochondrial fission via Drp1 reduction remains undetermined. Confirming the specificity of mdivi-1s effect via genetic targeting of Drp1 is an important future step, as pharmacological interventions carry the risk of unanticipated off-target effects, so the robustness of the mdivi-1 interaction with Drp1 may be further validated by confirming the effects with genetic interventions.

The primary limitation of the *in vitro* portion of the thesis was the time constraints, as this work was the final portion of the project, designed to investigate cellular mechanisms by which PQ may induce neurotoxicity and Drp1 inhibition may confer protection, beyond the established modulation of mitochondrial fission. Had

more time been available to continue lab work, further investigation of other cellular mechanisms which may interplay with autophagy and mitochondrial function could have been completed. Examination to determine how PQ altered autophagy flux and the subsequent abrogation of this effect by Drp1 inhibition in this model would also be beneficial to our understanding of how PD risk factors impact autophagy; in the α-synuclein model our lab found that Drp1 knockdown inhibited mTOR activity to promote autophagy flux (Fan *et al.*, 2019), however this has not been investigated in the PQ model.

A limitation of the entire study of this thesis was the reliance on immunohistochemical methods and image analysis; whilst these are useful assays and can allow for the maximal use of tissue samples from animal experiments, the data is never fully quantitative as it relies on the determination of analysis thresholds by the experimenter. Inclusion of additional quantitative methods, such as investigation of gene expression changes with qPCR, protein levels with immunoblotting and functional assays, such as oxygen consumption rate to assess mitochondrial function or live cell imaging of cells with fluorescently-tagged autophagy proteins to assess active autophagy, could increase the robustness of the findings.

5.4| Potential future work for the field

Further research into the potential of Drp1 inhibition as a therapeutic target for Parkinson's treatment should consider careful characterisation of the animal model used, investigation of multiple Drp1 inhibition methods and caveats to the monitoring of experimental outcomes.

The field of PD research currently lacks a clear, reproducible, relevant model of the disease. Whilst MPTP is a common chemical utilised to model PD pathology *in*

vivo, it lacks environmental relevance as, along with the 6-ODHA model, it is purely a tool for laboratory investigation and is not considered to be a tangible risk factor for disease development. There are a range of mutant *SNCA* transgenic rodent models, however the mutation of this gene is rare in patients (Douglas, Lewthwaite and Nicholl, 2007). Genetic models of PD which utilise modification of the *PARK* genes represent a very small proportion of actual PD cases (Douglas, Lewthwaite and Nicholl, 2007) and often lack the dopaminergic neuron death seen in PD patients (Chesselet, 2008; Fleming, Fernagut and Chesselet, 2005). Environmental risk factors contribute the greatest risk to disease development, though no single risk factor alone causes PD, and it is believed to result from the cumulative effects of toxic insults, which may conjoin with genetically pre-disposed risk. Whilst a number of environmental chemical models of PD exist, as discussed in Chapter 1.4, very few treatment paradigms are utilised by more than one lab, which raises concerns surrounding the reproducibility of neuropathology outside of the laboratory in which the model was developed. Newer work which utilises a combined approach to model PD, using co-administration of chemical risk factors or investigation of gene-environment interplay hold promise for future work. Co-administration of some environmental risk factors with dietary factors established enhanced PD risk. In a 2018 paper (Anselmi *et al.*, 2018), treatment of male Sprague-Dawley rats with PQ and lectin via gastric gavage reproduced behavioural and pathological changes characteristic of PD. After this treatment, α-synuclein was detected in the dorsal motor nucleus of the vagus and the SNpc, which corresponded with a significant reduction of nigrostriatal dopaminergic neurons. This treatment paradigm was shown to reproduce ascending vago-nigral α-synuclein pathology, supporting the Braak staging hypothesis (Braak *et al.*, 2003); autonomic dysfunction manifesting as reduced gastric motility and delayed gastric emptying is a

common prodromal feature of PD, resulting from enteric nervous system dysfunction, which precedes nigrostriatal degeneration and the onset of motor symptoms. Another recent study utilised subcutaneous osmotic minipump delivery of PQ (2.5mg/kg/day) to male Wistar rats, causing progressive development of pathologic PD features, including nigrostriatal degeneration, reduced motor performance and loss of striatal dopamine (Cristovao *et al.*, 2020). Increased microglial activation, reactive oxygen species and sustained α-synucleinopathy accompanied the nigrostriatal changes. As an improved model that recapitulates cardinal features of the disease, this holds promise for future experimental investigation of PQ and potential investigation of therapeutic methods. This model also benefits from the absence of systemic toxicity, which causes weight loss and premature mortality in some *in vivo* PQ models, including ours. PD research needs more representative, reproducible animal models in which the disease can be studied, and the development of improved models that recapitulate the functional and neuropathological changes of the disease in a progressive, degenerative manner is essential to the success of future pre-clinical research into potential therapeutic interventions.

Whilst my study utilised a single pharmacological inhibitor of Drp1, alongside genetic methods of functional manipulation, a number of other small molecules have been developed which target Drp1 and may therefore be efficacious in reducing toxic neuropathology.

Mdivi-1 has been investigated in almost 300 publications (Pubmed search, 07/10/2020) since its discovery by Cassidy-Stone et al (2008), with efficacy shown in multiple neurodegeneration models, including a variety of PD paradigms. In 2014 our lab published work demonstrating the effectiveness of mdivi-1 in both PINK1 knockout mice and the MPTP mouse model (Rappold *et al.*, 2014). In 2017, mdivi-1 was shown

to be effective at inducing neuroprotective effects in a mutant α-synuclein A53T rat model, including significantly reducing the A53T-increased Ser129 phosphorylated and proteinase K resistant α-synuclein, despite no change in the overall levels of the protein. Mdivi-1 also protected against the induction of abnormal mitochondrial morphology and synaptosomal dysfunction (Bido *et al.*, 2017). Although Bordt et al (2017) queried the association of mdivi-1 with Drp1, it relied heavily on Seahorse Bioanalyser data and has since been disputed by a number of publications, including a recent paper which utilised a wide range of techniques to support the conclusion that mdivi-1 is a direct inhibitor of Drp1 (Manczak *et al.*, 2019). An extensive review of mdivi-1 literature from 2017 found that the majority of papers agree with the proposed function of mdivi-1, used lower concentration of mdivi-1 for experiments than Bordt et al (2017), and utilised mdivi-1 from the same commercial sources diluted in DMSO as recommended. There remains dispute regarding the ability of mdivi-1 to inhibit Drp1 GTPase activity, as Bordt et al (2017) only confirmed the finding from Cassidy-Stone et al (2008) regarding its inhibition of yeast Dnm1, whilst failing to confirm its inhibitory capacity in mammalian Drp1. However, Numanate et al (2014) and Manczak et al (2019) both report confirmation of mdivi-1's inhibitory impact on Drp1 GTPase activity; thus further investigation should be undertaken to confirm this effect in human Drp1.

Qi et al (2013) designed the novel peptide-based Drp1 inhibitor P110, which impedes Drp1 GTPase activity to block the interaction of Drp1 with Fis1, a mitochondrial outer membrane fission interactor protein involved in Drp1 recruitment to the organelle. *In vitro*, P110 demonstrated neuroprotection by inhibiting mitochondrial fragmentation and ROS production, with subsequent improvement of mitochondrial membrane potential and mitochondrial integrity. P110 increased neuronal cell viability by reducing apoptotic and autophagic cell death, whilst reducing

neurite loss of primary dopaminergic neurons in which it was also investigated. Under basal conditions, P110 was shown to exert minimal effects on mitochondrial fission and cell viability, however it was also confirmed to require the presence of Drp1 to exert protective effects under oxidative stress conditions, confirming its specificity to Drp1 interactions. P110 reduced Drp1 GTPase activity by approximately 50%, which is close to the activity reduction demonstrated by expression of the dominant-negative $Drp1^{K38A}$ (Qi *et al.*, 2013). P110 was shown not to influence any other mitochondrial dynamic proteins and was also shown to inhibit Drp1 translocation to mitochondria and mitochondrial fission in SH-SY5Y cells subjected to a range of mitochondrial stressors, including MPP+, CCCP, rotenone and H_2O_2, correcting mitochondrial morphology and dysfunction by suppressing ROS production. P110 has relevance to our investigation of inhibiting Drp1 function in autophagy, as it was shown to reduce LC3-positive puncta and cleavage of LC3-I to LC3-II, suggesting inhibition of excessive autophagy which can result from excessive fission. Modulation of Drp1 by P110 provided promising results in a rotenone-induced rat model of olfactory bulb alteration (Zhang *et al.*, 2020). In this model subcutaneous injection of rotenone induced motor dysfunction, along with dopaminergic degeneration in the olfactory bulb and the striatum of male Sprague Dawley rats after three weeks daily treatment [1mg/kg/daily] (Zhang *et al.*, 2020). As anosmia is a prodromal non-motor symptom in PD, the olfactory bulb has been proposed as a potential port of entry for environmental risk factors, evidenced by the presence of disease-related protein aggregates and disturbed olfactory information processing. Alongside dopaminergic degeneration, rotenone induced increased nuclear translocation of $NF_\kappa B$ and expression of NLRP3 inflammasome components, accompanied by microglial activation and astrocyte activation in the olfactory bulb. P110 treatment abolished these effects, suggesting

that Drp1-dependent mitochondrial fission induces nuclear $NF_{\kappa}B$ translocation and NLRP3 inflammasome activation that may contribute to olfactory bulb disturbances. NLRP3 inflammasome activation cascades were linked to mitochondrial impairment in microglia by Sarkar et al (2017b) who found that inhibition of inflammasome activation failed to impact mitochondrial deficits, indicative that mitochondrial impairment may be upstream of inflammasome activation. Similar to my findings with the intraperitoneal rotenone paradigm, subcutaneous rotenone delivery resulted in significant reduction of rat body weight through the treatment period compared to the controls (Zhang *et al.*, 2020). P110 is a promising option for future treatments, as it is a bioavailable compound of a Drp1-derived peptide sequence conjugated to TAT, which has been shown to pass through the blood brain barrier and quickly enter cells (Bright *et al.*, 2004; Qi *et al.*, 2008). As delivery of neuroactive agents to the brain, due to difficulties crossing the protective blood brain barrier, is an obstacle in therapeutic development, the production of this permeant potential drug is a promising step forward. P110 was also effective in an MPTP mouse model, in which MPTP administration caused Drp1 activation and mitochondrial translocation *in vivo*, accompanied by loss of nigrostriatal dopaminergic neurons and terminals, and behavioural deficits. P110 treatment blocked Drp1 activation and subsequent mitochondrial translocation, however it was unable to reduce the neuroinflammatory changes, which included microglial activation and astrogliosis. P110 also modulated apoptotic protein levels, as MPTP-treatment increased p53, BAX and PUMA, and this effect was diminished by inhibition of Drp1 mitochondrial translocation, indicative that it may protect against pathology via inhibition of the p53-apoptotic pathways in neurons (Filichia *et al.*, 2016).

Dynasore is a non-competitive GTPase inhibitor which has been shown to be effective at inhibiting the function of Drp1, dynamin 1 and dynamin 2 (Macia *et al.*,

2006). In cultured hippocampal neurons, Dynasore completely and reversibly inhibited endocytosis (Newton, Kirchhausen and Murthy, 2006) and in a rat model of spinal cord injury it was shown to ameliorate motor dysfunction, reduce Drp1 expression and the initiation of apoptosis (Li *et al.*, 2016). Although results are promising, there has not yet been research into the efficacy of Dynasore in models of neurodegeneration.

DDQ is the final Drp1 targeted molecule currently described: it reduces Drp1 levels and interferes with Drp1 interaction with abnormal proteins, such as β-amyloid. In SH-SY5Y cells with Aβ, DDQ inhibited the interaction between Drp1 and Aβ, enhancing mitochondrial fusion, increasing synaptic proteins and reducing mitochondrial fission proteins (Kuruva *et al.*, 2017). The promise of multiple small molecule Drp1 inhibitors provide a collection of chemicals for use in future studies.

The neurorestorative effects of targeting mitochondrial fission, whether with mdivi-1 or other small molecules, remains undetermined to date and should be an important objective of future work. Whilst neuroprotective studies are so far promising, the neurorestorative potential of treatments is more valuable to understand the potential future application to the treatment of human disease.

There are a few considerations for targeting Drp1 therapeutically in disease, including the physiological dynamics of Drp1 levels and the presence of tissue-specific isoforms which may require precise targeting to confer health advantages. Drp1 levels flux up and down during normal mitochondrial dynamics in a healthy mitochondrial life cycle (Oliver and Reddy, 2019), so expression alone is an insufficient indicator of functional levels. Post-translational modifications of Drp1 can both up- and down-regulate activity, so investigation of these changes relies on specific targeting of the

modified residue to understand the function of the modification. Complete knockout of Drp1 is lethal (Ishihara *et al.*, 2009; Wakabayashi *et al.*, 2009) however partial reduction appears to be protective; we do not yet understand whether there is a minimum threshold of Drp1 required for function and at what point reduction of Drp1 beyond this becomes insufficient for its physiological role. We need further research to understand how PD risk factors may result in upregulation of Drp1 activity, as this understanding is important for our understanding of the implications of therapeutically targeting the protein.

5.5| Conclusion

In summary, this study provided the first evidence that PQ-induced changes in PD may relate to the induction of autophagy blockade, which can be attenuated by Drp1 inhibition. Furthermore, the combination of toxic insult by PQ administration and α-synuclein expression induced additional changes which were not abrogated by reduced Drp1 function in tfLC3 HeLa cells. Additional work to elucidate the finer mechanisms of Drp1s role in autophagy regulation, and to optimise the pesticide-based animal models of PD to further explore these effects *in vivo*, can advance our understanding of PD pathology and provide new therapeutic targets. This will facilitate the advancement of our understanding of the underlying mechanisms of PD, which may lead to their targeting to combat the progression and presentation of PD in patients.

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