The regulation and function of the Y chromosome gene, *SRY*, in experimental and clinical Parkinson's disease

Paulo Pinares-Garcia

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Abstract

Parkinson's disease (PD) is a debilitating neurodegenerative disorder caused by the progressive loss of dopamine neurons in the substantia nigra pars compacta (SNc). Whilst the cause of dopamine cell loss in PD remains unresolved, the male-sex is a prominent risk factor for PD. Men are twice as likely to develop PD, and the disease progresses more rapidly in men than women. Aside from the influences of sex hormones, emerging evidence indicates that sex-chromosome genes also contribute to the inherent male bias in PD. Indeed, the Y-chromosome gene, *SRY*, co-localises with DA neurons in the male brain, where it regulates dopamine biosynthesis and consequently motor function. Moreover, *Sry* mRNA expression is abnormally elevated in a human male cell culture model of PD, suggesting a role for SRY in the pathogenesis of male PD. Thus, to better understand the contribution of SRY in male susceptibility to PD, this thesis sought to investigate the regulation and function of SRY in experimental and clinical PD.

Chapter 2 examined the regulation and function of SRY in healthy male rats and in acute toxin-induced models of PD in male rats. In healthy male rats, reducing nigral *Sry* expression, via repeated *Sry* antisense oligonucleotide (ASO) infusion, transiently reduced nigrostriatal dopamine biosynthesis and consequently motor function. On the other hand, nigral *Sry* expression was highly elevated in acute 6-OHDA and rotenone-induced rat models of PD. Moreover, lowering nigral Sry expression with *Sry* ASO-infusion in male rats diminished motor deficits and nigral dopamine cell loss in 6-OHDA or rotenone-induced rat models of PD. The protective effect of the *Sry* ASO-infusion was associated with male-specific attenuation of DNA damage, mitochondrial degradation and neuroinflammation.

Studies in chapter 3 extended the findings from the acute PD models in chapter 2, by assessing the regulation and function of SRY in the chronic rotenone model of PD - which closely resembles the progressive behavioural decline and pathology of clinical PD. Notably, repeated intraperitoneal rotenone administration induced slow, progressive decline in motor function and nigral DA cells over 12 weeks, which was associated nigral *Sry* mRNA up-regulation prior to DA cell loss. Moreover, bilateral nigral *Sry* ASO-infusion at 4 weeks following the rotenone treatment diminished the progression of rotenone-induced motor deficits and dopamine cell loss, which was associated with suppression of key pathogenic

mechanisms. Taken together, studies in chapter 3 demonstrate that *Sry* ASO-infusion exerts male-specific neuroprotective effects at a clinically relevant time-point of neurodegeneration, highlighting the translational potential of SRY inhibition in male PD.

Chapter 4 explored the clinical relevance of the preclinical studies in chapters 2 and 3 by examining SRY protein expression in post-mortem SNc sections from male PD, LBD and age-matched controls. Here, I demonstrated that SRY-positive dopamine neurons are preferentially lost in male PD patients, and surprisingly in male LBD patients, compared to age-matched male controls. The results from chapter 4 provide important clinical evidence to support the notion that SRY contributes to the vulnerability of male dopamine neurons to injury.

Overall, my thesis has provided novel preclinical and clinical findings which indicate that dysregulation of the male-only gene, *SRY*, may underlie the predisposition of male to PD. Future studies that elucidate the temporal and spatial profile of SRY in the male brain and identify the mechanism(s) underlying the detrimental effect of SRY dysregulation will be vital in understanding sex-specific causes of PD, and establish SRY inhibition as a novel male-specific neuroprotective strategy for PD.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature:

Name: Paulo Pinares-Garcia

Date: 10th September 2018

Publications during enrolment

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Conference proceedings and presentations

Pre-clinical testing of nigral SRY inhibition in the chronic rotenone rat model of Parkinson's disease, *P Pinares-Garcia, H Loke, D Thyagarajan, V Harley, J Lee*, 37th Annual Scientific Meeting of the Australasian Neuroscience Society, 2017. (Poster).

Regulation and Function of the Y-chromosome gene SRY in acute and progressive experimental animal models of Parkinson's disease, *P Pinares-Garcia, H Loke, D Thyagarajan, V Harley, J Lee,* University of California, Los Angeles, 2016. (Oral)

Regulation and Function of the Y-chromosome gene SRY in acute and progressive experimental animal models of Parkinson's disease, *P Pinares-Garcia, H Loke, D Thyagarajan, V Harley, J Lee,* Annual Meeting for the Society for Neuroscience, 2016. (Poster)

A Genetic basis for the male susceptibility to Parkinson's disease, *J Lee, P Pinares-Garcia, D Czech, H Loke, S Ham, V Harley,* 25th ISN-APSN- ANS meeting, 2015. (Poster)

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SRY: A Genetic basis for male susceptibility to Parkinson's disease, *P Pinares-Garcia, H Loke, S Ham, E Vilain, V Harley, J Lee,* BiomedLink Symposium, 2015. (poster)

A Role for SRY in the Healthy and Injured Male Dopamine Pathway: Implication for Male Susceptibility to Parkinson's Disease, *P Pinares-Garcia, H Loke, D Czech, V Harley, J Lee*, Students of Brain Research (SOBR) Student Symposium, 2015. (poster)

SRY: A male-specific target for Parkinson's Disease, *P Pinares-Garcia, D Czech, H Loke, V Harley, J Lee,* Anatomy & Developmental Biology Student Symposium, 2014. (Poster)

A role for the testis-determining gene SRY in the male susceptibility to Parkinson's Disease, *P Pinares-Garcia, H Loke, D Czech, V Harley, J Lee*, MIMR-PHI Student Symposium, 2014. (Poster)

A Role for SRY in the Healthy and Injured Male Dopamine Pathway: Implication for Male Susceptibility to Parkinson's Disease, *P Pinares-Garcia, H Loke, D Czech, V Harley, J Lee, BiomedLink* Symposium, 2014. (poster; **won best poster presentation, Neuroscience category**)

A Role for SRY in the Healthy and Injured Male Dopamine Pathway, *P Pinares-Garcia, J Lee, J Correia, A Russ, D Czech, V Harley,* Prince Henry's Institute Student Symposium, 2013. (poster; **won best written abstract, honours category**)

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Dysregulation of SRY in the male brain: a genetic basis for sex-biased neurological disorders, H Loke, **P Pinares-Garcia**, D Czech, V Harley, J Lee, 25th ISN-APSN Biennial meeting (in conjunction with ANS), 2015. (Poster)

Dysregulation of SRY in the Male Brain: A Genetic Basis for Male-Biased Neurological Disorders, H Loke, **P Pinares-Garcia**, D Czech, V Harley, J Lee, ASMR Victorian Student Research Symposium, 2015. (Poster)

Awards and prizes

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Abbreviations

6-OHDA	6-hydroxydopamine
^{o}C	degrees Celsius
µg	microgram
µl	microlitres
µM	micromolar
AAV	adeno-associated virus
aCSF	artificial cerebrospinal fluid
ADHD	attention deficit hyperactivity disorder
ASD	autism spectrum disorder
ASO	antisense oligonucleotide
BAC	bacterial artificial chromosome
bp	base pair
CALB-1	calbindin-1
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CMA	chaperone mediated autophagy
COMT	catechol-O-methyl transferase
D1R	D1 receptor
D2R	D2 receptor
DA	dopamine
DAB	3, 3 -diaminobenzidine
DAPI	4', 6'-diamidino-2-phenyldole
DAT	dopamine transporter
DBH	dopamine beta-hydroxylase
DBS	deep brain stimulation
DDC	dopa decarboxylase
DJ-1	Daisuke-Junko 1
DNA	deoxyribonucleic acid
DOPAC	3',4'-dhiydroxyphenylacetic acid
dpc	*days post coitum*
DRD1	dopamine receptor D1
DRD2	dopamine receptor D2
dsDNA	double stranded DNA
ESC	embryonic stem cell
GABA	gamma-aminobutyric acid
GAD	glutamate decarboxylase
GAD65	glutamate decarboxylase 65
GAD67	glutamate decarboxylase 67
GADD45γ	growth arrest and DNA-damage-inducible protein gamma
GDNF	glial cell line-derived neurotrophic factor
GPi	globus pallidus interna

GSH	glutathione
h	hours
H&E	hemaotoxylin and eosin
hESC	human embryonic stem cell
hiPSC	human induced pluripotent stem cell
HMG	high mobility group
HRP	horseradish peroxidase
HSC70	heat shock cognate protein 70
HVA	homovanillic acid
ICV	intracerebroventricular
Il-1β	interleukin-1 beta
Il-10	interleukin -10
iNOS	inducible nitric oxide synthase
iPSC	induced pluripotent stem cell
kb	kilobase
LAMP-2A	lysosomal associated membrane protein 2A
LB	Lewy body
LBD	Lewy body disease
L-DOPA	L-3,4-dhihydroxyphenylalanine
LIDS	levodopa induced dyskinesias
LN	Lewy neurites
LRRK2	leucine-rich repeat kinase 2
M	molar
MAO-A	monoamine oxidase A
MAO-B	monoamine oxidase B
ml	millilitre
mM	millimole
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
NO	nitric oxide
OFT	open field test
ODN	oligodeoxynucleotide
OMM	outer mitochondrial membrane
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PET	positron emission tomography
PFC	prefrontal cortex
Pgc1α	peroxisome proliferator-activated receptor gamma coactivator 1
PINK1	PTEN-induced kinase 1
PUMA	p53 up-regulated modulator of apoptosis
qRT-PCR	quantitative reverse transcriptase PCR
RNA	ribonucleic acid
ROS	reactive oxygen species

RBD	rapid eye movement behaviour disorder
RT	room temperature
RT-PCR	reverse transcriptase PCR
SEM	standard error of the mean
siRNA	small interfering RNA
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulate
STN	subthalamic nucleus
SO	sense oligonucleotide
SOD1	superoxide dismutase 1
SOD2	superoxide dismutase 2
SOX	SRY-related HMG box
SOX3	SRY-related HMG box 3
SOX6	SRY-related HMG box 6
SOX9	SRY-related HMG box 9
SOX10	SRY-related HMG box 10
SPECT	Single-photon emission computed tomography
SRY	sex-determining region on the Y chromosome
TBP	TATA-box-binding protein
TH	tyrosine hydroxylase
TNFα	tumour necrosis factor alpha
TOM-20	translocase of outer membrane-20
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VMAT	vesicular monoamine transporter
VAMP	vesicle-associated membrane protein
VTA	ventral tegmental area

Chapter 1:
Literature review

1.1 Parkinson's disease (PD) is a debilitating neurodegenerative movement disorder

1.1.1 Prevalence of PD

PD is a second most common neurodegenerative disorder after dementia, which affects over 7 million people worldwide. The prevalence of PD is 2% of people over the age of 60, increasing to 4% over the age of 80, clearly highlights aging as a significant risk factor. Alongside the significant burden on patients and families, PD has a significant economic impact on society (Noyes et al., 2006, Huse et al., 2005); the US population with PD incurred medical expenses of approximately $14 billion in 2010 ($8.1 billion higher than expected for a similar population without PD) and is expected to double by 2040 (Kowal et al., 2013). In Australia, the total health system cost of PD was estimated to be approximately $570 million in 2014, an increase of $220 million from 2005 (Deloitte, 2015).

1.1.2 Motor symptoms of PD

PD is a debilitating movement disorder, characterized by hallmark motor symptoms that include resting tremors, rigidity (akinesia), slowness of movement (bradykinesia), and postural instability (Lang and Lozano, 1998). Other secondary motor symptoms involve gait and posture changes resulting in a shuffling, forwards gait (festination), decreased facial expressions, and difficulty in speech and chewing (Jankovic, 2008).

1.1.3 Non-motor symptoms of PD

PD is also associated with non-motor symptoms such as olfactory dysfunction, gastrointestinal dysfunction, sleep disturbances, rapid eye movement sleep behaviour disorder (RBD), depression, cognitive decline and dementia (Chaudhuri

et al., 2006). RBD, olfactory dysfunction, constipation and depression can develop more than 10 years prior to the onset of PD motor symptoms (Chaudhuri et al., 2006, Bohnen et al., 2008, Shiba et al., 2000, Abbott et al., 2001). Braak et al. (2003) have described a neuropathological staging which involves the staged loss of brainstem, olfactory, midbrain and cortical neurons, and associated Lewy body pathology in these regions. The neuropathological spread closely correlates with development of non-motor symptoms of PD, which may explain why non-motor symptoms of PD such as gastrointestinal and olfactory dysfunction can appear prior to dopamine (DA) cell loss-associated motor symptoms (Berg et al., 2015, Mahlknecht et al., 2015). These non- motor symptoms therefore have the potential for use as screening biomarkers to diagnose preclinical cases of PD.
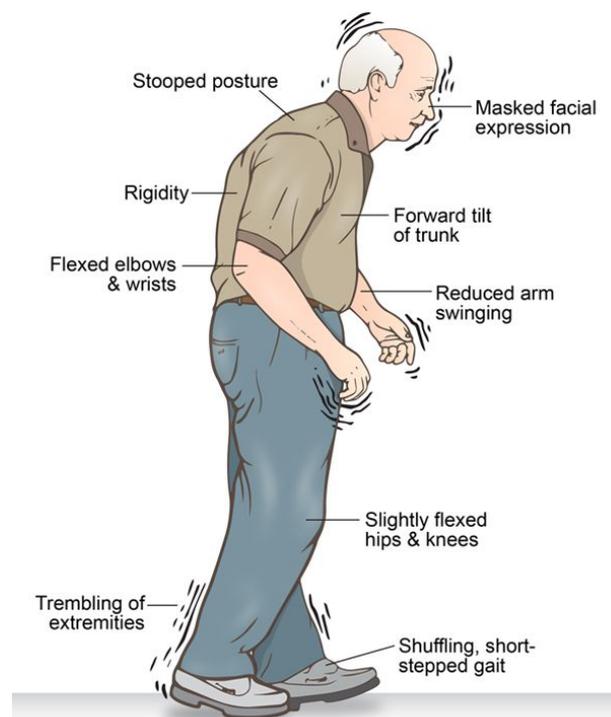
Figure 1.1. Cardinal motor symptoms of PD include resting tremors, rigidity (akinesia), slowness of movement (bradykinesia), shuffling gait and postural instability. UWorld (2017) *Typical appearance of Parkinson's disease* [jpeg]. Retrieved from https://tinyurl.com/yby5fkyc.

Cognitive impairment and dementia are increasingly associated with cortical degeneration during the late stages of PD. Cognitive decline is associated with increased cortical density of Lewy bodies and neurites in the hippocampus and amygdala of PD patients (Churchyard and Lees, 1997, Harding et al., 2002). Dementia is prevalent in up to 40% of the PD population over 65 years, six times greater than in healthy individuals (Emre, 2003) but in other studies has been reported to be as high as 83% in late stage (>20yrs since onset) PD patients (Hely et al., 2008).

1.2 Pathophysiology of PD

1.2.1 Symptoms of PD result from the loss of midbrain dopamine neurons

Motor symptoms of PD are associated with the progressive selective loss of DA neurons in the substantia nigra pars compacta (SNc) that project to the striatum, which is known as the nigrostriatal pathway (Fig. 1.2) (Fahn and Sulzer, 2004). Dopamine acts as a modulator of striatal tone on output of basal ganglia to the thalamus, which in turn projects to the motor cortex. The basal ganglia are a group of nuclei that are integrated in a feedback loop, via two opposing direct (excitatory) and indirect (inhibitory) pathways. These two pathways regulate total inhibitory GABAergic output to the thalamus. In normal conditions, DA release in the striatum excites striatal neurons via the direct pathway and inhibits striatal neurons via the indirect pathway, which reduces inhibitory input into the thalamus, and leads to increased motor activity. Conversely, progressive DA neuron degeneration in the SNc (Fig. 1.3) leads to a deficiency of DA. DA deficiency reduces the excitatory output of the direct pathway, and increases the inhibitory output of the indirect pathway, which results in the motor symptoms associated with PD (Albin et al., 1989, DeLong, 1990, Alexander et al., 1991, Calabresi et al., 2014).

The motor symptoms of PD manifest when more than 70% of DA neurons have been lost (Lloyd, 1977), suggesting that the SNc has a high degree of compensatory ability as an adaptive response to injury or depletion of DA neurons. This compensatory mechanism could occur through several mechanisms, including increased DA synthesis, transport and release by surviving neurons; increased density of presynaptic terminals, branching and neural sprouting; and forced metabolic activity and reduced reuptake, as adaptive responses to sustain the level of nigrostriatal DA levels (Lee et al., 2008, Song and Haber, 2000, Stanic et al.,

2003, Finkelstein et al., 2000). Within the SNc, specific regions appear more susceptible to cell death in PD. In particular, at the onset of PD motor symptoms, the lateral-ventral tier of the SNc is the most susceptible (90% of cells lost), followed by the medial ventral and dorsal tiers (60-70% cells lost) (Fearnley and Lees, 1991, Damier et al., 1999). This cell death is associated with striatal dopamine depletion which is most prevalent in the caudal regions of the striatum, with late stage PD patients showing greater than 98% DA depletion (Kish et al., 1988).
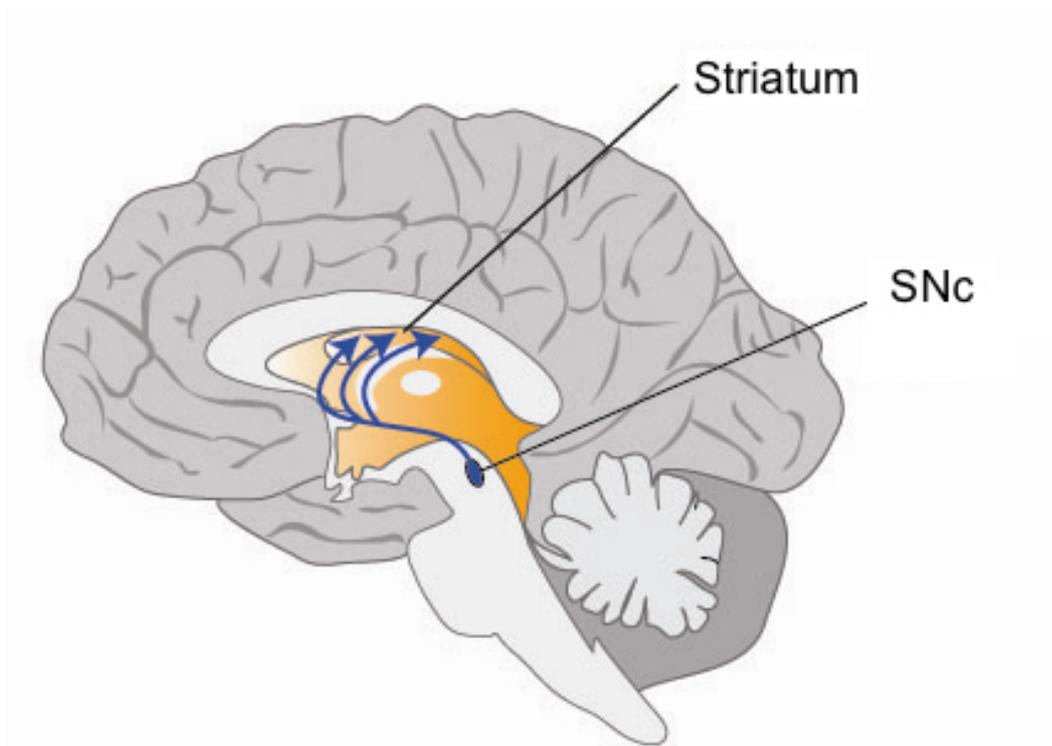
Figure 1.2. Sagittal diagram of human brain showing the nigrostriatal DA pathway (outlined in blue) which is affected in PD. Reproduced from Somayaji and Sulzer (2017)

Healthy   **PD**
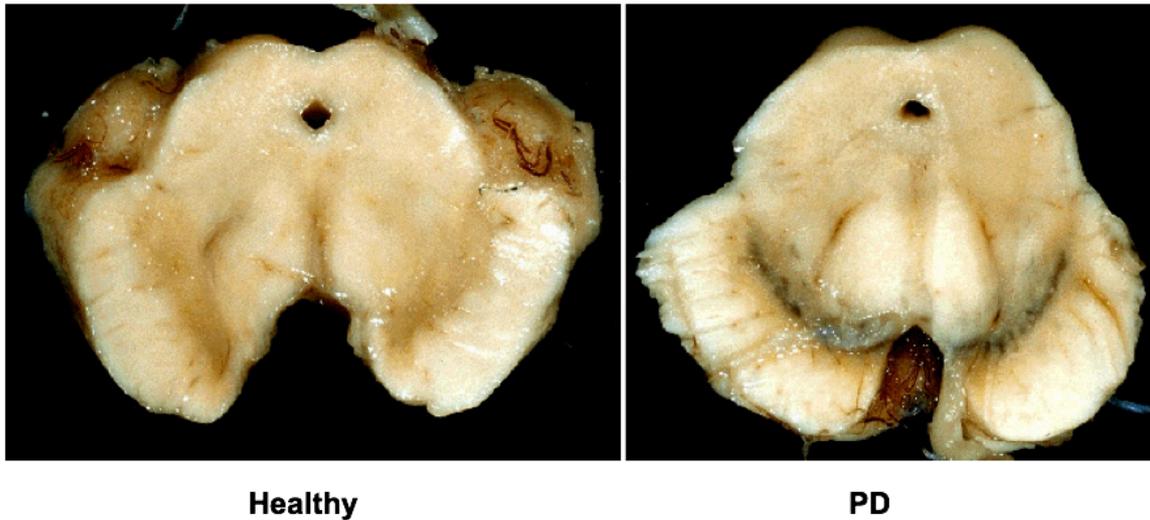
Figure 1.3. Neuromelanin-pigmented dopamine neurons are lost in human PD. DA neurons in the SNc are amongst the highest pigmented cells in the human brain, with the pigment composed of neuromelanin (Graham, 1979). The dark pigmented areas on both hemispheres in the normal brain outline the DA neurons within the SNc (left). Loss of DA neurons in PD is clearly indicated by loss of the dark pigmented areas (right).

1.2.2 Lewy bodies are a hallmark pathological feature of PD

Protein-rich inclusion bodies known as Lewy bodies are the hallmark pathology seen in both pre-symptomatic and symptomatic stages of PD (Braak et al., 2003). These inclusion bodies are primarily composed of aggregated, insoluble α-synuclein protein (Spillantini et al., 1997), and are present as threadlike Lewy neurites or granular spherical Lewy bodies (Fig. 1.4). α-synuclein is soluble in the cytoplasm, and binds with high affinity to the membranes of synaptic vesicles or to membranes rich in acidic phospholipids (Perrin et al., 2000, Davidson et al., 1998, Jo et al., 2000). Following a conformational change it self-aggregates with misfolded α-synuclein and additional proteins (e.g. ubiquitin, neurofilament protein, tau) to cause insoluble aggregations (Goedert, 2001). All PD-affected neurons eventually develop Lewy bodies and neurites over time (Braak et al., 2003).
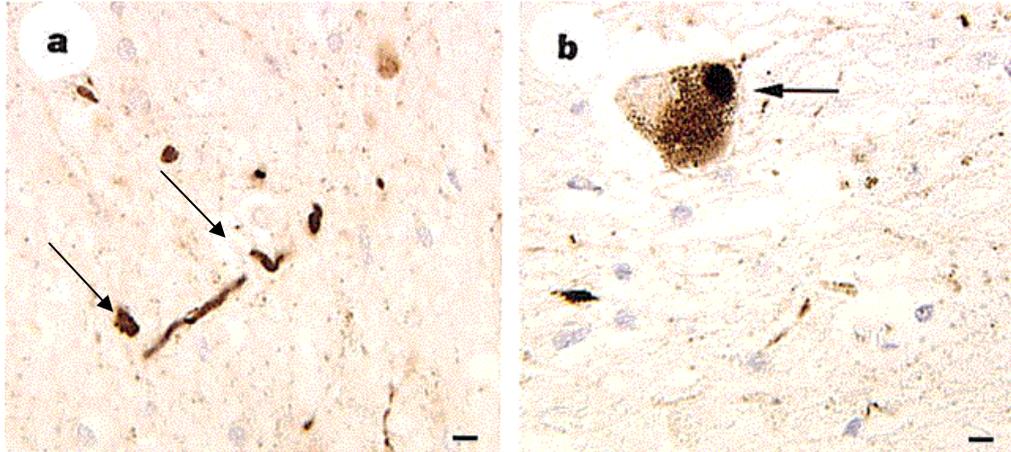
Figure 1.4. A) Lewy neurites in the SNc. **B)** Lewy body (arrow) in pigmented nerve cell of the SNc. Scale bar -10 µm. (Spillantini et al., 1997).

1.2.3 PD progresses through defined neuropathological stages

PD progresses through 6 defined neuropathological stages during the pre-symptomatic and symptomatic phases, and Lewy body and neurite pathology correlates with this progression in a predetermined pattern through these stages (Table 1.1) (Braak et al., 2003).

The first pathological changes appear within the dorsal motor nucleus of the vagal nerve and this damage spreads to the medulla oblongata, anterior olfactory nucleus, during stages 1-2. The lesions and pathology found during these stages support the findings from other studies which show olfactory dysfunction in PD patients in some cases many years prior to the onset of PD motor symptoms (Chaudhuri et al., 2006). During stages 3-4, the SNc and anterior temporal mesocortex become the site of progressive intraneuronal lesions, and during stage 4 the prevalent loss (>70%) of SNc DA neurons is apparent, which correlates with the emergence of the cardinal motor symptoms. During stages 5-6, the Lewy body and neurite pathology spreads to the neocortex, including the prefrontal, premotor

and sensory association areas- marking the largest topographical extent of neurodegenerative progression (Braak et al., 2003, Braak and Del Tredici, 2004).

Table 1.1. Neuropathological staging of PD progression

Stage	Site of progression	Pathology
1	medulla oblongata	Lesions in the dorsal IX/X motor nucleus and/or intermediate reticular zone, anterior olfactory nucleus
2	medulla oblongata and pontine tegmentum	Pathology of stage 1 plus lesions in caudal raphe nuclei, gigantocellular reticular nucleus, and coeruleus–subcoeruleus complex
3	midbrain	Pathology of stage 2 plus midbrain lesions, in particular in the SNc
4	basal prosencephalon and mesocortex	Pathology of stage 3 plus prosencephalic lesions. Cortical involvement is confined to the temporal mesocortex (transentorhinal region) and allocortex (CA2-plexus). The neocortex is unaffected.
5	neocortex	Pathology of stage 4 plus lesions in high order sensory association areas of the neocortex and prefrontal neocortex
6	neocortex	Pathology of stage 5 plus lesions in first order sensory association areas of the neocortex and premotor areas, occasionally mild changes in primary sensory areas and the primary motor field

Adapted from Braak et al. (2003).

1.3 Treatments for PD

1.3.1 Pharmacological

Currently, the hallmark symptomatic therapy for PD is levodopa (L-DOPA) a chemical synthesized from amino acid l-tyrosine, and a DA precursor (Yahr et al., 1969), which replenishes the deficiency of striatal DA caused by loss of nigral DA neurons. Carlsson et al. (1957) first showed that intraperitoneal 3,4-Dihydroxyphenylalanine (L-DOPA) infusion rescued parkinsonian-like immobility caused by reserpine in mice and rabbits. This effect was later shown to be due to the rescue against dopamine depletion caused by reserpine (Carlsson et al., 1958). In 1961, L-DOPA was first used in a clinical trial of 20 PD patients where it was injected intravenously, and showed immediate therapeutic effects (Birkmayer and Hornykiewicz, 1960).

While L-DOPA is effective in alleviating the debilitating motor symptoms of PD, complications arise with prolonged use in PD patients. L-DOPA induces the development of dyskinesias, which are abnormal involuntary movements that are increasingly debilitating over time. Levodopa-induced dyskinesias (LIDs) are prevalent in 40% of patients after 4-6 years of Levodopa treatment, depending on a number of factors including age of onset, severity of PD, and dose of L-DOPA treatment (Ahlskog and Muenter, 2001). The underlying mechanisms for L-DOPA-induced dyskinesias aren't well understood, but involve pulsatile overactivity of the striatal post-synaptic receptors. This pulsatile overactivity is likely to occur due to dysregulated dopamine release, caused by variability in delivery of L-DOPA, release and the short half-life of L-DOPA (Cenci, 2014, Jenner, 2008).

Dopamine agonists, such as ropinirole (D2 receptor agonist), pramipexole (D2 agonist) and apomorphine (D1,D2 agonist) directly activate the post-synaptic

DA receptors and are increasingly used in the early stages of PD to treat motor symptoms of PD (Fox et al., 2011). DA agonists produce less drug-induced dyskinesias than L-DOPA when used as a monotherapy or in conjunction with L-DOPA (Rascol et al., 2000, Hauser et al., 2007, Holloway et al., 2004, Stowe et al., 2008). A double–blind study comparing the occurrence of dyskinesias between PD patients treated with levodopa or ropinirole showed that after 5 years, 20% of the ropinirole treated patients developed dyskinesias, compared to 46% of the levodopa treated cohort (Rascol et al., 2000). However, DA agonists do not have the same level of symptomatic efficacy as L-DOPA treatment, and also produce non-motor side effects that include oedemas, somnolescence, dizziness and hallucinations (Stowe et al., 2008).

Selegiline and rasagiline are potent, irreversible monoamine oxidase inhibitors that maintain synaptic DA levels by blocking synaptic DA degradation (Youdim et al., 2001). These drugs inhibit monoamine oxidase A and B (MAO-A, MAO-B) that are located on the mitochondrial membrane, which catalyze the oxidative deamination of synaptically released DA (Bentué-Ferrer et al., 1996). These inhibitors are used as monotherapy and in conjunction with L-DOPA to reduce L-DOPA induced motor fluctuations (Riederer and Laux, 2011, Schapira, 2011). Studies also suggest a neuroprotective potential for monoamine oxidase inhibitors in *in vivo* models of PD, through increased levels of several neurotrophic factors (nerve growth factor, brain-derive neurotrophic factor, neurotrophin 3) (Youdim and Weinstock, 2001, Naoi and Maruyama, 2010). Rasagiline has been used in two clinical trials to assess its potential neuroprotective effects, but these results have remained inconclusive (Olanow et al., 2008).
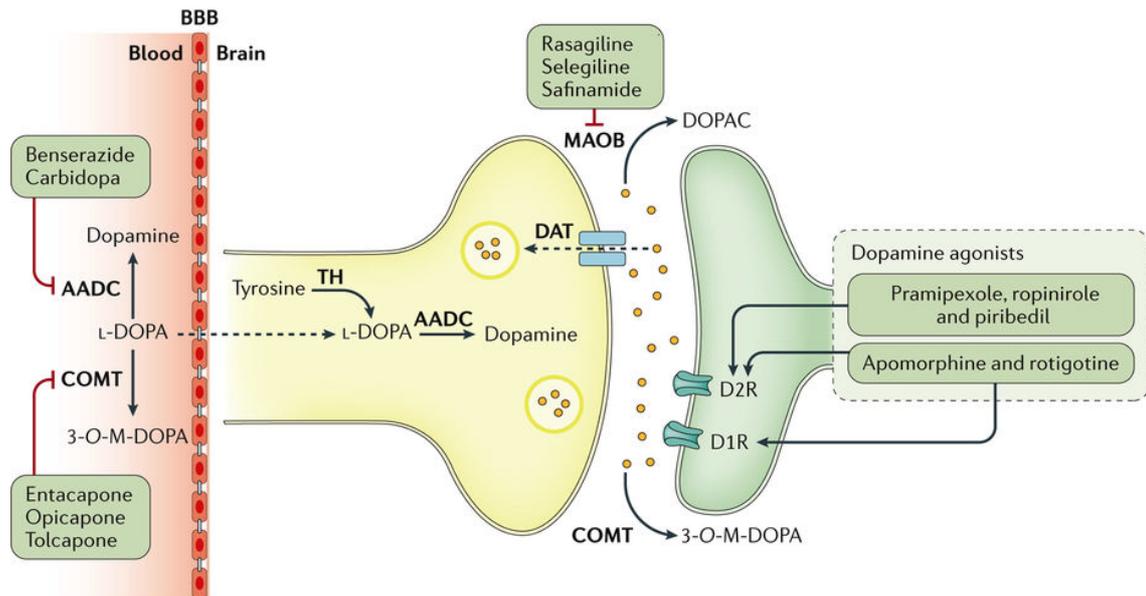
Figure 1.5. Pharmacological treatments for PD Pharmacological treatments include DA replenishment via L-DOPA, a precursor of DA, commonly combined with aromatic L-amino acid decarboxylase (AADC) or catechol-O-methyltransferase (COMT) inhibitors. MAO-B inhibitors maintain synaptic DA levels. DA agonists act on postsynaptic D1 and/or D2 receptors. Reproduced from Poewe et al. (2017).

1.3.2 Surgical

Within the last 20 years, high frequency deep brain stimulation (DBS) of the subthalamic nucleus (STN) or globus pallidus interna (GPi) by direct electrode placement has become the gold standard in surgical therapy in PD. DBS is surgically safe, and alleviates PD motor symptoms including tremor, rigidity and bradykinesia (Limousin et al., 1998, Benabid et al., 1994). In addition, DBS improves L-DOPA-induced dyskinesias in PD patients (Krack et al., 1997). Clinical studies have since shown that DBS therapy leads to marked improvements in motor function, such as a 70% improvement in rigidity and tremor, and a 50% akinesia improvement, over a 5 year period (Krack et al., 2003). Furthermore, DBS therapy provides a greater improvement in motor symptoms of PD patients when compared to patients receiving L-DOPA alone (Deuschl et al., 2006, Weaver et al., 2009).

The therapeutic mechanisms of DBS therapy remain unclear. Abnormal firing bursts, rhythmic oscillations and synchronous activity in neurons in the basal ganglia network and thalamus, particularly in the beta-band (10-30Hz) frequency, have been reported in non-human primate models of PD and in PD patients based on recorded local field potentials (Meissner et al., 2005, Levy et al., 2002, Brown et al., 2001). It is likely that the synchronised oscillations produced at the site of DBS electrode placement alter the dysfunctional firing patterns occurring in the basal ganglia network of the PD-affected brain (Hess et al., 2013). In PD patients, STN- DBS at high frequency (>70Hz) reduced pathological beta oscillations to reverse parkinsonian symptoms, while DBS at 20Hz exacerbates the synchronisation of pathological beta oscillations in the STN (Brown et al., 2004, Wingeier et al., 2006). Emerging studies suggest that the temporal pattern of DBS stimulation is highly critical for successful therapy, and modulation of these patterns could lead to increases in treatment efficacy (Hess et al., 2013, Brocker et al., 2013). Non-regular patterns of STN- DBS stimulation have been reported to relieve parkinsonian symptoms in PD patients to a greater degree than temporally regular patterns (Brocker et al., 2013).

The potential neuroprotective effect of DBS has been increasingly studied. PD is associated with glutamatergic overactivity in the STN, as a result of progressive DA cell loss (Benazzouz et al., 1993, Lozano et al., 2002). This overactivity leads to excess glutamate release onto nigral DA neurons which has an excitotoxic effect on nigral DA neurons. DBS could likely have a neuroprotective effect by alleviating STN overactivity (Rodriguez et al., 1998). In addition, STN-DBS treatment in the rat 6-OHDA model of PD improves parkinsonian symptoms, and correlated with an increase in BDNF protein levels in the SNc and motor (M1) cortex

and increase in BDNF mRNA in the SNc and GPi (Spieles-Engemann et al., 2011). BDNF is a known neurotrophic factor of DA neurons (Hyman and Hofer, 1991) and could likely play a neuroprotective role in the SNc. STN-DBS has demonstrated a putative neuroprotective effect in a progressive, A53T α-synuclein (aSyn)-overexpressing PD rat model, with STN-DBS treatment providing an increase in motor function in the single pellet reaching test and rearing behaviour. This improvement correlated with a 29% increase in tyrosine hydroxylase (TH; a DA marker and the rate limiting enzyme in the DA biosynthesis pathway) -positive SNc neurons compared to controls (Musacchio et al., 2017). STN-DBS has recently been suggested to have a neuroprotective role through the activation of BDNF-tropomyosin receptor kinase type B (trkB) signalling in the SNc in STN-DBS treated rats (Fischer et al., 2017).
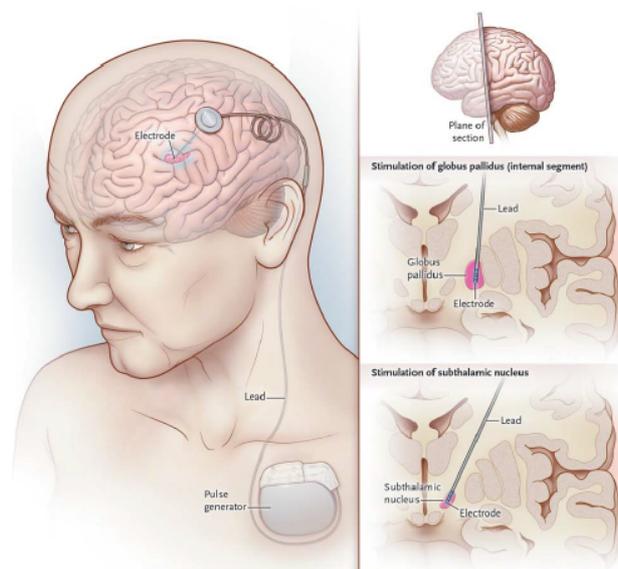
Figure 1.6. Deep brain stimulation of the subthalamic nucleus (STN) or globus pallidus interna (GPi) The DBS electrode is inserted through the skull, and implanted in the brain, with the electrode tip placed directly above the target region. An insulated wire then connects the electrode to the neurostimulator, implanted on the chest. Reproduced from Okun (2012).

1.3.3 Experimental and future therapeutic directions

Although pharmacological and surgical approaches in PD effectively treat motor symptoms, to date there is no disease modifying therapy for PD. Neuroprotective strategies that reduce or prevent DA cell loss therefore represent an unmet need in PD treatments. Hence, gene therapy and DA neuron transplantation strategies have been studied as experimental therapies in PD.

In PD, reduced inhibitory GABA input into the STN leads to abnormal STN overactivity (Miller and DeLong, 1987, Bergman et al., 1994). Adeno-associated viarl (AAV)-mediated Glutamic acid decarboxylase (GAD; catalyses synthesis of GABA) transfer into the STN improves motor function and protects against DA cell loss in 6-hydroxydopamine (6-OHDA) treated rats (Luo et al., 2002, Lee et al., 2005), and in clinical trials shows improvement of motor symptoms in late-stage PD patients compared to sham controls (Kaplitt et al., 2007, LeWitt et al., 2011).

AAV-mediated or lentiviral delivery of glial cell line- derived neurotrophic factor (GDNF), which is known to support DA neuron growth and survival, protects against DA cell loss in rat and non-human primate models of PD (Choi-Lundberg et al., 1997, Bjorklund et al., 2000, Kordower et al., 2000). Direct GDNF delivery into the putamen in human PD patients has shown improvement in motor symptoms and levodopa-induced dyskinesias (Gill et al., 2003). However, larger randomized controlled clinical trials failed to see any benefit in direct intraputamenal or intracerebroventricular (ICV) infusion of GDNF (Lang et al., 2006, Nutt et al., 2003). A lack of therapeutic benefit in these previous studies has been suggested to be due to poor delivery methods, and inability of ICV delivery to reach the target nigrostriatal area (Nutt et al., 2003)

Advances in stem cell technologies have facilitated cell transplantation into the nigrostriatal pathway as a potential strategy to restore nigral DA neuron populations. These include fetal mesencephalic stem cells, embryonic stem cells (ESCs), or induced pluripotent stem cells (iPSCs) (Kim and De Vellis, 2009, Trounson and McDonald, 2015). These emerging experimental therapies will be discussed below.

Pre-clinical studies revealed that striatal transplantation of rat or human embryonic ventral mesencephalic tissue improves motor symptoms in multiple rat and primate models of PD (Perlow et al., 1979, Dunnett et al., 1981, Bjorklund et al., 1980). In humans, transplantation of human fetal ventral mesencephalic tissue into the SNc of PD patients showed clinical benefit in PD motor symptoms and long-term survival of grafts for up to 24 years (Kefalopoulou et al., 2014, Hallett et al., 2014, Li et al., 2016). Despite the therapeutic effects achieved in these trials, others have reported minimal clinical benefit and emergence of off-medication, graft-induced dyskinesias (GIDs) in up to 50% of all patients (Freed et al., 2001, Olanow et al., 2003), which could be caused by the asymetric increase of DA function in the striatum (Ma et al., 2002). Asides from the high variability in clinical benefit and variability in survival, other obstacles to fetal cell transplantation as a viable approach included the large amount of tissues required (4 midbrain tissues from 6 to 9 week old human embryos for 1 PD patient; Olanow et al., 2003), and the poor survival of transplanted grafts, with autopsy findings from nigral tissue of grafted PD patients showing that less than 20% of transplanted cells survive (Hagell and Brundin, 2001).

Human embryonic stem cells (hESCs) are pluripotent, self-renewing cells isolated from pre-implantation blastocysts that can be differentiated into any cell

type, including DA neurons (Kirik et al., 2002). Successful differentiation of DA neurons requires specific *in vitro* protocols and treatment with morphogens, survival factors and growth factors to generate a high yield of DA neurons (Perrier et al., 2004, Kirik et al., 2002). Numerous pre-clinical studies have reported restoration of striatal denervation and improvements in motor function for at least 6 months following striatal transplantation of hESCs in 6-OHDA-induced rat models of PD (Ben‑Hur et al., 2004, Kriks et al., 2011, Grealish et al., 2014, Brederlau et al., 2006, Kirkeby et al., 2012). Similarly, therapeutic effects have been seen following transplantation of mouse-derived ESCs in 6-OHDA-treated rats (Kirik et al., 2002). Whilst transplantation-induced teratomas caused by residual undifferentiated ESCs were commonly seen in pre-clinical stufies (Kirik et al., 2002, Brederlau et al., 2006), the risk of teratoma formation can be minimised via cell sorting to purify DA neuron populations, and prolonging *in vitro* differentiation of ESCs (Brederlau et al., 2006, Hedlund et al., 2008). Given the advancements in the safe use of hESCs, the first open-label in-human studies are due to commence in 2019 (Barker et al., 2017).

The development of adult fibroblast-derived iPSCs (Takahashi and Yamanaka, 2006) has facilitated the generation of stem cells that offers advantages over existing stem cell sources. In particular, iPSCs are derived from a patient's own somatic cells, therefore eliminating the need for immunosuppressive therapy, and circumvents ethical limitations associated with the use of stem cells derived from human embryos. Mouse iPSC grafts transplanted into the striatum of 6-OHDA-treated rats can successfully differentiate into DA neurons, functionally integrate into the SNc, and improve 6-OHDA-induced motor deficits (Wernig et al., 2008). Promisingly, PD patient-derived iPSCs grafted into the striatum of 6-OHDA-lesioned rats have shown prominent DA cell survival and functional motor

improvement (Hargus et al., 2010). Recent pre-clinical studies revealed that human control and PD patient-derived iPSC transplantation in MPTP- lesioned primates rescued motor impairment, produced functional DA neurons, and survived without tumour formation for at least 24 months (Kikuchi et al., 2017, Hallett et al., 2015). Whilst IPSC-derived DA cell replacement strategies are a promising therapy in clinical PD, it is important to determine clinically safe differentiation protocols that minimise the generation of non-DA neurons that can cause graft-induced dyskinesias, and to refine protocols for increased reproducibility of iPSC graft populations (Kikuchi et al., 2017, Hallett et al., 2015).

In summary, current pharmacological approaches for PD include the gold standard L-DOPA therapy to replenish DA levels, and the use of DA agonists and/ or COMT, AADC, and MAO inhibitors to maintain synaptic DA levels. Moreover, current surgical treatments for PD include STN or GPi DBS therapy. Whilst these treatments effectively treat motor symptoms of PD, prolonged use come with complications such as L-DOPA induced dyskinesias. Furthermore, they fail to treat the characteristic progressive loss of DA neurons in PD. Experimental therapies to replenish DA cell populations using ESCs and iPSCs show promising results in pre-clinical animal models of PD, effectively treating motor symptoms and restoring loss of DA neuron populations. Based on the success of these pre-clinical studies, several independent groups have begun to pursue clinical trials with ESC and iPSC-derived DA neurons.

1.4 Pathogenesis mechanisms underlying DA cell loss in PD

The pathogenesis mechanisms underlying the progressive decline of nigral DA neurons and disease symptoms in PD require further study. However, to date mechanisms implicated to have a role in the pathogenesis of PD include oxidative stress, mitochondrial dysfunction, dysregulated or disrupted autophagy, neuroinflammation and DNA damage.

1.4.1 Oxidative stress

Oxidative stress refers to the imbalance between the production of reactive oxygen species (ROS) and cellular antioxidant activity to detoxify their toxic effects, through either increased ROS production or impaired antioxidant function. ROS include superoxide (O_2^{2-}), hydroxyl radial ($\cdot OH$) and hydrogen peroxide (H_2O_2). Superoxide is produced in the mitochondrial complexes I and III, which can be converted to the less toxic H_2O_2 by the mitochondrial enzyme manganese superoxide dismutase (MnSOD) (Murphy, 2009). Superoxide can react with nitric oxide (NO), which is abundant through the brain, to produce peroxynitrite ($ONOO^-$), a highly reactive oxidant that can induce lipid peroxidation (Beckman and Koppenol, 1996, Szabó et al., 2007). H_2O_2 is normally detoxified by the antioxidants catalase and glutathione (GSH), but in the presence of ferrous iron (Fe^{2+}) can also be converted through the fenton reaction into $\cdot OH$. These ROS are implicated in oxidative damage that includes DNA strand breaks, lipid peroxidation, and protein oxidation (Dias et al., 2013).

Increasing evidence suggests that oxidative stress has a significant role in the pathogenesis of PD. Post-mortem nigral tissues from PD patients show reduced levels of antioxidants such as GSH (Sofic et al., 1992, Dexter et al., 1989), increased

levels of lipid peroxidation markers such as 4-HNE (4-hydroxynonenal), lipid hydroperoxide and MDA (malondialdehyde) (Dexter et al., 1989, Dexter et al., 1994, Yoritaka et al., 1996), markers of oxidative damage to protein (protein carbonyls) and DNA (8-hydroxy-2-deoxyguanosine) (Alam et al., 1997a, Alam et al., 1997b), and increased iron content compared to controls. Increased iron in the SNc contributes to the exacerbation of oxidative damage via the Fenton reaction, where ferrous iron interacts with H_2O_2 to generate hydroxyl radicals (Sofic et al., 1988, Oakley et al., 2007).

Nigral DA neurons may be particularly vulnerable to oxidative stress due to the production of ROS during normal DA metabolism. The metabolism of DA via MAO-B into 3,4-dihydroxyphenylacetic acid (DOPAC) produces H_2O_2 as a byproduct (Cohen, 1987). DA is also capable, through spontaneous auto-oxidation or through the enzyme prostaglandin H synthase, of forming highly reactive DA quinones (Hastings, 1995). DA quinone is capable of modifying proteins localised to complex I and III in the mitochondrial electron transport chain (Van Laar et al., 2009), and can increase mitochondrial respiration that leads to increased ROS formation (Berman and Hastings, 1999). DA quinone is also capable of modifying alpha synuclein to form insoluble protein fibrils, which suggests that DA oxidation may also have a role in Lewy body formation (LaVoie et al., 2005, Conway et al., 2001).

1.4.2 Mitochondrial dysfunction

Mitochondria are complex and dynamic organelles that generate cellular energy, and are also involved in the normal metabolism of lipids and amino acids. Under normal conditions, mitochondrial respiration produces O_2^{2-} and H_2O_2 as by-products

which are readily detoxified by antioxidant activity. However under pathological conditions, the increased production of these ROS can lead to cell toxicity (Dias et al., 2013). Mitochondrial dysfunction was first implicated to have a key role in PD pathogenesis following the discovery that the toxin MPP+ (1-methyl-4-phenylpyridinium), which inhibits mitochondrial complex I (a component of mitochondrial electron transport chain), caused irreversible motor symptoms and pathology clinically similar to idiopathic PD when accidentally injected intravenously (Nicklas et al., 1987, Ramsay et al., 1986, Langston et al., 1983). Other known toxins that inhibit mitochondrial complex I include rotenone, paraquat, maneb and pyridaben, and these also cause a PD phenotype and DA cell deficits in PD models, to suggest that mitochondrial dysfunction plays a critical role in PD pathogenesis (summarised later in animal models of PD). Mitochondrial complex I activity in the post mortem SNc of PD patients is reduced 30-40% (Schapira et al., 1990a, Mizuno et al., 1989). This reduction is specific to complex I in the SNc and in the frontal cortex (Parker et al., 2008), and does not occur in other neurodegenerative disorders (Schapira et al., 1990b).

The notion that mitochondrial dysfunction is a key pathogenic process is supported by studies looking at the function of genes associated with familial PD. Mutations in the protein deglycase Daisuke Junko-1 (DJ-1) gene cause an autosomal recessive form of familial PD (Bonifati et al., 2003). DJ-1 knockout mice show reduced scavenging of mitochondrial H_2O_2 that is produced during mitochondrial respiration (Andres-Mateos et al., 2007). A recent study has shown in rats and post mortem SNc PD tissue that α-synuclein can bind to TOM-20 (mitochondrial pre-protein translocase of outer membrane; a marker of functional mitochondria) and prevent its interaction with TOM-22. Lack of TOM-20: TOM-22

binding inhibits transport of proteins into the mitochondria, which can cause increased ROS generation, and inhibition of mitochondrial activity (Di Maio et al., 2016).

The autosomal recessive PD-associated genes PTEN-induced putative kinase 1 (PINK1) and parkin are known to interact with mitochondrial fission and fusion machinery components including dynamin-related protein 1 (Drp1), Mitofusin, Mitochondrial Dynamin Like GTPase (OPA1), and Drp1-interacting protein Mitochondrial fission 1 (Fis1), to modulate normal mitochondrial dynamics (Yang et al., 2008). In addition, PINK1 and parkin have been implicated in promoting mitophagy, the selective degradation of damaged mitochondria (Narendra et al., 2012).Complex I inhibition has been observed in knockout mouse models of PINK1 (an autosomal recessive PD-linked gene), that leads to increased sensitivity to apoptotic stress (Morais et al., 2009). In addition, defects in mitochondrial morphology are seen in DA neurons in drosophila PINK1 and parkin mutants (Clark et al., 2006, Park et al., 2006, Yang et al., 2006). The full interactions involved in the PINK1/parkin pathway remain unclear, but in summary, PINK1 accumulates on the outer mitochondrial membrane (OMM) of damaged mitochondria, and recruits and activates parkin to the OMM. In turn, parkin mediates mitophagy, the autophagic degradation of mitochondria, through the ubiquitination of mitochondrial substrates that include Miro and mitofusin 1 and 2 (Narendra et al., 2010, Gegg et al., 2010, Tanaka et al., 2010).

Given the crucial role of the PINK1/parkin pathway in regulation of mitophagy, this quality control process likely prevents an increase in ROS production caused by the toxic accumulation of dysfunctional mitochondria, as a protective mechanism against cell death (Narendra et al., 2010, Przedborski, 2017).

1.4.3 Autophagy

Autophagy describes a self-degradation process through which cell components such as misfolded proteins and damaged organelles can be removed, and is divided into 3 subtypes: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy, each differing in the mechanism used to deliver substrates to lysosomes. CMA is a selective degradation pathway, where substrates containing a unique pentapeptide motif (QFERQ) are recognised by and bound to the heat shock cognate protein 70 (hsc70). This chaperone-substrate complex is then delivered to the lysosome membrane, and interacts with lysosomal-associated membrane protein type A (LAMP2A) where the substrate is translocated and internalized into the lysosome for degradation (Dice, 2007, Bandyopadhyay et al., 2008). In microautophagy, cytoplasmic substrates (such as aggregated proteins, damaged organelles) are non-selectively directly engulfed and degraded by lysosomes (Li et al., 2012). Macroautophagy is a degradation pathway that involves sequestration of substrates into autophagosomes, and fusion with lysosomes to allow cargo degradation (Rubinsztein et al., 2012). Given that autophagy is involved in the clearance of dysfunctional organelles and aggregated intracellular proteins, the role of dysregulated autophagy in α-synuclein aggregation in PD has been investigated.

α-synuclein is a known substrate of CMA, containing the motif recognised by hsc70 (Cuervo et al. 2004). Levels of Heat shock cognate 70 kDa protein (Hsc70) and Lysosome-associated membrane protein 2 (LAMP-2A) are significantly decreased in the SNc of PD patients, providing evidence that inhibition of CMA is associated with human PD (Alvarez-Erviti et al., 2010). A53T and A30P α-synuclein mutations prevent CMA degradation and facilitate aggregation-mediated

neurotoxicity by blocking LAMP-2A-associated lysosomal translocation *in vitro* (Martinez-Vicente et al., 2008). Deletion of Autophagy related 7 (Atg7), a gene required for autophagosome formation, causes presynaptic aggregation of α-synuclein protein *in vivo,* further implicating dysregulated autophagy in the accumulation of α-synuclein (Friedman et al., 2012).

Other PD-associated genes, including LRRK2, DJ-1 and VPS35 have also been implicated to have a role in autophagic dysfunction. Over-expression of Lrrk2 (a PD-associated gene) increases autophagosome formation (Gómez-Suaga et al., 2011). In contrast, siRNA-mediated knockdown of LRRK2 in a genomic reporter cell model has been reported to increase autophagy and reduces cell death under stress conditions (Alegre-Abarrategui et al., 2009). The autosomal-dominant G2019S mutation of LRRK2 inhibits lysosomal translocation that prevents CMA-mediated α-synuclein degradation (Cuervo et al., 2004, Orenstein et al., 2013). DJ-1 is a known substrate of CMA (Wang et al., 2016). VPS35 deficient mice have been reported to develop α-synuclein aggregations in the SNc, caused by impaired LAMP-2A activity (Tang et al., 2015).

1.4.4 Neuroinflammation

Traditionally, inflammation was seen as a biological response to signal cell injury. However, emerging evidence suggests that neuroinflammatory responses such as microglial activation directly contributes to nigral degeneration in PD. Microglia are the resident immune cells in the brain, and in response to inflammation they undergo a change in morphology, referred to as its reactive, phagocytotic state.

Human post-mortem studies were the first to show evidence for a link between neuroinflammation and PD, where an increase in reactive microglia were localised to the SNc and striatum of idiopathic PD patients (McGeer et al., 1988,

Mogi et al., 1995). The SNc has a dense microglia population compared to other brain regions (Lawson et al., 1990). Levels of pro-inflammatory cytokines measured from cerebrospinal fluid and human post-mortem SNc tissue, including Interleukin (IL)-1β, IL-2, IL-4, IL-6, IFN-γ, epidermal growth factor (EGF), tumor growth factor (TGF) and tumour necrosis factor (TNF) are elevated in patients with PD compared to controls (Mogi et al., 1996, Hunot et al., 1999). Increased levels of pro-inflammatory cytokines caused by over-activation of microglia can in turn induce an immunostimulation in glial cells to produce reactive oxygen species, including nitric oxide and superoxide (Colton and Gilbert, 1987, Moss and Bates, 2001, Boje and Arora, 1992, Chao et al., 1992), which suggests that in its reactive state, microglia are capable of exacerbating neuroinflammation-associated oxidative stress.

A microarray study comparing the gene expression profiles between the lateral tier and medial tiers of the SNc in human PD cases found that the lateral tier, which is selectively more vulnerable to cell death (Fearnley and Lees, 1991), showed higher expression of pro-inflammatory cytokines, including the TNF receptor superfamily genes in glial cells of the lateral tier compared to the medial tier. Additionally, they showed reduced expression of glutathione in the lateral tier, suggesting that the lateral tier in particular is particularly vulnerable to oxidative stress (Duke et al., 2007).

Epidemiological studies also provide supporting evidence to suggest a role for neuroinflammation in PD. The risk of PD incidence is 45% lower in regular users of non-aspirin non-steroidal anti-inflammatory drugs (NSAIDS) (Chen et al., 2005), however these findings have been refuted with subsequent studies showing little or no effect of NSAIDS on incidence of PD (Hernán et al., 2006, Hancock et al., 2007).

A link between neuroinflammation and PD has also been established in animal models of PD, which show that microglial activation found in the SNc and striatum of MPTP treated mice (Kurkowska-Jastrzębska et al., 1999), and 6-OHDA-treated rats (Cicchetti et al., 2002) leads to DA cell death through the increase of glial-derived ROS production, and reduced production of trophic factors.

1.4.5 DNA damage

DNA damage refers to alterations of DNA structure, such as DNA strand breaks and base excision, that occur when the metabolic processes that take place in cells also produce ROS. Under normal conditions, DNA alterations are repaired through base excision mechanisms, and the toxic effects of ROS are attenuated through antioxidant activity that respond to elevated ROS (Canugovi et al., 2013). DNA damage can be induced by increased oxidative stress and mitochondrial dysfunction, that affect both genomic and mitochondrial DNA (Sanders et al., 2014).

Increasing lines of evidence show that DNA damage can play a role in neurodegeneration of DA cells in PD. 8-hydroxyguanosine (8-OHdG), a byproduct of nucleic acid oxidative damage, has greater immunoreactivity in SNc neurons of PD patients compared to controls (Zhang et al., 1999, Alam et al., 1997a). Increased levels of 8-OHdG has also been shown in the mitochondrial DNA of PD patients (Alam et al., 1997a, Shimura-Miura et al., 1999). There are higher levels of mitochondrial DNA breaks in nigral DA cells of PD cases than controls (Bender et al., 2006, Kraytsberg et al., 2006). To date however, whether DNA damage is a cause of SNc degeneration or a consequence of cell death that occurs in the PD affected SNc remains unclear.

Taken together, these studies indicate that several cell death mechanisms, including oxidative stress, mitochondrial dysfunction, dysregulated autophagy,

neuroinflammation and DNA damage are in the pathogenesis of PD. Misfolding of proteins such as α-synuclein causes aggregations called Lewy bodies, which have been associated with progression of disease. Studies show that oxidative damage is closely linked to mitochondrial dysfunction, and both promote ROS-mediated toxicity of DA cells which can in turn mediate neuroinflammatory and DNA-damage responses. These studies also suggest that these mechanisms do not act in isolation, but instead as a pathogenesis cascade. Understanding the common downstream molecular pathways that may occur will give insight into the initial trigger(s) that underlie pathogenesis of DA neurons in PD.
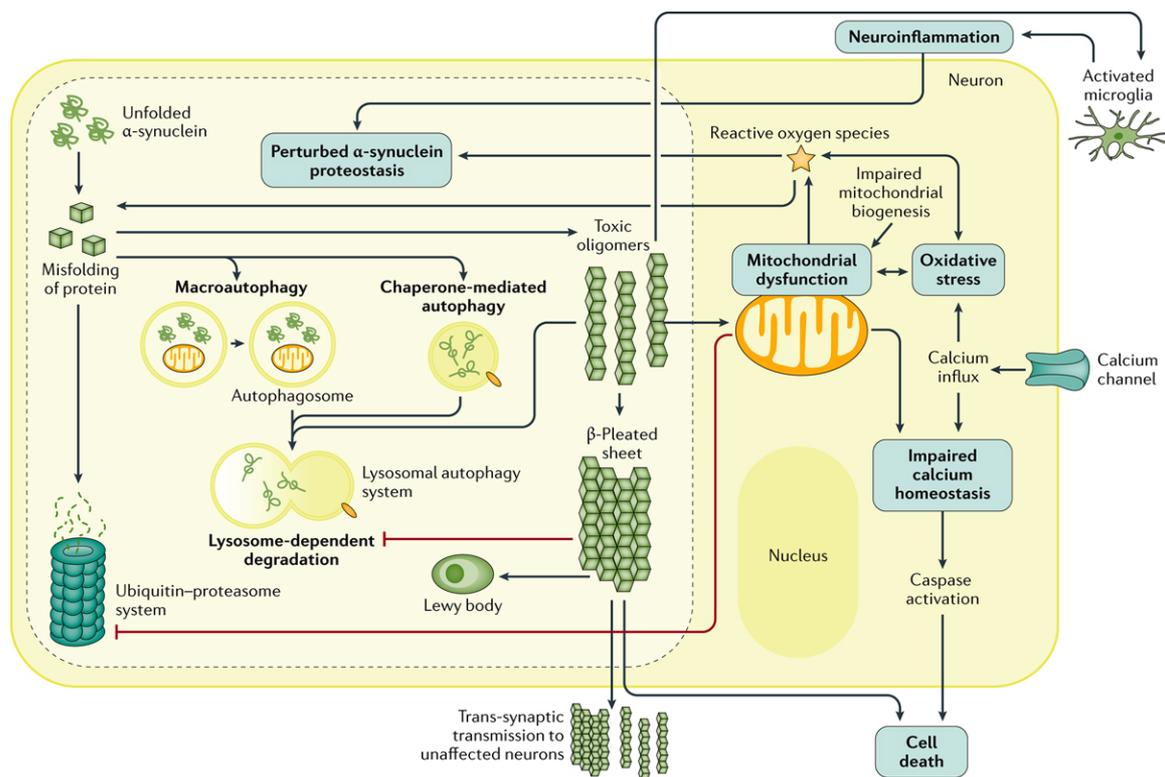
Figure 1.7. Pathogenesis mechanisms implicated in Parkinson's disease PD-associated pathogenesis mechanisms include oxidative stress mitochondrial dysfunction, neuroinflammation, dysregulated autophagy and mitophagy, and DNA damage. These pathways often share common molecular interactions, eventually leading to DA cell death. Reproduced from Poewe et al. (2017).

1.5 Animal models of PD

The development of animal models of PD has played a crucial role in our understanding of the etiology, pathology and pathogenic mechanisms in PD, and also allow for the testing of potential neuroprotective strategies against PD. Current animal models can be split into two groups: neurotoxic and genetic models. Despite the continuing development of these two forms of models, to date none completely mimics the progression of motor symptoms, pathogenic mechanisms, DA cell loss, Lewy body pathology and non-DA related aspects of the disease seen in clinical PD (table 1.2).

1.5.1 Toxin-based models of PD

6-OHDA model of PD

6-hydroxydopamine (6-OHDA) is the most widely used neurotoxin in *in vivo* and *in vitro* research models for PD. The 6-OHDA model was first established by Ungerstedt (1968) where 6-OHDA was injected into the rat SNc, and caused an anterograde degeneration of the nigrostriatal pathway, and 6-OHDA–induced motor deficits. Due to its low cost and high reproducibility, this model has since become well characterised in rats, mice and primates (Blandini et al., 2008). 6-OHDA is structurally similar to DA, but an additional hydroxyl group makes 6-OHDA neurotoxic to DA neurons. 6-OHDA has high affinity for the DA transporter (DAT), which allows for the selectively damage of DA neurons (Bove et al., 2005, Sachs and Jonsson, 1975). 6-OHDA is unable to cross the blood-brain barrier, therefore requiring direct administration into the nigrostriatal system for neurotoxicity to occur. 6-OHDA accumulates in DA neurons and inhibits mitochondrial activity, but can also be oxidised into quinones and ROS in the form of hydroxyl radicals (Bove et al.,

2005, Blum et al., 2001). These ROS initiate stress response systems, which lead to oxidative stress in surrounding cells, ultimately causing DA cell death (reviewed earlier).

Numerous studies indicate that the degree of nigrostriatal lesion and subsequent decline in motor function depends on the site of injection and dose of 6-OHDA. For instance, direct injection into the SNc produces neurodegeneration within 12 hours, followed by loss of striatal terminals over 1-7 days (Jeon et al., 1995). Striatal 6-OHDA lesions are capable of producing progressive, retrograde loss of SNc DA neurons from 1-2 weeks following lesion, and progress over 8-16 weeks (Sauer and Oertel, 1994). Direct injections are typically performed unilaterally, as bilateral administration of 6-OHDA is commonly fatal (Ungerstedt, 1971). Additionally, unilateral injection allows using the un-lesioned contralateral hemisphere as an internal control. Behavioural analysis following the post-6-OHDA administration of amphetamine or DA agonists can assess the asymmetrical rotations that occur, to estimate the degree of unilateral DA cell loss (Ungerstedt and Arbuthnott, 1970). While the 6-OHDA model mimics the behaviour deficits, DA depletion and DA neuron loss in PD, 6-OHDA lesions do not replicate formation of α-synuclein Lewy bodies in the SNc, the hallmark pathology in clinical PD. In addition, 6-OHDA does not cause lesions at the brainstem and olfactory regions associated with PD, nor replicate the progressive motor deficits and DA cell loss seen in clinical PD.

MPTP model of PD

The toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was first discovered in the 1980's following the accidental intravenous injection of synthetic

meperidine contaminated with MPTP by a group of drug users, who developed symptoms resembling idiopathic PD (Davis et al., 1979, Langston et al., 1983). Due to its lipophilic structure, MPTP can readily pass through the blood-brain barrier. MPTP readily enters the brain and is metabolized by MAO-B to the active metabolite form MPP^+ in astrocytes. MPP^+ is a substrate of DAT and accumulates in the mitochondria of DA neurons where it inhibits complex 1, which induces free radical production that leads to cell damage and death via oxidative stress mechanisms (Du et al., 2001, Sriram et al., 1997).

Whilst MPTP is readily used to model PD in mice and non-human primates, and is commonly administered systemically (Przedborski et al., 2001), rats are resistant to MPTP and require the stereotaxic administration of the metabolite MPP+ for nigrostriatal lesions to occur (Chiueh et al., 1984, Przedborski et al., 2001) MPTP mimics the DA depletion, DA neuron loss and behaviour deficits seen in clinical PD, and causes an increase in oxidative stress and mitochondrial dysfunction pathogenesis mechanisms seen in human PD (Nicklas et al., 1985). MPTP does not cause the formation of α-synuclein inclusions (Halliday et al., 2009).

Paraquat model of PD

Paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) is a widely used herbicide with a structural similarity to MPP+. When converted to its cation PQ+ state, paraquat is a substrate of DAT (Rappold et al., 2011). Paraquat causes neurotoxicity through redox cycling, to generate ROS including superoxide, hydrogen peroxide, and hydroxyl radicals which exacerbates oxidative stress (Przedborski and Ischiropoulos, 2005, Day et al., 1999). Intraperitoneal injection of paraquat in mice causes age and dose-dependent DA depletion, nigrostriatal lesions and motor

deficits (McCormack et al., 2002, Thiruchelvam et al., 2003). Significantly, paraquat administration in mice is associated with α-synuclein aggregations in SNc DA neurons (Manning-Bog et al., 2002), which makes the paraquat model valuable for the study of mechanisms of onset of Lewy body pathology in PD.

Rotenone model of PD

The pesticide rotenone is a neurotoxin that, similarly to MPTP, has a lipophilic structure which allows it to readily cross the blood-brain barrier. Rotenone causes toxicity through inhibition of mitochondrial complex 1, which leads to over-production of ROS. Rotenone-induced animal models of PD replicate almost all hallmarks of PD, including behavioural deficits, DA depletion, nigral DA cell loss, α-synuclein aggregations, pathology in non-dopaminergic peripheral regions and non-motor symptoms such as gastrointestinal dysfunction (Betarbet et al., 2000, Sherer et al., 2003b, Greenamyre et al., 2003).

Direct stereotaxic injection of rotenone into the medial forebrain bundle causes nigral DA cell loss and striatal DA depletion (Heikkila et al., 1985). Systemic intravenous infusion of low doses (2-3 mg/kg/day) of rotenone for 1- 3 weeks through osmotic mini-pumps produces nigrostriatal lesions, Lewy-body like inclusions and progressive motor deficits (Betarbet et al., 2000).

Chronic intraperitoneal rotenone injections (2 mg/kg) for 6 weeks shows evidence of Lewy body-like inclusions in the myenteric plexus and a progressive loss in gastrointestinal motility, which replicates peripheral, enteric nervous system symptoms and pathology seen in clinical PD (Drolet et al., 2009). Lower doses of systemic rotenone over 2 months show a further progressive, dose dependent effect on motor function, nigrostriatal deficits and DA levels (Alam and Schmidt, 2002).

The variability in percentage of cohorts that show rotenone-mediated toxicity and the reproducibility and consistency of lesion size have been has been the main limitations of the systemic rotenone model (Betarbet et al., 2000, Fleming et al., 2004).

Cannon et al (2009) described a highly reproducible rotenone model of PD, which showed that daily intraperitoneal rotenone treatment (3mg/kg) in rats induced relatively uniform bilateral nigrostriatal lesions in all rats studied. Rotenone treatment led to a progressive motor deficit, loss of 45% of nigral DA neurons, loss of striatal DA terminals which was associated with nigral α-synuclein aggregation. Thus, the reproducible features of this modified chronic rotenone rat model of PD make it ideal for the assessment of experimental disease-modifying therapies (Cannon et al., 2009).

Table 1.2 Toxin-induced animal models of PD

Neurotoxin	Motor deficits	DA cell death	LB pathology	Non-motor symptoms	References
6-OHDA (rat)	+	+	-	-	(Bove et al., 2005, Blum et al., 2001).
MPTP (mouse, primate)	+	+	-	-	(Przedborski et al., 2001, Schober, 2004)
Paraquat (rat, mouse)	+	+	+	-	(Manning-Bog et al., 2002),
Rotenone (acute, rat)	+	+	+	+	(Heikkila et al., 1985)
Rotenone (chronic, rat)	+	+ (progressive)	+	+	(Cannon et al., 2009, Drolet et al., 2009)
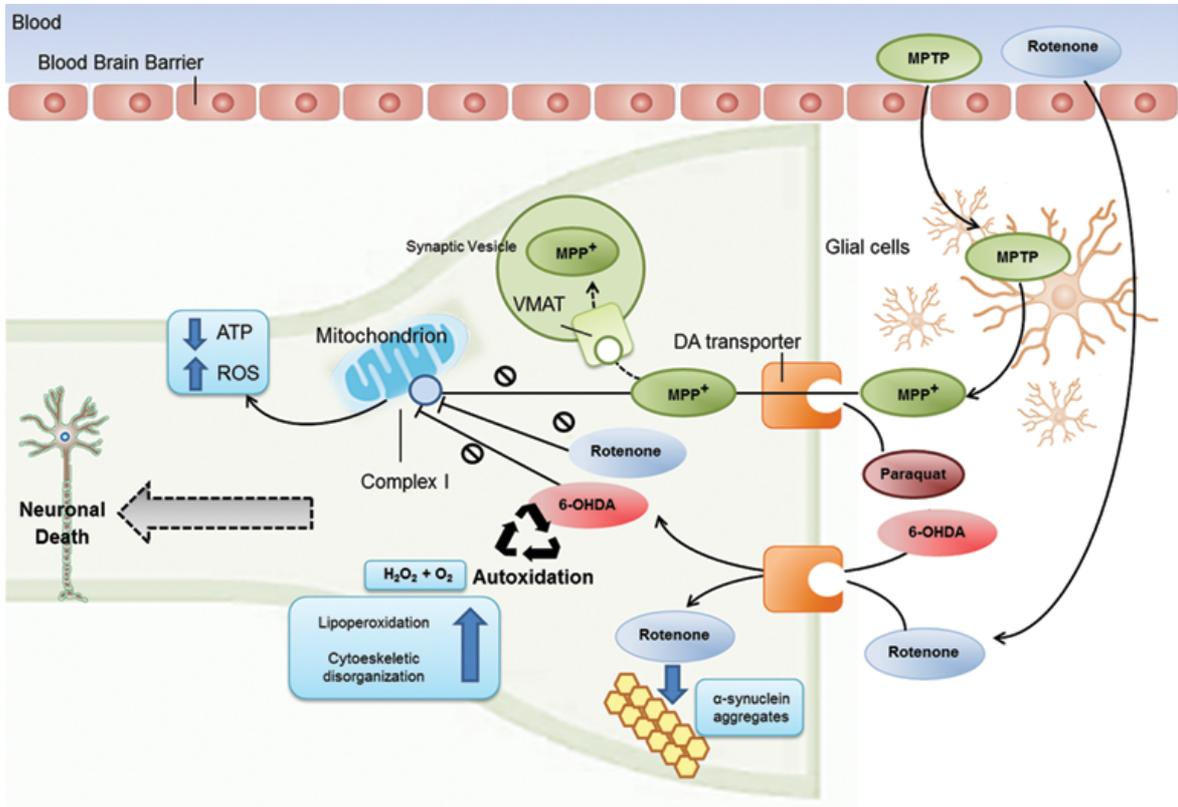
Figure 1.8. Toxin-based animal models of PD MPTP crosses the blood-brain barrier and metabolized to MPP+ by MAO-B in microglia. MPP+ is then taken up by DAT where it impairs mitochondrial respiration by mitochondrial complex 1, causing oxidative stress. 6-OHDA and paraquat cross DA cell membrane through DAT, causing toxicity through mitochondrial dysfunction-induced ROS and quinone production that leads to nigrostriatal neurodegeneration. Due to its lipophilic structure, rotenone crosses the blood brain barrier, inducing the formation of α-synuclein aggregates and inhibits mitochondrial complex I activity with the subsequent production of ROS and quinones. Reproduced from Blesa et al. (2016).

1.5.2 Genetic-based models of PD

Currently, genetic forms of PD account for 15% of all PD cases, although this figure is likely to increase as novel genes continue to be identified (Klein and Westenberger, 2012, Verstraeten et al., 2015, Trinh and Farrer, 2013). The identification of PD-associated genes has advanced the study of molecular mechanisms that lead to PD. New genetic models of PD continue to be generated to study the common pathways between familial and idiopathic PD that initiate the cascade of pathogenic mechanisms. Advances in genetic manipulation have facilitated the use of transgenic approaches to drive overexpression of PD genes, targeted deletions to create knockout models, and viral vector-mediated approaches to introduce gene mutations (Dawson et al., 2010, Blesa et al., 2012). Whilst the precise mechanisms underlying the genetic forms of PD will be discussed in detail in section 1.6.2 of this thesis, this section will summarise the current genetic animal models available. These include the overexpression of autosomal dominant genes (α-synuclein, Leucine-rich repeat kinase 2; LRRK2) and knockout models of autosomal recessive genes (Parkin, PINK1, DJ-1) in rodents.

α-synuclein transgenic models of PD

α-synuclein is located pre-synaptically and regulates Soluble NSF attachment protein receptor (SNARE) complex assembly, which mediates fusion of synaptic vesicles to the presynaptic membrane (Burré et al., 2010). Mutations in α-synuclein that lead to A53T, A30P, and E46K substitutions cause autosomal dominant PD, and lead to over-expression of α-synuclein protein which is associated with increased Lewy body pathology. Multiplication of the α-synuclein locus is also associated with increased PD (Singleton et al., 2003).

A study by Masliah et al. (2000) was the first to develop a transgenic mouse model to overexpress wild-type human α-synuclein. Despite the presence of motor deficits and presence of α-synuclein aggregations in the SNc, hippocampus and neocortex, the main limitation to this transgenic mouse model is the lack of nigral DA cell degeneration. Further pre-clinical studies have used different promoters to drive expression of the transgene (Neumann et al., 2002, Giasson et al., 2002, Lee et al., 2002). Whilst these α-synuclein transgenic mouse models have successfully reproduced the hallmark motor deficits and LB pathology, the majority have not been able to reproduce the progressive DA cell loss seen in clinical PD (see Table 1.3).

The use of the bacterial artificial chromosome (BAC) approach has been an alternative approach to create genetic models of PD. Due to their large insert size, BACs can accommodate the transgene's native promoter and regulatory factors, to provide a more stable vector than other approaches (Shizuya et al., 1992). Janezic et al. (2013) reported that BAC-mediated WT human α-synuclein overexpression in mice exhibits deficits in DA release, motor deficits, and α-synuclein protein aggregate formation. However, nigral DA neuron loss was modest, with 18-month old mice displaying 30% loss of DA neurons.

Recently, adeno-associated virus (AAV) approaches have been used to drive the overexpression of WT and mutant forms of α-synuclein as a model of PD in mice, rats and primates. AAV-mediated A30P overexpression, via vector injection into the midbrain of rats, induces DA cell loss, but fails to reproduce motor deficits (Klein et al., 2002). Conversely, AAV-mediated overexpression of human WT and A53T α-synuclein in rats, mice and primates via delivery into the SNc induces spontaneous motor deficits, striatal DA terminal loss, Lewy body pathology, and crucially, a progressive 30-80% loss of nigral DA neurons (Decressac et al., 2012,

Ip et al., 2017, Klein et al., 2002). Taken together, these studies suggest that the AAV-mediated A53T overexpressing α-synuclein rodent and primate models closely resemble the hallmark clinical symptoms and pathology seen in human PD.

LRRK2 transgenic models of PD

Mutations to the *LRRK2* (leucine rich repeat kinase 2) gene are the most common genetic cause for autosomal dominant PD (Berg et al., 2005), with the most common mutations including G2019S, R1441G and R1441C. Multiple pre-clinical studies have looked to model PD using LRRK2 knockout and transgenic overexpression approaches, however results have been inconsistent and varied in both mice and rats (Xiong et al., 2017). For instance, knockout of LRRK2 in mice has been reported to cause α-synuclein aggregation, alongside increased apoptosis, neuroinflammation and oxidative DNA damage, without any changes to motor function or DA cell loss (Tong et al., 2010). BAC-transgenic mice generated to carry WT, R1441C, or G2019S missense mutations have been shown to display motor deficits and DA depletion, without nigral DA cell neurodegeneration or α-synuclein aggregation (Li et al., 2009, Li et al., 2010). To date, two transgenic LRRK2 mouse models have successfully shown nigral DA cell loss. Transgenic (G2019S) mice display age-dependent 50% loss of DA cells and associated motor deficits, despite a lack of α-synuclein aggregation (Chen et al., 2012). Another study has reported that G2019S transgenic mice develop modest nigral DA cell loss, without loss in motor function or α-synuclein aggregation (Ramonet et al., 2011).

In many cases, LRRK2 models fail to reproduce the hallmark nigrostriatal lesions and LB pathology, and to date no LRRK2 model reproduces all the hallmark characteristics of PD, suggesting that the LRRK2 mouse model is not a strong

model. This may be due to technical variability involving different levels of LRRK2 expression, or additional compensatory mechanisms. The use of additional genetic manipulations and/ or toxins in combination with LRRK2 overexpression may provide a more effective model of PD.

Table 1.3 Autosomal dominant animal models of PD

Promoter	Motor deficits	DA cell death	LB pathology	References
α-synuclein				
PDGF- β (WT) (mouse)	+	-	+	(Masliah and Hashimoto, 2002)
mThy1 (A30P) mouse)	+	-	+	(Neumann et al., 2002)
mPrP (A53T) (mouse)	+	=	+	(Giasson et al., 2002, Lee et al., 2002)
BAC (WT) (mouse)	+	Progressive DA cell degeneration	+	(Janezic et al., 2013)
LRRK2				
BAC (mouse; WT; G2019S; R1441C)	+	-	-	(Li et al., 2009)
G2019S (mouse)	+	+	-	(Chen et al., 2012)
G2019S (mouse)	-	+	-	(Ramonet et al., 2011)

Pink1 and parkin knockout models of PD

Mutations in parkin and PINK1, genes associated with mitochondrial maintenance, are linked to autosomal recessive forms of PD (see Table 1.4). PINK1 and parkin knockout mouse models display very similar parkinsonian phenotypes. Though these models reproduce striatal DA depletion, they do not exhibit nigral DA cell loss,

nor LB pathology. Despite this, these knockout models all show a reduction in mitochondrial activity, indicating that these models could be used to further understand pathogenic mechanisms of PD (Gautier et al., 2008, Kitada et al., 2007, Gispert et al., 2009, Goldberg et al., 2003, Itier et al., 2003). However, the lack of nigrostriatal lesion or LB pathology in these knockout models makes them inadequate models to test neuroprotective strategies.

DJ-1 knockout models of PD

Missense mutations to the DJ-1 gene, involved in α-synuclein chaperone activity, have been implicated in autosomal recessive, early onset PD. Knockout mice models have been reported to show DA depletion and motor deficits, but are not able to reproduce nigral DA cell loss, nor LB pathology (Goldberg et al., 2005). DJ-1 knockout mice treated with MPTP showed a hypersensitivity to the toxin-induced deficits, which demonstrate a motor deficit, striatal DAT loss and loss in nigral DA cells that are attenuated with AAV-mediated delivery of DJ-1 (Kim et al., 2005). Rousseaux et al. (2012) report a DJ-1 knockout mouse model, crossed with C57-BL6 mouse line, which shows mild motor deficits, and progressive, age dependent loss of nigral DA cells. Though this model requires further characterisation, this could be a valuable tool to test the preclinical stages of PD, and potential neuroprotective strategies.

Table 1.4 Autosomal recessive animal models of PD

Gene	Motor deficits	DA cell death	LB pathology	References
PINK1 (KO)	+	No DA cell degeneration	-	(Gautier et al., 2008, Gispert et al., 2009, Kitada et al., 2009)
Parkin (KO)	+	No DA cell degeneration	-	(Goldberg et al., 2003, Itier et al., 2003)
DJ-1 (KO)	+	No DA cell degeneration	-	(Goldberg et al., 2005, Kim et al., 2005)
DJ-1 KO-C57BL6	+	Progressive DA cell degeneration	-	(Rousseaux et al., 2012)

In summary, despite the continuous development of toxin and genetic models of PD, no single model can fully reproduce all hallmark aspects of clinical PD. Recent studies show promise in approaches using viral-mediated expression of PD-linked genes, and the combination of transgenic mouse models and DA toxin delivery. Hence, using multiple models may likely be the key strategy develop a more clinically relevant model of PD.

1.6 Aetiology of PD

Currently the underlying cause(s) of PD are to date not well understood, with over 85% of PD cases having idiopathic origin. The remaining 10-15% of cases arise from genetic mutations that are known as familial forms of PD (Thomas and Beal, 2007, Lesage and Brice, 2009). It is therefore likely that PD arises due to a combination of environmental and genetic factors, which will be discussed in this section.

1.6.1 Environmental factors

Predisposing factors

Aging is the most significant risk factor for idiopathic PD. PD affects 1% in the population over 60, and rises to almost 5% of the population over 85 (Nussbaum and Ellis 2003, de Lau and Breteler, 2006) . Additionally, there is increasing evidence that links a range of environmental factors with increased risk of developing PD. These include a link between increased PD risk and head trauma (Lehman et al., 2012), anaemia (Savica et al., 2009), and the use of herbicides and pesticides including paraquat, maneb, permethirn and rotenone (Tanner, 2010, Tanner et al., 2011, Semchuk et al., 1992, Costello et al., 2009, Dick et al., 2007). These toxins are known to induce mitochondrial dysfunction and oxidative stress, pathogenic mechanisms that are capable of exacerbating cell death.

Protective factors

The association between cigarette smoking and PD has been well documented, with several studies showing a clear temporal and dose-dependent association between smoking and reduced risk of developing PD (Kiyohara and Kusuhara, 2011, Thacker et al., 2007). The neuroprotective potential of nicotine has been investigated *in vivo*,

and studies show that nicotine administration in 6-OHDA rat models of PD protects against toxin-induced motor deficits and nigral DA cell loss, and is able to reduce levodopa-induced abnormal involuntary movements (Huang et al., 2009, Bordia et al., 2008). Nicotine may have neuroprotective potential through stimulation of nicotinic acetylcholine receptors, or through the inhibition of MAO-B activity (Fowler et al., 1996, Quik et al., 2009).

Similarly, studied have shown the potential protective effect of caffeine consumption in PD, with increased caffeine intake associated with reduced incidence of PD (Hernán et al., 2002, Ross et al., 2000). Caffeine protects against nigral DA neuron loss in paraquat and maneb mouse models of PD. Caffeine is an antagonist of adenosine receptors, which modulate GABA and glutamate release within the indirect pathway of the basal ganglia network. This neuroprotective effect could therefore be potentially mediated through blockade of these receptors (Kachroo et al., 2010, Xu et al., 2005).

The use of non-steroidal anti-inflammatory drugs (NSAIDs) has been increasingly associated with reduced risk for PD, with regular users of NSAIDs (greater than twice a week) reporting 45% less risk of PD than non- users (Chen et al., 2005, Wahner et al., 2007). Further clinical studies have shown an association between ibuprofen use and reduced risk of PD, but no effect with other NSAIDs (Gao et al., 2011), to suggest that ibuprofen in particular may have potential as a neuroprotective strategy in PD.

1.6.2 Genetic factors

In at least 10-15% of cases, PD is caused by a single gene mutation with either autosomal dominant or recessive inheritance (Klein and Westenberger, 2012,

Verstraeten et al., 2015, Trinh and Farrer, 2013). Through linkage analysis, genomic sequencing and genetic association approaches, mutations in 3 genes (α-synuclein, LRRK2, and VPS35) are now known to cause autosomal dominant forms of familial PD, while mutations in six other genes (PINK1, DJ-1, Parkin, ATP13A2, FBX07, and PLA2G6) are known to causes recessive forms of PD. Parkin, PINK1 and DJ-1 mutations cause early-onset autosomal recessive parkinsonism, where onset of disease occurs between ages 20 to 50, and affects between 5 to 10% of all PD cases (Golbe, 1991). Among early onset PD patients, parkin mutations are the most common, present in 45% of early onset PD cases (Lücking et al., 2000). PINK1 and DJ-1 mutations are less common, present in less than 4% and 2% of early-onset PD cases respectively (Kumazawa et al., 2008, Hague et al., 2003, Hedrich et al., 2004, Schrag and Schott, 2006).

1.6.2a Autosomal dominant PD

α-synuclein

α-synuclein encodes a 140 amino acid length, natively unfolded presynaptic protein of the synuclein family, comprised of 3 regions: a lipid-binding terminus, a central, NAC (non-Aβ component), hydrophobic terminus which is not seen in other proteins in the synuclein family, and a carboxyl terminus (Maroteaux et al., 1988). α-synuclein is abundantly expressed in presynaptic terminals throughout the brain, positioned closely to synaptic vesicles (Maroteaux et al., 1988). α-synuclein mediates assembly of SNARE complexes, which have a crucial role in mediating the fusion of neurotransmitter-containing vesicles to the presynaptic membrane. α-synuclein promotes complex assembly by directly binding to phospholipids via the N-terminus and to the C-terminus of the SNARE protein synaptobrevin-2/ vesicle-

associated membrane protein 2 (VAMP2) (Burré et al., 2010). α-synuclein has a key role in the pathophysiology of PD as a major component of Lewy bodies and neurites (Spillantini et al., 1997, Baba et al., 1998).

A total of 5 α-synuclein point mutations are known to date. The A53T missense mutation is localised to the protein region where the secondary structure is an α-helical formation, bound by β-sheets. The A-T amino acid substitution disrupts the α helix, extending β sheet structure of the protein. The A30P mutation is localised to the terminal position, and likely affects binding to synaptic vesicles. In addition, the A-P substitution disrupts the α helical structure and extends the β sheet, making protein more susceptible to self-aggregate (El-Agnaf et al., 1998, Polymeropoulos et al., 1997). The E46K mutation increases α-synuclein fibril formation, which promotes aggregate formation (Choi et al., 2004, Greenbaum et al., 2005). The G51D mutation is located on the α-synuclein NAC-terminus domain, and causes α-synuclein to assemble into fibrils that are higher in toxicity than WT α-synuclein (Lesage et al., 2013). The H50Q mutation, similarly to A30P and A53T mutations, disrupts the α helical structure (Appel-Cresswell et al., 2013).

Following the A53T identification in Italian and Greek families (Polymeropoulos et al., 1997), the A30P mutation was detected in a single German family (Krüger et al., 1998), and the E46K mutation in a single Spanish family (Zarranz et al., 2004). Most recently, the G51D mutation has been reported in unrelated French and British families, as well as a Japanese female patient (Kiely et al., 2013, Lesage et al., 2013, Tokutake et al., 2014), and H50Q in two single patients of English and Welsh ancestry (Proukakis et al., 2013, Appel-Cresswell et al., 2013).

Multiplication of WT α-synuclein has also been associated with increased disease severity , earlier age of onset, and faster progression of symptoms in PD patients (Singleton et al., 2003, Chartier-Harlin et al., 2004, Ferese et al., 2015). Quadruplication of α-synuclein has been seen in 7 unrelated families, and typically present with earlier age of onset and more severe progression than cases with α-synuclein triplication, indicating that severity of disease correlates with dosage of α-synuclein (Ferese et al., 2015, Ikeuchi et al., 2008).

LRRK2

Leucine-rich repeat kinase 2 (LRRK2) encodes a large multi-domain protein belonging to the Ras of complex (ROCO) protein family. LRRK2 has two central enzymatic domains (GTPase and serine/threonine kinase) as well as domains that mediate protein-protein interactions. LRRK2 is expressed throughout several tissues including the liver, lungs, heart, kidneys and the brain (Paisan-Ruiz et al., 2004, Westerlund et al., 2008, Giasson et al., 2006). Wild type mammalian LRRK2 regulates neurite length and neuron survival in developing neurons (MacLeod et al., 2006, Parisiadou et al., 2009).

LRRK2 has been implicated to have a role in regulation of mitochondrial fusion/fission dynamics, interacting with the mitochondrial fission factor Dynamin-like protein 1 (DLP1). LRRK2 expression promotes DLP1 recruitment to mitochondria, and induces mitochondrial fission and fragmentation (Niu et al., 2012, Wang et al., 2012). LRRK2 interacts with members of the dynamin GTPase superfamily involved in mitochondrial fusion, including mitofusin 1, 2 and OPA1 (Stafa et al., 2013). LRRK2 is also implicated to have a role in the autophagy/ lysosomal pathway, by regulating Ca^{2+} dependent activation of a CaMKK/adenosine

monophosphate (AMP)-activated protein kinase (AMPK) pathway, which when overexpressed leads to increased autophagosome production (Gómez-Suaga et al., 2011). Loss of LRRK2 causes impairment of the autophagy-lysosomal pathway, leading to accumulation of aggregated α-synuclein protein (Tong et al., 2010), and has been also been implicated in chaperone-mediated autophagic dysfunction (Orenstein et al., 2013).

Mutations of LRRK2 are the most common cause of autosomal dominant PD, accounting for 4- 10% of familial PD cases, dependent on population cohorts studied (Berg et al., 2005, Di Fonzo et al., 2005, Zimprich et al., 2004, Healy et al., 2008), and 3.6% of sporadic PD cases (Paisan-Ruiz et al., 2008). Currently, over 100 mutations (miss-sense or non-sense) of LRRK2 have been reported (Rubio et al., 2012). To date however, only 7 mutations have confirmed autosomally dominant PD pathogenicity via linkage studies. These include Asn1437His, Arg1441Cys, Arg1441Gly, Arg1441His, Tyr1699Cys, Gly2019Ser, and Ile2020Thr (Paisan-Ruiz et al., 2004, Di Fonzo et al., 2006, Zimprich et al., 2004, Zabetian et al., 2005, Di Fonzo et al., 2005, Aasly et al., 2010). The most common and well-studied mutation Gly2019Ser is detected in 1.6% of idiopathic PD cases (Gilks et al., 2005), and 3-6% of familial PD cases of North American and European origin (Di Fonzo et al., 2005, Kachergus et al., 2005, Healy et al., 2008, Nichols et al., 2005, Di Fonzo et al., 2006). Gly2019Ser is more prevalent in familial PD populations of Ashkenazi Jews, with up to 30% of all cases carrying the mutation (Ozelius et al., 2006) and North African Arab kindred (40%) (Lesage et al., 2006). Conversely, the Gly2019Ser mutation remains rare in Asian kindred, prevalent in <0.1% of an Asian population studied (Tan et al., 2005). The Gly2019Ser mutation has age-dependent penetrance, varying from 28% at 59 years to 50% at 60 years, and 74% at 79 years

(Healy et al., 2008). The Gly2019Ser mutation enhances kinase activity, suggesting that this mutation causes a gain of function phenotype to enhance neurotoxicity (West et al., 2005). Further studies are required to characterise the function of other LRRK2 mutations in the context of PD pathogenesis.

VPS35

Vacuolar protein sorting 35 (VPS35) encodes a protein component of the retromer complex, which plays a role in regulating retrograde transport of proteins from endosomes to the trans-Golgi network, and recycling of transmembrane protein cargo (Attar and Cullen, 2010). The D620N mutation of VPS35 causes autosomal-dominant PD, shown in two independent studies comprised of Austrian and Swiss kindred (Zimprich et al., 2011, Vilariño-Güell et al., 2011). Whilst the pathogenesis mechanisms underlying VPS35 mutations are not fully understood, viral-mediated overexpression of human WT and D620N VPS35 in primary cortical neurons and in the rat SNc leads to neuronal death (Tsika et al., 2014), indicating that the mutation likely has a toxic gain of function. The D620N mutation disrupts the function of VPS35 via altering the transport of endosomes containing CI-M6PR (a capthesin D receptor) to the perinuclear subcellular localization. Consequently, this mutation alters trafficking of capthesin D, a protease that is involved in degradation of α-synuclein, which can potentially lead to altered α-synuclein aggregation (Follett et al., 2014). The D620N VPS35 mutation has been implicated to have a role in impaired autophagy through inhibition of autophagosome formation (Zavodszky et al., 2014). Overexpression of the D620N mutation in mice reduces LAMP-2A expression, which results in accumulation of α-synuclein (Tang et al., 2015). VPS35

mutations are rare, comprising 1% of familial PD cases and <0.2% of sporadic PD (Lesage et al., 2012, Zimprich et al., 2011).

1.6.2b Autosomal recessive PD

Parkin

Parkin belongs to the family of ubiquitin E3 ligases, and catalyses the attachment of ubiquitin to protein targets, marking them for proteasome-mediated destruction (Shimura et al., 2000). Parkin also has a role in mitochondrial maintenance, where it induces autophagy of dysfunctional mitochondria, via ubiquitination of mitochondrial membrane proteins (Narendra et al., 2008). Parkin mutations cause a loss of function of the protein via inactivation of the E3 ligase function, failure of target protein ubiquitination and consequent build-up of proteins normally degraded by ubiquitin-proteosome pathway (Shimura et al., 2000). Candidate protein substrates of parkin remain unclear, but to date parkin has been implicated in the ubiquitination of α-synuclein, which when impaired causes α-synuclein accumulation (Shimura et al., 2001).

To date, 147 mutations of parkin have been reported, and are comprised of point mutations, exonic deletions, insertions and duplications (Abbas et al., 1999, Hedrich et al., 2001, West et al., 2002, Foroud et al., 2003, Nuytemans et al., 2010, Grünewald et al., 2010). Mutations in parkin were first reported in Japanese kindred with cases displaying autosomal recessive juvenile PD (ARJP) (Kitada et al., 1998). Parkin mutations are the most common cause for ARJP, responsible for 50% of ARJP cases under 25 years, and 7% of cases between the ages of 30-45 (Lücking et al., 2000, Periquet et al., 2003).

PINK1

PTEN-induced putative kinase 1 (PINK1) contains an N-terminal mitochondrial targeting sequence, a serine/threonine kinase domain, and a C-terminal domain. PINK1 has a role in mitochondrial maintenance, with close functional links to Parkin. PINK1 is imported into mitochondria, and accumulates on the OMM via TOM7 in association with the TOM complex (Hasson et al., 2013). On the OMM, PINK1 phosphorylates ubiquitin which activates Parkin's ubiquitin ligase activity (Matsuda et al., 2010) to ubiquitinate OMM proteins (Sarraf et al., 2013). This leads to proteasomal degradation of OMM proteins (Tanaka et al., 2010, Chan et al., 2011, Yoshii et al., 2011) and to selective autophagy of damaged mitochondria (Narendra et al., 2008).

PINK1 mutations are the second most common cause of autosomal recessive early onset PD, responsible for 2-4% of Caucasian sporadic PD cases, and between 4-9% of Asian populations The locus containing PINK1 was first identified in 2001 (Valente et al., 2001), within 4 affected members of a large Italian kindred. Identification of the gene and its function remained unknown until 2 mutations of the PINK1 kinase domain were reported in 1 Spanish and 2 Italian families (Valente et al., 2004). The Spanish kindred showed a G309D missense mutation at the kinase domain, and the two Italian families a W437X substitution which truncated the final 145 amino acids of the C-terminus in the kinase domain. To date, 10 missense, nonsense, copy number, frameshift and deletion mutations have been reported (Li et al., 2005, Camargos et al., 2009, Ibáñez et al., 2006, Bonifati et al., 2005, Kumazawa et al., 2008, Hatano et al., 2004). These mutations cause a loss of function effect on PINK1 protein, as recruitment of parkin by PINK1 is abolished which in turn causes accumulation of dysfunctional mitochondria

(Matsuda et al., 2010). Patients present with early onset of disease, slow disease progression, absence of dementia and good response to levodopa treatment. To date, limited neuropathology information has been reported, with brainstem LB pathology and cell loss in the SNc.

DJ-1

Protein deglycase DJ-1 (Daisuke-Junko-1) is a multifunctional protein, involved in transcriptional activation, amino acid repair, molecular chaperone, oxidative stress, and mitochondrial function (Richarme et al., 2015). DJ-1 functions as a redox regulated molecular chaperone which is activated in oxidative environments, and inhibits alpha synuclein fibril aggregation, a function which is abolished in the Leu166Pro mutation (Shendelman et al., 2004, Zhou et al., 2006). DJ-1 is localised to the mitochondria, and scavenges H_2O_2, functioning as an atypical peroxiredoxin-like peroxidase (Andres-Mateos et al., 2007). DJ-1 has been identified as an oxidative stress sensor (Mitsumoto and Nakagawa, 2001, Canet-Aviles et al., 2004). DJ-1 transcriptionally activates PINK1 by binding to Foxo3a, indicating that DJ-1 may modulate the PINK1-Parkin pathway (Requejo-Aguilar et al., 2015). DJ-1 mutations are rare, accounting for 1-2% of early onset PD cases. DJ-1 was first associated with autosomal recessive early-onset PD through the identification of a missense (Leu166Pro) and a large exon deletion mutation found in Dutch and Italian families (Bonifati et al., 2003). DJ-1 loss of function mutations cause abnormal mitochondrial fragmentation, caused by elevated ROS levels (Mitsumoto and Nakagawa, 2001). DJ-1 loss of function mutations cause increased glycolysis and cell proliferation, and one downstream effect may be reduced glutathione expression (Requejo-Aguilar et al., 2015).

GBA1

The glucocerebrosidase (GCase) protein is encoded by the GBA1 gene. GBA is a lysosomal enzyme, required for the metabolism of glucocerebroside (an intermediate of glycolipid metabolism) to ceramide. Homozygous mutations cause Gaucher's disease, a lysosomal storage disorder caused by the accumulation of glucocerebroside in macrophages (Tsuji et al., 1987). The mechanism through which mutations of GBA1 increase PD risk remain unclear. In cultured cortical neurons, loss of GCase leads to reduced protein degradation by lysosomes, causing increased α-synuclein aggregation (Mazzulli et al., 2011). Increased α-synuclein aggregation is also seen in dopaminergic iPS cells derived from patients with Gaucher's disease (Mazzulli et al., 2011). GBA1 mutations have been implicated in impairment of lysosomal activity and clearance of autophagosomes, as well as impairment in mitochondrial activity leading to generation of ROS (Migdalska-Richards and Schapira, 2016) Single heterozygous mutations, most commonly N370S and L444P variants, increase the risk of developing PD 20 to 30-fold and are more commonly present in the Ashkenazi Jewish population (Aharon-Peretz et al., 2004).

ATP13A2

ATP13A2 is an enzyme involved in the transport of inorganic cations such as zinc and manganese. Mutations in this gene cause an autosomal recessive form of PD, also called Kufor-Rakeb syndrome, which presents as early onset parkinsonism with dementia in a total of 11 unrelated families of Jordanian and Chilean heritage (Najim-al-Din et al., 1994, Ramirez et al., 2006). Though the underlying mechanisms

of the mutations are still unclear, 14 mutations have been implicated in impairment of the autophagy-lysosomal pathway and mitochondrial function (Park et al., 2015).

FBX07

The FBX07 gene encodes a member of the F-box protein family, which form a part of the ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box). FBX07 has a role in mitochondrial maintenance via interaction with PINK1 and parkin (Burchell et al., 2013). Loss of function mutations of FBXO7 have been implicated in loss of parkin-mediated mitophagy, resulting in mitochondrial dysfunction (Burchell et al., 2013).

PLA2G6

PLA2G6 encodes a A2 phospholipase enzyme, which functions to metabolize phospholipids. PLA2G6 mutations cause infantile neuroaxonal dystrophy, and neurodegeneration with brain iron accumulation. PLA2G6 cases have been linked to forms of early onset dystonia parkinsonism. However, the underlying mechanisms of PLA2G6 mutations remain unclear (Paisan-Ruiz et al., 2009).

Table 1.4. Autosomal dominant and recessive linked genes in familial Parkinson's disease

Autosomal Dominant PD					
Symbol	Gene	Gene function	Form of PD	Neuropathology	Reference
PARK1	*SNCA*	Encodes alpha synuclein protein. Role in regulating vesicle transmission in presynaptic terminal	Early onset PD	Neuronal degeneration in SNc, widespread LB pathology	(Polymeropoulos et al., 1997)
PARK8	*LRRK2*	Encodes a large protein with multiple domains. Roles include neuronal maintenance, mitochondrial function, and autophagy	Idiopathic PD	Neuronal degeneration in SNc. Tau protein neurofibrillary tangles	(Zimprich et al., 2004, Paisan-Ruiz et al., 2004)
PARK17	*VPS35*	Encodes component of Retromer complex. Role in regulating endosome trafficking and membrane protein recycling	Idiopathic PD	N/A	(Zimprich et al., 2011, Vilariño-Güell et al., 2011)
Autosomal recessive PD					
PARK2	*Parkin*	Encodes an E3 ubiquitin ligase, involved in proteasome-dependent protein degradation	Early onset PD	Neuronal degeneration in SNc, neurofibrillary tangles, Lewy bodies are absent	(Kitada et al., 1998)

PARK6	*PINK1*	Encodes PTEN-induced putative kinase 1. Localised to mitochondria, and regulates mitophagy with close functional links to DJ-1	Early onset PD	Neuronal degeneration in SNc, and brainstem LB formation	(Valente et al., 2004)
PARK7	*DJ-1*	Encodes the multifunctional protein DJ-1. Involved in amino acid repair, stress sensor, redox-activated chaperone, and mitochondrial function	Early onset PD	N/A	(Bonifati et al., 2003)
GBA1	*GBA*	Encodes GBA gene- involved in lysosomal storage	Early onset PD	Cognitive decline	(Sidransky et al., 2009)

1.7 Male sex is a strong risk factor for PD

Whilst the underlying cause for PD remains unclear, known risk factors for PD include aging, environmental and genetic factors. Asides from aging, studies indicate that the male-sex is the most significant risk factor.

1.7.1 Sex differences in clinical and animal models of PD

Clinical basis for sex differences in PD

Clinical and epidemiological studies demonstrate that the male sex is a significant risk factor for PD. Multiple studies report a significantly higher incidence rate of PD in men compared to women, with a relative risk of 1.5-3.7, depending on race and ethnicity (Wooten et al., 2004, Haaxma et al., 2007, Taylor et al., 2007, Baldereschi et al., 2000, Van Den Eeden et al., 2003). In a recent meta-analysis of 27 studies, Hirsch et al. (2016) reports that the incidence of PD is higher in men than women in the 60 to 69 (58.2 vs 30.3 per 100,000 person-years) and 70 to 79 (162.6 vs 93.3 per 100,000 person-years) age ranges. The prevalence of PD is 2-fold higher in men than women (Elbaz et al., 2002), which has been reported to be as high as 3-fold higher in the 50 to 59 age group (Pringsheim et al., 2014). The onset of motor symptoms occurs 2 years later in women than men (Haaxma et al., 2007). Women are more likely to present with milder motor deficits than men, inferring that PD symptoms progress more severely in men than women (Haaxma et al., 2007).

Non-motor symptoms of PD that are more commonly and severely presented in women compared to men include mood and apathy problems, fatigue, sadness, anxiety, depression, constipation, restless leg syndrome, and pain (Martinez-Martin et al., 2012, Solla et al., 2012), whilst daytime sleepiness, salivation problems, rigidity, rapid eye movement behaviour disorder, dexterity and sexual dysfunction

are more severe in men than women (Martinez-Martin et al., 2012, Scott et al., 2000, Baba et al., 2005). In patients with more than 5 years since disease diagnosis, men scored more poorly in cognitive tests than women, showing poorer results in verbal fluency, and facial recognition of emotions, whilst women present with poorer visuospatial recognition (Miller and Cronin-Golomb).

Together, epidemiological and clinical data indicates there is a male-sex bias in PD, with males having a higher incidence, greater symptom severity, and faster progression of PD compared to women.

Animal models of PD replicate sex bias in PD

Sex differences observed in human PD have been replicated in animal models of PD. 6-OHDA-induced lesion of the nigrostriatal pathway in male rats caused a greater loss of nigral DA neurons and depletion of striatal DA levels compared to females (Murray et al., 2003, McArthur et al., 2007, Tamas et al., 2005). Moroz (2003) showed that male rats continued to show greater loss of nigral TH neurons 5 weeks following 6-OHDA lesion of the medial forebrain bundle compared to females, indicating that the sex difference is not due to increased rate of degeneration. Similarly, intraperitoneal MPTP treatment caused greater loss of motor function, nigral DA neurons and striatal DA levels in male mice compared to females (Dluzen and McDermott, 2000, Miller et al., 1998, Dluzen et al., 1996b, Antzoulatos et al., 2010). In contrast, Ookubo et al. (2009) showed that MPTP treatment in mice led to a greater loss of nigral TH protein expression, striatal DA and DAT expression in females compared to males. In their study, Ookubo et al (2009) performed a more severe MPTP lesion (4x 20mg/kg intraperitoneal injections at 2 hr intervals), whereas other studies typically use milder 10mg/kg doses.

Therefore, the variability in their findings may likely be explained by differences in MPTP dose.

Taken together, these findings indicate that animal models of PD reproduce the sex difference seen in epidemiological and clinical studies, which show a male-sex bias in PD.

Sex differences in the healthy and injured DA system

Various clinical and pre-clinical studies have reported clear sex differences in the healthy and injured nigrostriatal DA system. For example, there is an intrinsic sex difference in DA neurons in the SNc, with male rodents having 20% higher number of DA neurons compared to females (Dewing et al., 2006, Murray et al., 2003, McArthur et al., 2007). Although males have higher number of DA neurons than females, there are no sex differences in extracellular DA content (Murray et al., 2003, Robinson et al., 1990, Walker et al., 2000). This suggests that there are compensatory mechanisms in the female DA biosynthesis pathway to maintain equal levels of DA content between sexes. In support, [^{18}F] Fluorodopa- positron emission tomography (PET) analysis (an index of presynaptic dopamine synthesis) showed that healthy premenopausal women have higher DA synthesis capacity compared to men (Laakso et al., 2002). Furthermore, [^{11}C]raclopride PET analysis showed that striatal D2 receptor affinity (K_d) was lower in healthy women compared to men, suggesting that there is increased release of endogenous DA in women (Pohjalainen et al., 1998). Bhatt and Dluzen (2005) reported that baseline striatal DOPAC production is lower in female mice than males, suggesting that females have reduced DA uptake. This sex bias is not due to differences in enzyme activity, as neither in catechol-o-methyl transferase (COMT; an enzyme that methylates DA)

nor monoamine oxidase A or B (enzymes that catalyse oxidation of DA) levels or activity show no differences between male and female mice (Huotari et al., 2002, Unzeta et al., 1994). High frequency electrical stimulation of the medial forebrain bundle in rats induced greater striatal DA release in females compared to males, which was also observed *in vitro* via stimulation of coronal brain slices (Walker et al., 2000). In addition, increased DA terminal and synapse density has been observed in the female embryonic rat caudate nucleus compared to males (Ovtscharoff et al., 1992, Trips et al., 1998), although this has not been studied in adults. In summary, these results indicate there are sex differences in multiple components of the DA biosynthesis pathway, which may underlie a compensatory mechanism to maintain equal overall DA content.

Striking sex differences have also been reported in clinical PD, in particular in cellular pathways involved in PD pathogenesis. For instance, plasma concentrations of α-synuclein are reduced in males with late stage PD compared to male age-matched controls (Caranci et al., 2013). However, such differences in α-synuclein were not observed between women, suggesting that plasma α-synuclein may be a male-specific biomarker for PD (Caranci et al., 2013). Microarray analysis of nigral DA neurons from post-mortem control brains revealed that genes that are involved in signal transduction and neuronal maturation are up-regulated in women, whilst genes that have been implicated in PD pathogenesis (α-synuclein, PINK1) are up-regulated in men (Cantuti-Castelvetri et al., 2007). Studies from Simunovic and colleagues have shown that in the post-mortem PD brain, genes involved in pathways implicated to have a role in PD pathogenesis such as apoptosis, oxidative phosphorylation, and synaptic transmission are downregulated in DA neurons in male PD and controls compared to females, whilst genes related to mitochondrial

respiration were upregulated in male PD and controls compared to females (Simunovic et al., 2009, Simunovic et al., 2010).

Similarly, evidence from experimental models of PD demonstrate robust sex differences in markers and sensors of PD pathogenesis. 6-OHDA treatment in male mesencephalic cultured neurons produced higher levels of ROS, leading to increased oxidative stress- induced apoptotic and necrotic cell death in male neurons compared to females (Misiak, 2010). Baseline levels of PON2, a potent antioxidant located on the mitochondria and expressed in the striatum, is reduced in the healthy male mouse brain compared to females (Giordano et al., 2014). Subcutaneous rotenone treatment in mice led to a greater increase in Iba1 (a marker of active microglia) and TNF-α (a pro-inflammatory cytokine) levels in the female SNc compared to males (Mitra et al., 2015). In addition, intraperitoneal MPTP treatment in mice causes increased iNOS protein expression in the striatum in males compared to females (Joniec et al., 2009). Striatal levels of glutaredoxin (Grx1; a key antioxidant enzyme involved in the maintenance and activity of mitochondrial complex 1) are increased in male mice in response to MPTP treatment, compared to females (Kenchappa et al., 2004). Higher levels of 8-OHdG, a by-product of oxidative DNA damage, are excreted by healthy men compared to women (Loft et al., 1992). Taken together, these studies demonstrate pronounced sex differences in the expression of cellular pathways implicated in PD pathogenesis, which may underlie the male susceptibility to PD.

1.7.2 Biological factors underlying sex differences in PD

The male sex bias in PD has been classically explained by prevailing levels of sex hormones between men and women. In particular, the neuroprotective effect of

oestrogen in females has been clearly demonstrated. However, there is evidence to indicate that genetic factors, including sex-chromosome genes, also play an additional role that contribute to the sex differences in PD.

Hormonal factors

Numerous clinical and pre-clinical studies have demonstrated that 17B-estradiol E2 (oestrogen) is neuroprotective against PD in females. For example, onset of menopause, ovariectomy, withdrawal of hormone replacement therapy, and oestrogen-low periods during the female menstrual cycle have been linked with increased severity of PD symptoms in female PD patients (Quinn and Marsden, 1986, Shulman, 2007, Benedetti et al., 2001). Conversely, increased levels of oestrogen via hormone replacement therapy after menopause leads to reduced PD symptom severity in females (Tsang et al., 2000b, Saunders-Pullman et al., 1999b), and post-menopause oestrogen treatment has been associated with reduced risk of developing PD (Currie et al., 2004).

The neuroprotective potential of oestrogen treatment has also been explored in animal models of PD. Ovariectomy in healthy non-human female primates caused a significant reduction in nigral DA neuron density when compared to intact females (Leranth et al., 2000). Conversely, oestrogen replacement therapy at 10 days post ovariectomy, rescued DA neuron density to above-control levels, suggesting a restorative role for oestrogen (Leranth et al., 2000). Submaximal doses of 6-OHDA or MPTP treatment in ovariectomised female rats and mice caused a greater loss of striatal DA than gonad-intact females, with oestrogen treatment restoring striatal DA to control levels (Dluzen et al., 1996a, Gillies et al., 2004, Murray et al., 2003). The neuroprotective effect of oestrogen is dependent on dose of 6-OHDA, with

oestrogen having no effect with higher doses of 6-OHDA that cause lesions greater than 60% (Murray et al., 2003). In contrast, submaximal 6-OHDA treatment in gonadectomised male rats attenuated losses in striatal DA levels compared to gonad-intact male controls, whilst oestrogen treatment in 6-OHDA- gonadectomised male rats caused striatal DA content to decrease to levels of intact male controls (Murray et al., 2003) indicating that estrogen does not have a neuroprotective effect in males.

On the other hand, exogenous testosterone administration in 6-OHDA-treated gonadectomised male rats had no effect on striatal DA content (Murray et al., 2003, Gillies et al., 2004). Similarly, testosterone treatment had no effect on striatal DA levels in gonadectomised male CD-1 mice lesioned with methamphetamine compared to male controls (Gao and Dluzen, 2001). However, testosterone administration in mice following a mild methamphetamine-induced nigral lesion caused increased DA depletion in gonadectomised and gonad-intact male controls (Lewis and Dluzen, 2008), suggesting that testosterone may have a detrimental role in males. Therefore, the effects of testosterone treatment on DA neurons in males remain unclear, however this may be due to differences in lesion size and length of treatment between studies.

Collectively, these clinical and pre-clinical findings demonstrate the key role of oestrogen as a neuroprotective factor in female DA neurons, whilst on the other hand, hormonal factors may have a detrimental role in males.

Genetic factors

Asides from hormonal factors, recent evidence indicates that genetic differences between sexes can also contribute the sex differences in PD. Beyer et al. (1991)

showed that dissociated rat mesencephalic cells cultured in the absence of sex hormones at E14, show sex differences in the number of DA neurons, with 25% more TH-ir cells in female cultures compared to males. Conversely, embryonic midbrain mouse cells cultured prior to the onset of sex hormone production developed higher endogenous DA levels when cultured from XY rather than XX embryos (Carruth et al., 2002). Dewing et al. (2003) has reported a sex-specific expression of at least 7 genes in the developing male and female brain, with 3 (Xist, Eif2s3x) enhanced in the female brain, and 4 (Dby, Eif2s3y, Cyp7b, rorα4) enhanced in the male brain when cultivated at 10.5 *dpc* (prior to the onset of sex hormone expression).

San Luciano et al. (2012) studied single nucleotide polymorphisms of the IL-6 promoter (-174G>C) and estrogen receptor beta (1730G>A) in caucasian male and female PD patients, and showed that GG phenotype of the -174G>C mutation increased the risk of PD two-fold in non-Jewish men with PD, which was not seen in females. A prior study reported increased risk of PD due to the -174G>C IL-6 promoter mutation was most pronounced in patients with early onset PD (Håkansson et al., 2005). Additionally, AA phenotype of the 1730G>A mutation reducing the risk of PD by half in the female population with Ashkenazi Jewish descent (San Luciano et al., 2012).

These findings support the notion that innate sex-specific genetic differences in the nigrostriatal DA pathway may also contribute to the sex differences in PD. Classically, the neuroprotective effects of oestrogen were thought to solely underlie the sex differences in PD. However, recent evidence demonstrate that genetic factors, such as sex-chromosome genes (Carruth et al., 2002, Dewing et al., 2006, Dewing et al., 2003, McCarthy and Arnold, 2011), contribute to inherent sex

differences in the DA pathway, which could explain the male sex bias in PD. One ideal candidate of study is the Y-chromosome gene, *SRY*, based on its role in male-sex determination, its localisation in male DA neurons, and its role in regulating motor function in males (Dewing et al., 2006).

1.8 Y-chromosome gene *SRY*: A risk factor for male PD?

1.8.1 *SRY* is the key male-sex determining gene

SRY (sex-determining Region of the Y chromosome) is the key gene in the male sex-determining pathway located on the Y chromosome, which encodes a male specific transcription factor (Fig 1.9) (Sinclair et al., 1990). SRY expression is sufficient to cause sex reversal in XX transgenic mouse embryos (Koopman et al., 1991). *Sry* mutations result in development of the female phenotype, indicating that DNA binding activity is required for testis formation (Harley et al., 1992). *SRY* is an intronless gene encoding a 204 amino acid length protein (Su and Lau, 1993). *SRY* contains a DNA-binding High Mobility Group (HMG) box and shares homology with other HMG box-containing SOX genes (Sinclair et al., 1990). Due to the structure of the HMG box, SRY binds to linear DNA and causes a sharp bend (Ferrari, 1992).

SRY binds to and activates the testis specific enhancer of *Sox9* (*TESCO*) synergistically with steroidogenic factor 1 (SF1) (Sekido and Lovell-Badge, 2008, Sekido, 2004). SF1 and SOX9 then cooperatively maintain *Sox9* expression. Once sufficient SOX9 levels are reached, auto-regulation of *Sox9* initiates, and the precursor cells differentiate into Sertoli cells, facilitating testis formation. In the absence of *Sry*, testes do not form and the female phenotype is switched on, initiating development of ovaries (Sekido and Lovell-Badge, 2008). In the gonad, SRY expression is regulated by growth arrest and DNA damage gamma (GADD45γ), a stress response protein expressed in gonadal somatic cells (Gierl et al., 2012, Warr et al., 2012). In brief, the signalling cascade involves GADD45γ alongside MAP3K4 promoting p38MAPK signalling, which phosphorylates and activates GATA4. GATA4 in turn binds to and transactivates the SRY promoter, to initiate the male sex pathway (Gierl et al., 2012). Humans and mice have one copy

of *SRY* on the Y-chromosome, and rat strains have 6 copies. All loci are functional, and multiple copies are expressed in the testis and adrenal glands at different ages (Turner et al., 2007).

SRY is expressed between 10.5 to 12.5 days *post coitum* in the bipotential gonad of the mouse and 6 weeks post gestation in humans, where it is expressed in the genital ridges (Bernard et al., 2012). SRY initiates a male specific molecular pathway, but also represses the female pathway (Harley et al., 1992).
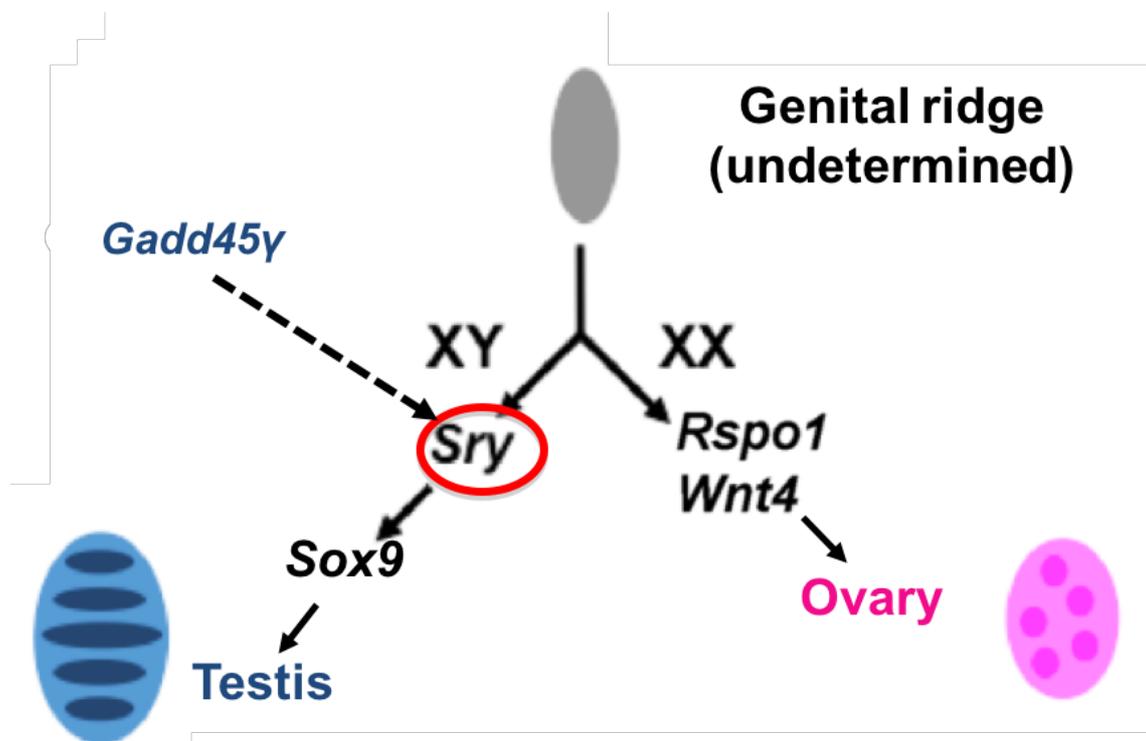
Figure 1.9. *SRY* is the key male-sex determining gene. SRY binds to and activates the testis specific enhancer of *Sox9* to initiates testis formation. In the absence of SRY, testes do not form and the female phenotype is switched on, to initiate development of ovaries. GADD45γ is a known DNA damage marker, and regulates SRY via the MAP3K4/ p38MAPK pathway. Reproduced from Matson et al (2011).

1.8.2 SRY expression and function in non-reproductive tissues

Classically, the role of *SRY* was thought be limited to sex determination, due to its function in testes development. However, *Sry* mRNA and protein is also expressed in non-reproductive tissues such as the heart, adrenal glands, liver, lungs, kidneys and brain in males (Milsted et al., 2004, Clepet, 1993, Dewing et al., 2006), indicating that SRY may have a role outside of male sex determination (Fig 1.10A). In the liver, SRY directly binds to promoter region of the SAGA complex associated factor 29 (Sgf29) gene, a gene involved in c-Myc-mediated hepatocarcinogenesis (Murakami et al., 2014a). Knockdown of SRY in rodent male hepatocellular carcinoma (rHCC) cell lines reduces invasiveness and tumoregenicity of rHCC cells, demonstrating a role for SRY as an oncogenic protein (Murakami et al., 2014a). SRY is overexpressed in male HCC patients, and *Sry*-overexpressing male mice are more susceptible to N-nitrosodiethylamine-induced hepatocarcinogenesis (Liu et al., 2017).

Peripheral expression of SRY has also been implicated to have a role in catecholamine-dependent functions in males (Ely et al., 2010). Viral vector-mediated overexpression of *Sry* in the adrenal medulla of male normotensive WKY rats increases adrenal TH and plasma noradrenaline levels, which led to an increase in blood pressure compared to controls (Ely et al., 2007). In addition, *Sry* over-expression in the kidney in male WKY rats increased renal TH, plasma noradrenaline content, urinary dopamine and increased blood pressure compared to controls (Ely et al., 2009). To identify downstream blood-pressure-mediating target genes of SRY, Milsted et al. (2010) examined genes of the renin-angiotensin system (RAS), which are known to regulate blood pressure. *Sry*-expressing plasmid vectors were transfected into Chinese hamster ovary (CHO-K1) cells (which

endogenously express renin-angiotensin system genes) that contained luciferase reporter constructs of rat angiotensinogen, renin, ACE, and ACE2 genes. They showed that SRY expression upregulated promoter activity of angiotensinogen, renin, and ACE, and downregulated ACE2 activity, to suggest that SRY regulates blood pressure via the renin-angiotensin pathway in males, which could underlie sex differences in hypertension (Ely et al., 2010).

In summary, in addition to the gonads SRY is also expressed in non-reproductive tissues, where it mediates non-reproductive functions such as regulation of catecholamine levels and blood pressure in males.

1.8.3 SRY is expressed in the male brain

Initial studies revealed that SRY is expressed in several regions of the male human and rodent brain, including the pre-frontal cortex (PFC), temporal cortex, and hypothalamus (Mayer et al., 1998, Lahr et al., 1995). Subsequent quantitative PCR and in situ hybridization studies in rodents demonstrated that *Sry* mRNA is expressed in brain regions rich in catecholaminergic bodies and nerve terminals, such as the SNc, ventral tegmental area (VTA), locus coeruleus (LC) and hypothalamus in males (Dewing et al., 2006, Milsted et al., 2004) (Fig 1.11B). In the rodent male SNc, SRY co-localized with a subpopulation of TH -positive neurons, in both the nuclei (expected site of SRY function) and the cytoplasm (Dewing et al., 2006). Consistent with the results in rodents, SRY protein was localized in both nuclei and cytoplasm of TH positive cells in the post-mortem human male, but not female, SNc (Czech et al., 2012) (Fig 1.10). SRY also co-localised with TH-positive neurons in the VTA, adjacent to the SNc. VTA is crucial for mediating reward and addictive behaviours (Wise, 2009). Thus, the expression of SRY in the VTA may

explain sex differences originating in this region (Gillies et al., 2014b). Moreover, SRY protein co-localised with GAD-67 (a marker of GABAergic neurons) positive neurons in the SNr (Czech et al., 2012), indicating a role for *SRY* for both DA and GABA-mediated functions in the male brain.

Mayer et al. (2000) showed that in the male mouse brain, *Sry* transcripts are in a circular (i.e. untranslatable) form during the pre-natal period (e11-e19) whilst postnatally, *Sry* transcripts changed to its active linear form. This result indicates that *Sry* is developmentally regulated, and that based on its post-natal linear form, SRY could have a function in the male brain.

Taken together, SRY is expressed in brain regions such as the SNc, VTA and PFC, where it co-localises with DA and GABAergic neurons. Given that degeneration or dysfunction of these brain regions underlie neurological disorders such as PD, Attention deficit hyperactivity disorder (ADHD) and schizophrenia, dysregulation of SRY may explain the male susceptibility to these disorders.
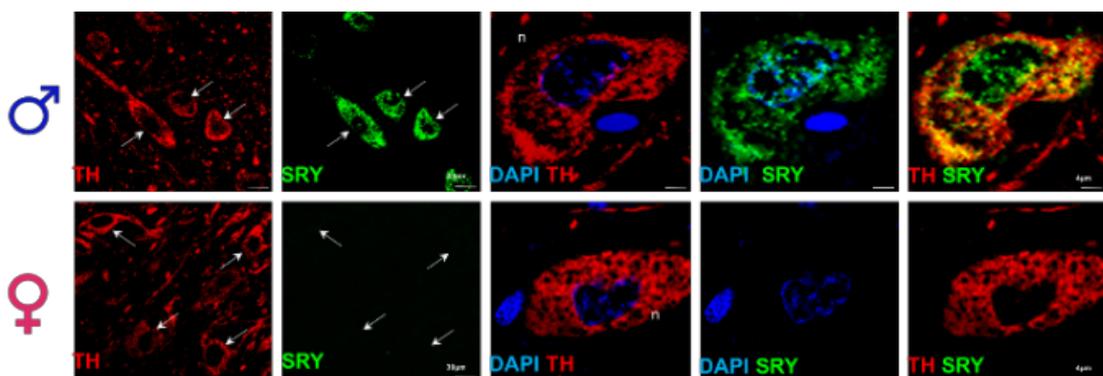
Figure 1.10. SRY co-localizes with DA neurons in the human male SNc. TH+ and SRY+ neurons in the human post-mortem male and female SNc (Czech *et al.,* 2012).

Figure 1.11. A: Distribution and function of SRY in the brain and peripheral tissues in males. **B:** Distribution of *Sry* mRNA in noradrenergic (left) and dopaminergic (right) pathway in the rodent brain. Line drawings of sagittal sections of the rat brain, depicting *Sry* mRNA (blue circle), and catecholaminergic cell bodies (red dot) and axon projections (red line and arrow). The sites depicted are taken from observations in rat and mouse (Xu et al., 2002, Zhang et al., 2010, Ely et al., 2007). DA, dopamine; FC, frontal cortex; HC, hippocampus; HY, hypothalamus; LC, locus coeruleus; MAO-A, monoamine oxidase A; ME, median eminence; MFB, medial rebrain bundle; NA, noradrenaline; RAS, renin-angiotensin system; ST, striatum; SN, substantia nigra; TH, tyrosine hydroxylase VTA, ventral tegmental area. Reproduced from Lee and Harley (2012).

1.8.4 SRY regulates dopamine biosynthesis in males

In line with the localization of SRY protein in catecholamine neurons, SRY has been implicated to have a role in the control of DA biosynthesis in male DA neurons, *in vitro* and *in vivo*. Reporter assays in rat-derived PC12 cells found that SRY regulates the transcription of *TH* through binding at the AP1 site of the TH promoter region (Milsted et al., 2004). Additionally, Wu et al. (2009) found evidence for a functional SRY binding site on the promoter of MAO-A (an enzyme that degrades DA) in a human neuroblastoma BE(2)C cell line, to show that SRY directly regulates MAO-A transcription (Wu et al., 2009). Collectively, these studies suggest a role for SRY in the regulation of DA synthesis and catabolic genes in male DA neurons. SRY has been reported to interact with a functional binding site on the ER-β promoter, to down-regulate ER-β transcription *in vitro* (Tao et al., 2012).

Czech et al. (2012) showed that reducing SRY levels via siRNA in a human male DA cell line M17 (which expresses endogenous SRY and DA machinery) was associated with a marked reduction in *TH*, *MAO-A*, dopa-decarboxylase *(DDC)*, and dopamine ß-hydroxylase (*DBH*) mRNA (all components of DA biosynthesis pathway), compared to non-specific siRNA treated cells. Conversely, SRY over-expression via *SRY* plasmid vectors in M17 cells significantly increased levels of *TH* (3-fold) and *MAOA* (2.5 fold) mRNA and protein levels, as well as elevated *DDC* and *DBH* levels, compared to control vectors. As a consequence of this up-regulation, SRY over-expression increased extracellular DA levels (Czech et al., 2012). Taken together, these studies indicate that *SRY* positively regulates multiple components of the DA biosynthesis and metabolic pathway to maintain DA levels in males, indicative of a male-specific, hormone-independent genetic mechanism for DA regulation.

1.8.5 The role of *SRY* in the healthy and injured DA pathway in males

The physiological role of SRY in the male brain was first demonstrated by a paper by Dewing et al. (2006). Dewing and colleagues assessed the consequence of reducing nigral SRY expression, via daily repeated infusion of *Sry* antisense oligodeoxynucleotides (ASO) in male rats. Repeated ASO-infusion led to a reduction in the numbers of TH-positive neurons and TH protein levels in the SNc compared to the control side. The ASO-mediated reduction of TH-positive neurons was associated with significantly reduced motor deficits in the contralateral forelimb, in both the akinesia and limb-use asymmetry tests compared to controls. The ASO-mediated effect on TH-positive neurons were transient and reversible, upon withdrawal of ASO-infusion, which indicates that motor deficits were due to down-regulation of DA transmission as opposed to cell death. Importantly, these locomotor and biochemical changes were not seen in female rats, indicating that the effect of *Sry* ASO-infusions were male-specific. Thus, these results showed that SRY positively regulates the nigrostriatal DA pathway, and consequently voluntary movement in healthy males.

Given the role for SRY in regulation of DA biosynthesis and voluntary movement, and the susceptibility of males to PD, our group assessed the regulation of SRY in a toxin-induced *in vitro* model of PD (Czech et al., 2014). In response to 6-OHDA treatment, endogenous *SRY* mRNA expression was highly increased (9-fold) in human M17 cells, which was most pronounced at lower concentrations of 6-OHDA - i.e. before cells were undergoing active cell death. *SRY* up-regulation was concomitant with an increase in *GADD45*γ mRNA, a DNA damage inducible factor and a regulator of *SRY* in the developing male gonad. The p38 antagonist, SB202190, significantly reduced 6-OHDA-mediated up-regulation of *SRY*, whilst the

p38 agonist, anisomycin increased *SRY* expression, indicating that GADD45γ-mediated phosphorylation of the p38-MAPK is required for *SRY* up-regulation in a human male DA cell line (Czech et al., 2014). These results show that *SRY* is up-regulated in response to DNA damage in an *in vitro* model of PD, suggesting a role for SRY in PD pathogenesis.

Based on the findings that:

- *SRY* co-localizes with DA neurons in the SNc in males

- *SRY* regulates multiple components of the DA machinery in males

- *SRY* regulates motor function in males

- *SRY* is up-regulated in an *in vitro* model of PD

I hypothesize that:

> **Dysregulation of the Y chromosome gene, *SRY*,**
> **contributes to the susceptibility of males to PD.**

1.9 Aims

The aims of this thesis are to:

1. Determine the regulation and function of *Sry* in acute toxin-induced rat models of PD (Chapter 2)

2. Determine the regulation and function of *Sry* in the chronic rotenone rat model of PD (Chapter 3)

3. Assess human SRY expression in post-mortem male Parkinson's disease and age-matched control brains (Chapter 4)

Chapter 2:
The regulation and function of *Sry*
in acute toxin-induced rat models of Parkinson's disease

2.1 Introduction

Parkinson's disease is a progressive, neurodegenerative disorder characterized by symptoms of bradykinesia (slowness in movement), akinesia (inability to initiate movement) shuffling gait, stooped posture and resting tremors (Lang and Lozano, 1998). The motor symptoms of PD emerge when the progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) exceeds more than 50% (Fearnley and Lees, 1991, Lang and Lozano, 1998). Whilst current therapies based on DA replacement strategies effectively treat motor symptoms, they do not slow down or halt the progression of PD which highlights the unmet need for development of disease-modifying therapies.

The underlying cause(s) of PD are not well understood. The genetic form of PD account for 10 to 15% of PD cases such as mutations that cause monogenic forms of PD and susceptibility polymorphisms that increase risk of PD (Klein and Westenberger, 2012). However, the remaining 85% of PD cases have an idiopathic origin, which suggests that the aetiology of PD could arise from a combination of genetic and environmental factors (Klein and Westenberger, 2012). Epidemiological studies have shown that environmental predisposing factors such as aging (Nussbaum and Ellis 2003, de Lau and Breteler, 2006) and exposure to pesticides (Semchuk et al., 1992, Ascherio et al., 2006) increase the risk of developing PD, whilst protective factors such as cigarette smoking, coffee consumption and use of nonsteroidal anti-inflammatory drugs (Hernán et al., 2002, Chen et al., 2003) have been shown to reduce the risk of PD.

Aside from aging, the male-sex is the most significant risk factor for PD. There is a clear male-sex bias in PD, as men have a 2-fold higher incidence of developing PD, and 1.5 to 3.7-fold greater prevalence of PD compared to women (Baldereschi

et al., 2000, Wooten et al., 2004, Pringsheim et al., 2014, Van Den Eeden et al., 2003). Men with PD also have an earlier age of onset (Haaxma et al., 2007, Wooten et al., 2004), and faster disease progression (Haaxma et al., 2007) than women with PD. Evidence from animal models of PD reproduce the sex differences observed in human PD. For instance, 6-OHDA-induced loss of nigral DA neurons and depletion of striatal DA levels are more pronounced in male rodents compared to females (Murray et al., 2003, McArthur et al., 2007, Tamas et al., 2005). Similarly, MPTP treatment cause greater losses in motor function, nigral DA neurons and striatal DA levels in male mice compared to the female counterparts (Dluzen and McDermott, 2000, Miller et al., 1998, Dluzen et al., 1996b, Antzoulatos et al., 2010). Thus, a better understanding of the biological mechanisms underlying the male susceptibility in PD may help identify novel mechanism(s) underlying nigral DA cell loss.

The prevailing view of sex differences in PD is that it arises solely from the neuroprotective actions of oestrogen in females. For instance, onset of menopause, bilateral ovariectomy, and oestrogen-low periods during the menstrual cycle, and removal of oestrogen replacement therapy have been associated in with increased PD symptom severity in women with PD (Quinn and Marsden, 1986, Shulman, 2007, Benedetti et al., 2001, Sandyk, 1989). Oestrogen replacement therapy in post-menopausal women with PD can reduce severity of symptoms if given during early stages of PD (Tsang et al., 2000b, Saunders-Pullman et al., 1999b), and has been associated with decreased risk of developing PD (Currie et al., 2004). Similarly, neuroprotective actions of oestrogen have also been demonstrated in animal models of PD. For instance, ovariectomy in healthy non-human female primates caused reduced density of nigral DA neurons compared to intact females

(Leranth et al., 2000), whilst oestrogen replacement in ovariectomised females rescued nigral DA cell density to above-control levels, suggesting a restorative role for oestrogen in females (Leranth et al., 2000). 6-OHDA or MPTP treatment in ovariectomised female rodents leads to greater reduction in striatal DA levels compared to intact females, which are restored to control levels via oestrogen treatment (Dluzen et al., 1996a, Gillies et al., 2004, Murray et al., 2003). In contrast to the well-established protective influence of oestrogen on female DA neurons, the effect of exogenous or circulating hormones on adult male DA neurons is minimal if not harmful (Dluzen et al., 1994, Murray et al., 2003, Tamas et al., 2005, McArthur et al., 2007).

Emerging evidence, however, suggests that sex-specific genetic factors also contribute to the inherent sex differences in the healthy and diseased DA system (Carruth et al., 2002, Davies, 2014, McCarthy and Arnold, 2011). For instance, studies in cultured midbrain DA neurons (which lack sex hormones in the media) indicate that sex differences in the total number of DA neurons are independent of differences in sex hormones (Beyer et al., 1991, Lieb et al., 1995, Carruth et al., 2002). Microarray analysis of single cell laser captured DA neurons from human SNc sections revealed that the expression of PD pathogenesis genes (e.g. α-synuclein, PINK-1) was higher in males (Cantuti-Castelvetri et al., 2007), whilst the expression of genes associated with oxidative phosphorylation and synaptic transmission was lower in the SNc of male, compared to female PD patients (Simunovic et al., 2010). Thus, detriment in male nigrostriatal function is unlikely to be explained by lower oestrogen levels, but it may rather reflect a cell-intrinsic property of the single male DA neuron. Although the genetic factors driving the sex dimorphism in the nigrostriatal DA system are unknown, sex-linked genes are ideal

candidates. I propose that the Y-chromosome gene, *Sry*, contributes to this intrinsic susceptibility of male DA neurons, predisposing males to developing PD.

Sex-determining region Y (SRY) protein is a transcription factor encoded by the *Sry* gene that is responsible for the initiation of male sex determination in mammals. (Sinclair et al., 1990, Koopman et al., 1990). In the embryonic bipotential gonad, SRY transcriptionally activates the testis specific enhancer of *Sox9* (*TESCO*) synergistically with steroidogenic factor 1 (SF1) (Sekido and Lovell-Badge, 2008), to initiate differentiation of precursor cells into Sertoli cells (Sekido and Lovell-Badge, 2008). SRY expression is regulated via a signalling cascade that involves Growth arrest and DNA-damage-inducible protein 45 (GADD45γ; a stress response sensor protein and DNA damage marker) and Mitogen-activated protein kinase 4 (MAP3K4) that together transduce the p38-MAPK pathway, which phosphorylates and activates GATA binding protein 4 (GATA4). GATA4 then binds to and activates the SRY promoter to initiate male sex determination (Gierl et al., 2012, Warr et al., 2012).

Sry is also expressed in the non-reproductive tissues of adult males such as the heart, adrenal glands, kidneys and brain (Clepet et al., 1993). In the male brain, SRY is expressed in dopamine-abundant regions such as the SNc and ventral tegmental area (Czech et al., 2012, Dewing et al., 2006) where it co-localises with DA neurons (Fig.1.10) (Czech et al., 2012). Furthermore, SRY regulates *in vitro* transcription of DA biosynthesis and catabolism enzymes, tyrosine hydroxylase (TH) and monoamine oxidase-A (MAO-A) (Czech et al., 2012, Wu et al., 2009, Milsted et al., 2004). Direct actions of *Sry* in the male brain were first demonstrated by Dewing et al. (2006), which assessed the consequence of reducing SRY expression in the rat SNc, via repeated injection of *Sry* antisense oligonucleotides, on nigrostriatal DA

and motor function in males. Knockdown of nigral SRY led to a male-specific reduction in TH-positive neurons in the SNc, and consequently reduced motor function. Together, these results showed that SRY directly regulates the nigrostriatal DA pathway and voluntary movement in healthy males, independent of hormonal influences.

Given the role of SRY in male DA neurons, our group investigated whether *Sry* expression was dysregulated in an experimental model of PD (Czech et al., 2014). Treatment with the dopamine toxin 6-OHDA highly increased *Sry* mRNA expression in the human male cell line, M17. The *Sry* up-regulation was concomitant with increased *Gadd45γ* expression, a known regulator of SRY in the embryonic gonad (Gierl et al., 2012, Warr et al., 2012), as well as a marker for DNA-damage induced stress. The up-regulation of *Sry* was most pronounced at lower concentrations of 6-OHDA - i.e. before DA cells were undergoing active cell death. 6-OHDA treatment led to a steady increase in GADD45γ and phosphorylation of p38-MAPK protein that preceded the increase in SRY protein levels. Moreover, treatment with the p38 antagonist, SB202190 diminished 6-OHDA-mediated up-regulation of *Sry*, whilst the p38 agonist, anisomycin enhanced 6-OHDA induced *Sry* expression, indicating that phosphorylation of p38-MAPK is required for *Sry* up-regulation in male DA cells (Czech et al., 2014). Together, these results indicate that *Sry* up-regulation is mediated via the p38-MAPK pathway in an *in vitro* model of PD, suggesting a role for SRY in the pathogenesis of PD.

Based on the findings that:

- SRY co-localizes with DA neurons in the male SNc (Czech et al., 2012, Dewing et al., 2006)

- *Sry* regulates multiple components of the DA machinery in the male SNc (Czech et al., 2012, Wu et al., 2009, Milsted et al., 2004)

- SRY regulates voluntary movement in males (Dewing et al., 2006)

- *Sry* is up-regulated in experimental models of PD (Czech et al., 2014).

I hypothesise that:

- In the healthy male SNc, SRY positively regulates DA biosynthesis and consequently voluntary movement.

- In the injured male SNc, up-regulation of SRY expression is detrimental and exacerbates the loss of DA neurons in males.

To test these hypotheses, the aims of Chapter 2 are:

1. To assess the effect of reducing nigral SRY expression on nigrostriatal DA biosynthesis and motor function in healthy male rats

2. To assess the effect of reducing nigral SRY expression on nigrostriatal and motor degeneration in acute toxin-induced rat models of PD.

2.2 Materials and Methods

Animals

All methods conformed to the Australian NHMRC published code of practice for the use of animals in research and were approved by the Monash University Animal Ethics Committee (MMCB13/02 and MMCB15/18). Adult Long-Evans male and female rats weighing between 280 and 350 g were used. Animals were housed in a 12h light: dark cycle room and had access to food and water *ad libitum.*

Stereotaxic implantation of cannula in the rat SNc

Unilateral guide cannula (22 gauge, Plastics One) directed at the right SNc was implanted at 5.3 mm posterior, 2mm lateral from bregma, and 6.0 mm ventral to the surface of dura. The guide cannula was secured to the skull with stainless-steel screws and dental cement. Dummy cannulae that protruded <0.5 mm beyond the opening were placed in the guide cannulae.

Repeated intranigral *Sry* antisense oligonucleotide (ASO) infusions in rats

Nigral SRY expression in male rats was reduced by repeated intranigral *Sry* ASO-infusions, as previously described (Dewing et al., 2006). *Sry* ASO used for infusion was a cocktail of 3 distinct ASO designed to correspond to the rat *Sry* initiation codon at 3 distinct positions for optimal knockdown efficacy (Table 2.1) and added in equal proportions (described by Dewing et al., 2006), whilst the control sense oligonucleotide (SO) cocktail corresponded to the 3 complement sequences of the three ASO (Table 2.1). ASO (or SO) were HPLC-purified (Invitrogen) and dissolved in artificial cerebrospinal fluid (aCSF) to a final concentration of 2μg/μL. Intranigral ASO (or SO) infusions were made at a rate of 0.5μL/min followed by a 2 min

equilibration period, during which the needle remained in place. All rats were infused unilaterally with ASO or SO daily (2µg in 1µL in aCSF) for 10 consecutive days.

Oligonucleotide	Sequence	Target Region of mRNA
ASO 1	GCGCTTGACATGGCCCTCCAT	+1 to +21
ASO 2	CATGGGGCGCTTGACATGGCCCT	+5 to +27
ASO 3	GGCCCTCCATGCTATCTAGA	-10 to +10
SO 1	ATGGAGGGCCATGTCAAGCGC	+1 to +21
SO 2	AGGGCCATGTCAAGCGCCCCATG	+5 to +27
SO 3	TCTAGATAGCATGGAGGGCC	-10 to +10

Table 2.1. Base sequences and positions of rat antisense (ASO) and sense (SO) oligonucleotides. Blue indicates that the residue is phosphorothioated.

Toxin-induced rat models of PD

Regulation of Sry in toxin-induced rat models of PD

To determine the regulation of SRY expression in *in vivo* models of PD, the effects of DA toxins 6-hydroxydopamine or rotenone were assessed in male rats. In brief, a single nigral injection of 6-OHDA (30µg dissolved in 1.5µL of 0.1% ascorbic acid saline) or rotenone (30µg in dissolved 1.5µL of 1% DMSO/saline) was administered into the right SNc in male rats. Motor function was assessed by the limb-use asymmetry test at various time points post-surgery (0, 1, 2, 4, 7, 14 and 21 days post 6-OHDA or at 0 or 7 days post rotenone). At each behavioural time point, rats were killed and the brains were processed for measurement of nigral mRNA levels (Study performed by Hannah Loke and used with permission).

Function of Sry in toxin-induced rat models of PD

To determine the function of SRY in PD models, the effect of reducing nigral SRY expression in the 6-OHDA or rotenone-induced rat models of PD was assessed. One group of male rats received a single nigral injection of 6-OHDA (30µg) into the right SNc in male rats at 4 hours prior to the first *Sry* ASO-infusion, whilst another group of male rats received a single 6-OHDA injection at 4 hours following the last *Sry* ASO-infusion. A third group of male rats received a single nigral injection of rotenone (30µg) into the right SNc at 4 hours prior to the first *Sry* ASO-infusion. A group of female rats received a single intranigral injection of 6-OHDA (30µg) at 4 hours prior to the first *Sry* ASO-infusion as negative controls. Motor behaviour was assessed at pre-infusion (pre), 2 hours following the last *Sry* ASO-infusion (day 0), and at 7, 14, and 21 days post 6-OHDA or rotenone injection. Animals were culled at 21 days post 6-OHDA or rotenone injection for nigrostriatal mRNA and protein measurements.

Motor behaviour

Limb-use asymmetry test

The limb-use asymmetry test assessed spontaneous forelimb usage during vertical explorations in rats that have received unilateral drug injection. Motor impairment is indicated by a reduction in limb-use contralateral (i.e. left) to the site of drug injection (Fig. 2.1B). The rat was lowered into a clear cylinder and forelimb contacts during vertical explorations were video recorded until a total of 30 touches were reached. The data was expressed as the percentage of left (impaired) forepaw contacts; where symmetric paw use (left ≈ right) was a measure of unimpaired limb use.

Rotarod test

Rotarod test evaluated balance and motor coordination of rodents by assessing the ability of rodents to stay balanced on a rotating platform (Ugo Basile, Italy; Fig.2.1C). Briefly, animals were trained for 3 consecutive days before the day of testing at a fixed speed (10 rpm) for 5 minutes. On the day of the testing, rats were placed on the moving rotarod which accelerated from 10 to 40 rpm over 3 minutes. The time after which the rat fell was determined and 4 experimental readings were averaged to obtain a single value for each animal.

Amphetamine-induced rotational test

Rotational behaviour was measured by placing rats in a circular cage where they were tethered to an automated rotometer system and injected with amphetamine (2 mg/kg, intraperitoneally; Fig. 2.1D). The total number of rotations was measured for 90 mins at 10 minute intervals. The data are expressed as net rotations per minute, where rotation toward the side of the lesion was given a positive value.
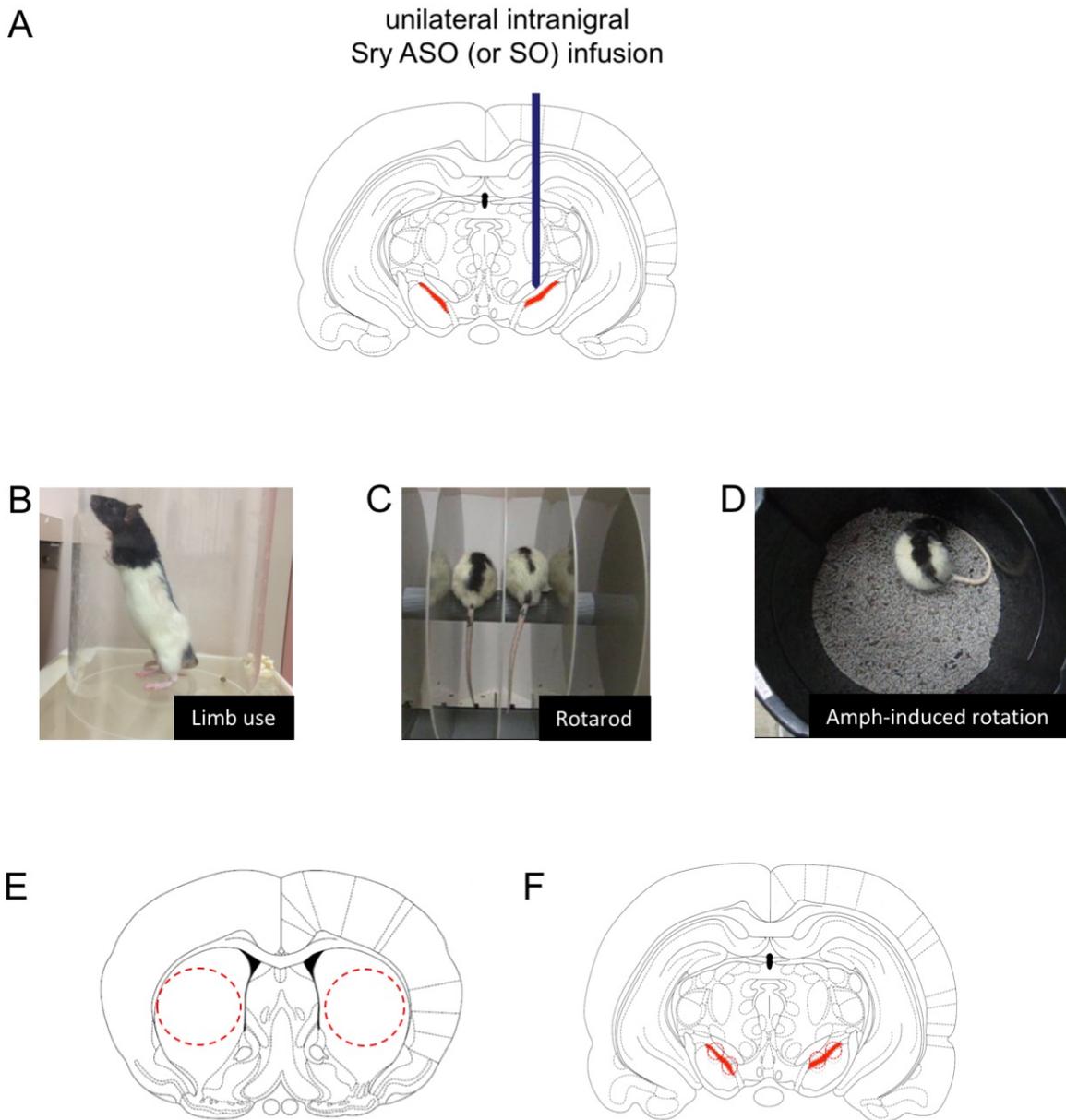
Figure 2.1. A) Coronal section of the rat midbrain showing the placement of the guide cannula above the right SNc (red) for repeated unilateral *Sry* antisense oligonucleotide (ASO) or control sense oligonucleotide (SO) infusions. Photos of **B)** the limb-use asymmetry test and **C)** the rotarod test. **(D and E)** Coronal sections of the **D)** rat striatum for DA/DOPAC measurements, and **E)** rat SNc for nigral mRNA and protein measurements.

Tissue processing and Histology

Rat brains were either intracardially perfused and processed for immunohistochemistry or isolated fresh and processed for western blot or qRT-PCR. Coronal sections were cut serially through the striatum (16µm thickness; Fig. 2.1E) and SNc (20 or 40µm thickness; Fig. 2.1F) and stored at -80°C for immunohistochemistry (n≥7/group). Between each series, a 200µm slab was collected to isolate SNc tissue for nigral mRNA (n=10/group) or nigral protein measurements via western blot (n≥12/group), or striatal tissue for DA and DOPAC measurements (n=10/group).

Western blotting

Total protein was isolated from the rat SNc and amount of protein was quantified by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 10 µg of protein was run on a 10% acrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Primary sheep anti-TH primary antibody (1:1000, Pelfreez) was applied overnight at 4°C. Secondary AlexaFluor488 antibody (1:200, Invitrogen) was incubated for 1h at room temperature. PVDF membranes were scanned using a Typhoon Trio variable mode imager (GE Healthcare).

Measurement of striatal DA and DOPAC

The striata was isolated and the levels of dopamine and DOPAC were measured, as described previously (Lee et al., 2008). In brief, the striata was homogenized and sonicated in perchloric acid (0.45N). The homogenate was centrifuged at (15,000*g*, 10 min, 4°C) and the supernatant was filtered through a syringe filter (0.22µm). DA and DOPAC (a metabolite of DA) were separated and quantified in the filtrate.

Filtrate (20µL) was manually injected into reverse Phase C–18 high performance liquid chromatography (HPLC) column coupled with electrochemical detector (Waters, Milford, Massachusetts). The striatal concentrations of DA and DOPAC were calculated as ng/mg tissue, and expressed as percentage of control (left) side.

TH and DAT immunohistochemistry and stereology

TH immunohistochemistry was performed by incubating 40µm-thick SNc sections in sheep anti-TH primary antibody (Pelfreeze, 1:2000, overnight at 4°C), followed by a biotinylated secondary antibody (goat, anti-sheep IgG, 1:1000, Vector Labs, USA) and reacted with cobalt and nickel-intensified diaminobenzidine (DAB, Sigma-Aldrich). DAB-immunostained sections were counterstained with neutral red. DAT immunohistochemistry was performed by incubating 16µm-thick striatal sections in rat anti-DAT primary antibody (Chemicon, 1:2000, 78 hrs at 4°C) followed by a biotinylated secondary antibody (rabbit, anti-rat IgG, 1:500, Vector Labs) and reacted with DAB. DAB-immunostained sections were analysed by bright-field microscopy, using an Olympus microscope equipped with Olympus cellSens image analysis software (v.1.7.1). TH-immunoreactive and neutral-red positive cell bodies or DAT-immunoreactive terminals were quantified stereologically on 5 regularly spaced sections covering the whole SNc or striatum. The fractionator design for estimating the number of TH-immunoreactive neurons and the number of striatal DAT-immunoreactive axonal varicosities were performed as previously (Lee et al., 2008, Stanic et al., 2003).

TOM-20 and TH co-immunofluorescence

TOM-20 and TH co-immunofluorescence was performed by incubating 5 regularly spaced 20μm-thick sections covering the whole SNc in sheep anti-TH and rabbit anti-TOM-20 primary antibody overnight at 4°C (anti-TH, 1:1000, Pelfreeze; anti-TOM-20, 1:200, Santa Cruz), followed by a 1hr incubation in donkey anti-rabbit 594 and donkey anti-sheep 488 (1:500, 1hr at RT, Invitrogen) and DAPI (1:1000, Invitrogen). Immunofluorescence staining was visualised by confocal microscopy (Nikon C1 macro laser confocal, Shinagawa-ku, Tokyo, Japan) equipped with NIS-Elements image software. For quantification of TH, TOM-20 and DAPI-positive labelling, confocal images at 20x magnification of SNc were analysed using IMARISx64 software (v7.6.5). Fluorescent intensity values of TH and TOM-20 above background threshold were given for all DAPI positive cells, and were quantified for co-immunostaining.

TUNEL and iNOS staining

TUNEL staining was performed by incubating 5 regularly spaced 20μm-thick sections covering the whole SNc in TdT enzyme and nucleotide mixture (1hr at 37°C, In Situ Cell Death Detection Kit, Roche), followed by incubation in anti-fluorescein antibody-peroxidase POD (30min at 37°C, Roche) reacted with cobalt and nickel-intensified DAB. Sections were counterstained with cresyl violet (Sigma-Aldrich Inc). iNOS immunohistochemistry was performed by incubating 20μm-thick SNc sections in mouse anti-iNOS primary antibody (Santa Cruz SC-7271, 1:200, overnight at 4°C), followed by a biotinylated secondary antibody (rat, anti-mouse IgG, 1:1000, Vector Labs, USA) and reacted with cobalt and nickel-intensified diaminobenzidine (DAB, Sigma-Aldrich). DAB-immunostained sections were

counterstained with neutral red. TUNEL-positive or iNOS-positive SNc sections were analysed by bright-field microscopy (Olympus BX53F, Japan) equipped with Olympus cellSens image analysis software (v.1.7.1) and quantified stereologically on regularly spaced sections covering the whole SNc. Neurons with deep black nuclei were identified as TUNEL-positive or iNOS-positive neurons.

Statistical Analysis

All values (including graphs) are expressed as the mean ± S.E.M. All data was analysed using tools within Graphpad Prism 6. Motor behaviour studies of the treatment groups across the days of testing was analysed by two-way analysis of variance (ANOVA) and post hoc Bonferroni test. Histological and biochemical studies were analysed using two-tailed unpaired Student t-test or one-way ANOVA, where appropriate. The exact P-values of the ANOVAs are given in the figure legends. Probability level of 5% ($p<0.05$) was considered significant for all statistical tests.

2.3 Results

2.3.1 SRY regulates motor function and nigrostriatal DA biosynthesis in healthy male rats

A study by Dewing and colleagues previously showed that SRY directly regulates DA biosynthesis and consequently voluntary movement in male rats (Dewing et al., 2006). To confirm and extend these findings, the first aim of this chapter determined the effect of reducing nigral SRY expression on motor behaviour and DA transmission in healthy male rats. Nigral SRY expression was reduced via repeated daily *Sry* antisense oligonucleotide (ASO) or control sense (SO) infusion into the male rat SNc for 10 days. Rats were culled at the end of Sry ASO-infusion, and SNc and striatal tissue was isolated for mRNA and protein measurements, DA cell counts, and striatal DA and DOPAC levels (Fig. 2.1D and E).

Motor function assessed at baseline revealed that there was no significant bias in left or right limb use, nor any difference in the rotarod test between the two treatment groups (Fig. 2.2B and C, pre). As previously demonstrated (Dewing et al., 2006), repeated ASO-infusion in male rats reduced motor function compared to sense oligonucleotide (SO)-infused male rats in the limb-use ($P<0.05$ vs pre and SO; Fig. 2.2B, left) and rotarod test ($P<0.05$ vs pre and SO; Fig. 2.2C, left) . SO-infusion had no effect on motor function in either the left-limb or rotarod test (Fig. 2.2B and C, left). In contrast, motor function was unaffected by the ASO-infusion in female rats, indicating that the ASO-mediated effect was male specific (Fig. 2.2B and C, right).

To determine the efficacy of SRY knockdown by ASO-infusion, nigral mRNA expression was measured following day 10 of ASO-infusion. Repeated ASO-infusion reduced nigral *Sry* mRNA expression (51% of SO, $P<0.01$ vs SO; Fig.

2.2D), but not that of *Sry* homologs *Sox3* or *Sox6* (Fig. 2.2D), indicating that the ASO cocktail was specific to *Sry*. ASO-infusion also reduced nigral *Th* (49% of SO, $P<0.01$ vs SO; Fig. 2.2D), *Mao-a* (57% of SO, $P<0.05$ vs SO; Fig. 2.2D), *Ddc* (48% of SO, $P<0.05$ vs SO; Fig. 2.2D), and *D2r* (55% of SO, $P<0.05$ vs SO; Fig. 2.2D) mRNA expression, total number of nigral TH-positive cells (77% of SO, $P<0.05$ vs SO; Fig. 2.2E), striatal DA (66% of SO, $P<0.05$ vs SO; Fig. 2.2F) and DOPAC (62% of SO, $P<0.05$ vs SO; Fig. 2.2F) content in male rats compared to SO-infused male controls, as % of control side.

The ASO-induced reduction in motor function was reversible, as limb use returned to baseline levels at 7 days following the last ASO-infusion (Fig. 2.3B). This result suggests that reduced motor function did not result from DA cell death, but instead due to a transient reduction in nigral DA transmission.

Taken together, these results support and extend findings by Dewing and colleagues (Dewing et al., 2006) and show that SRY regulates nigral DA biosynthesis genes and striatal DA levels, and consequently voluntary movement in healthy male rats.
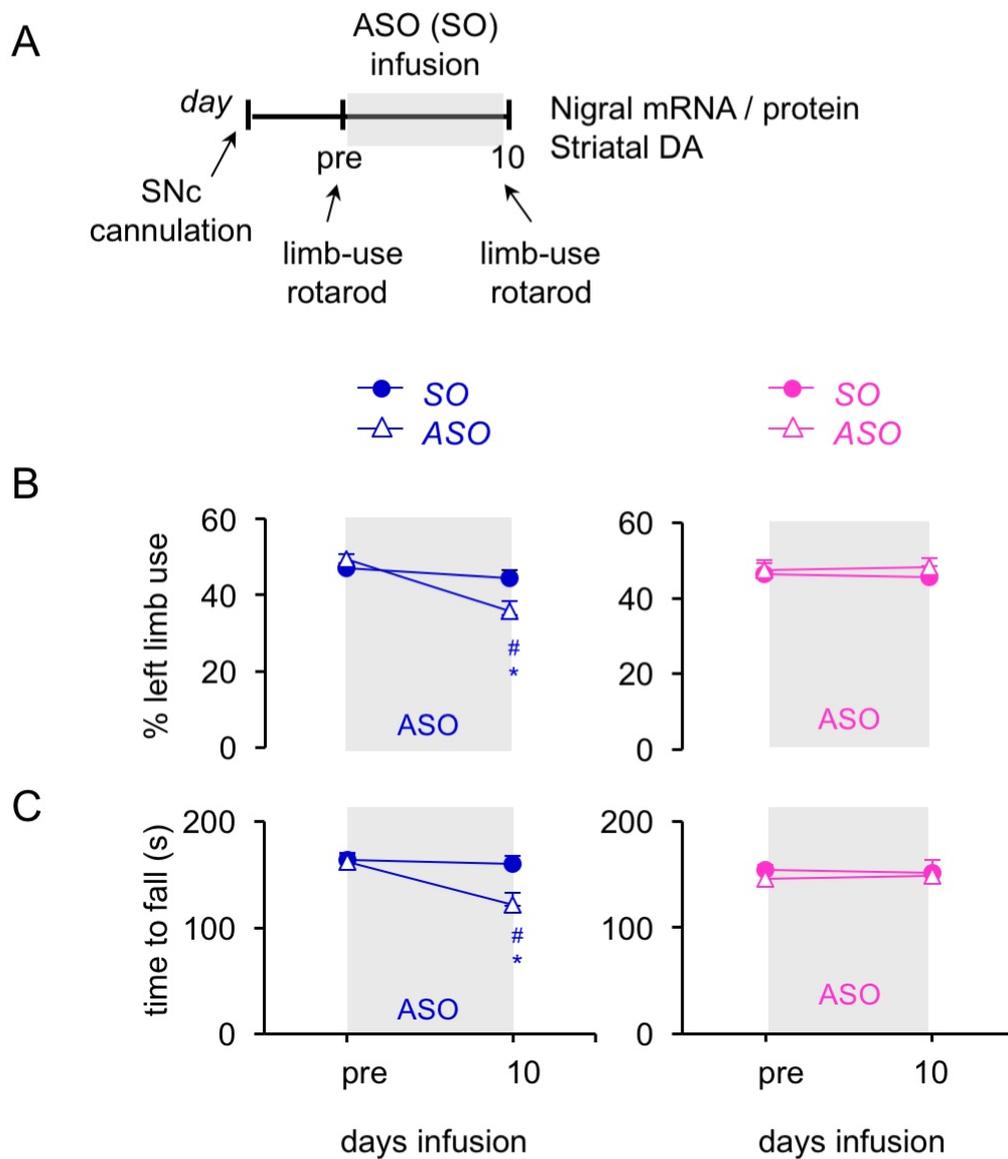
Figure 2.2. Nigral SRY positively regulates motor function and nigrostriatal DA biosynthesis in male rats. A) *Sry* ASO (or SO) was infused into the right SNc (once daily for 10 days) in male or female rats. Motor function was assessed at pre ASO-infusion (pre) or day 10 of ASO-infusion. Rats were culled on day 10 and post-mortem samples were assessed for nigral mRNA expression and striatal DA/DOPAC content. **(B-C)** Effect of ASO (or SO) infusion on motor function in the **B)** limb-use asymmetry and **C)** rotarod tests in male (left; blue) or female (right; pink) rats (n=10/group; two-way ANOVA, ** $P < 0.01$, * $P < 0.05$ vs. SO; # $P < 0.05$ vs. pre).
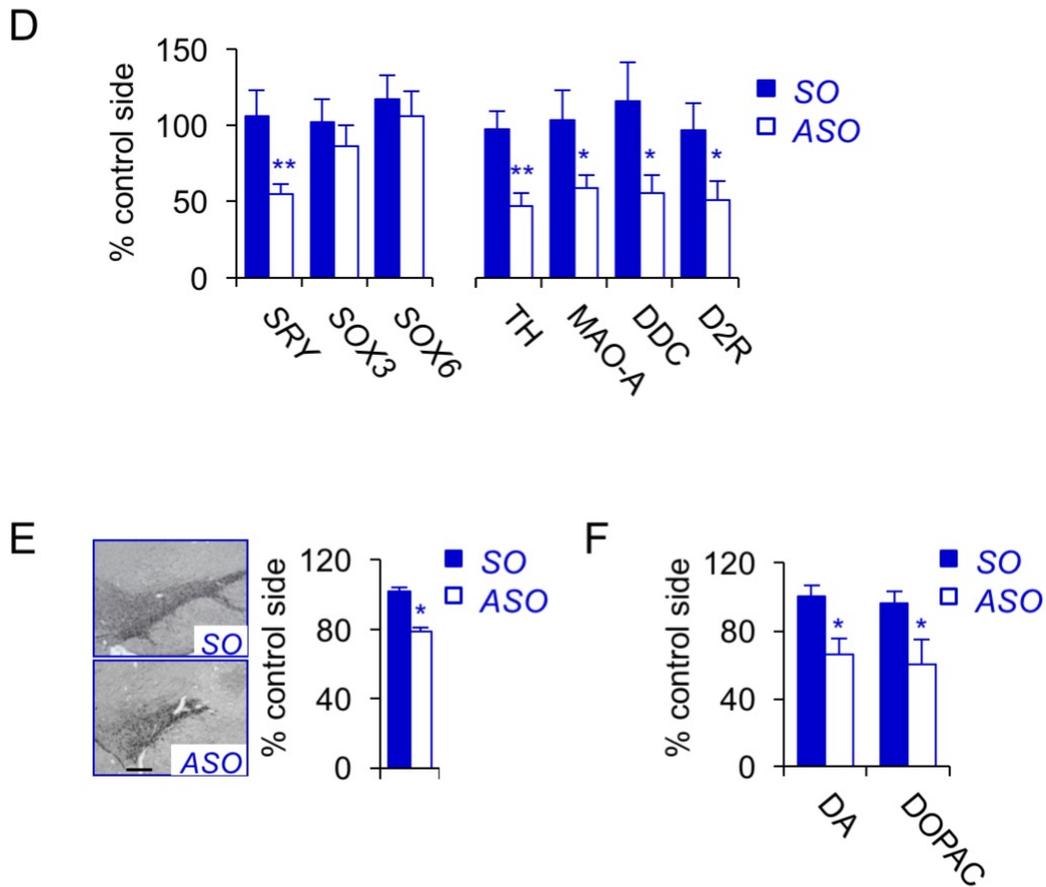
Figure 2.2 (continued) Following the last behavioural test on day 10, male rat brains were processed for **D)** nigral *mRNA* expression, **E)** nigral TH-positive cell counts (scale=400µm) and **F)** striatal DA and DOPAC levels as % of intact side (n=10/group; unpaired t-test, ** $P < 0.01$, * $P < 0.05$ vs. SO).
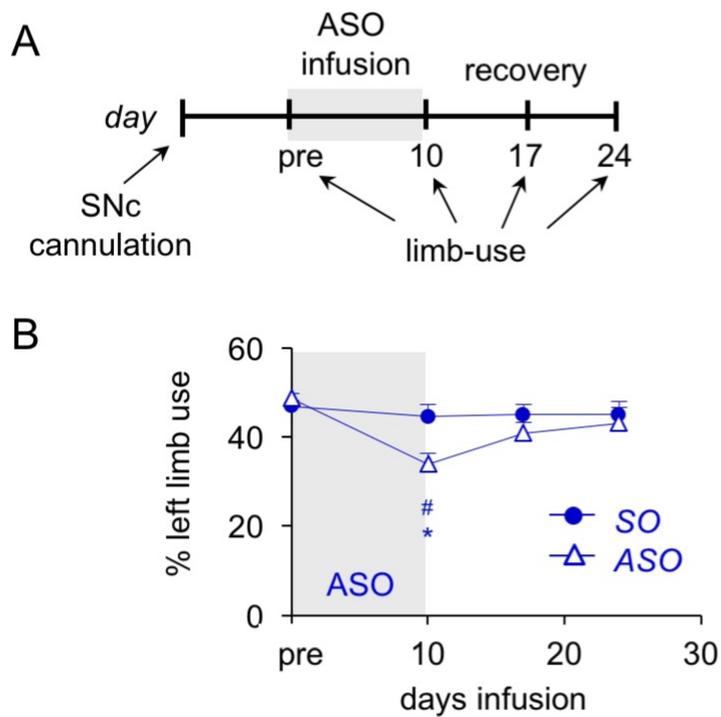
Figure 2.3. ASO-induced reduction of motor function in male rats is transient.
A) Effect of ASO (or SO) infusion on motor function in the **B)** limb-use asymmetry test at pre ASO-infusion (pre), day 10 of ASO-infusion, 7 or 14 days following the last ASO-infusion. (n=5/group; two-way ANOVA, * $P < 0.05$ vs. SO; # $P < 0.05$ vs. day 0).

2.3.2 Acute 6-OHDA or rotenone injection induces dose-dependent reduction in motor function in male rats

To establish the acute toxin-induced rat models of PD, the effect of acute intranigral injection of 6-OHDA or rotenone toxin was assessed at varying doses in male rats. The limb-use asymmetry test revealed that there were no significant left or right biases in limb use in any of the treatment groups prior to the intranigral injection of 6-OHDA (Fig. 2.4C, day 0). Similarly, the vehicle injected group did not show any significant difference in limb use at any of the days post-injection (Fig. 2.4C). A 6-OHDA-induced deficit in limb use was observed in both the 15µg (44% of vehicle, $P < 0.05$ vs vehicle; Fig. 2.4C) and 30 µg (13% of vehicle, $P < 0.05$ vs vehicle; Fig. 2.4C) treatment groups compared to vehicle, 7 days after the 6-OHDA injection. At day 14, the 6-OHDA-induced deficit in limb use persisted in the 15µg treatment group (42.4% of vehicle, $P < 0.05$ vs vehicle; Fig. 2.4C) and with 30µg treatment (10.5% of vehicle, $P < 0.05$ vs vehicle; Fig. 2.4C). By day 21 post 6-OHDA, 15µg treatment was not significantly different to vehicle (Fig. 2.4C). However, 30µg treatment showed a persistent deficit in limb use (14.6% of vehicle, $P < 0.05$ vs vehicle; Fig. 2.4C).

Similarly, there were no significant biases in limb use in any of the treatment groups prior to the intranigral rotenone injection (Fig. 2.4D, day 0). The vehicle group did not show any difference in limb use at any of the days post-injection (Fig. 2.4D). 7 days after the rotenone injection, a significant deficit in limb-use was observed in the 30µg rotenone treatment group compared to vehicle (28.8% of vehicle, $P < 0.05$ vs vehicle; Fig. 2.4D). At day 21 post injection, the rotenone-induced deficit in limb-use persisted in the 30µg

treatment group (32.4% of vehicle, $P < 0.05$ vs vehicle; Fig. 2.4D). However, 15μg rotenone injection did not produce a significant deficit in limb-use at any time point post-injection.

In summary, intranigral injection of 6-OHDA or rotenone at 30μg led to a significant, persistent motor deficit in limb-use, compared to the modest deficits seen with 15μg 6-OHDA or rotenone injection. Based on these results, I chose to use 30μg 6-OHDA and 30μg rotenone to test the effect of *Sry* ASO-infusion in aim 2 of chapter 2.
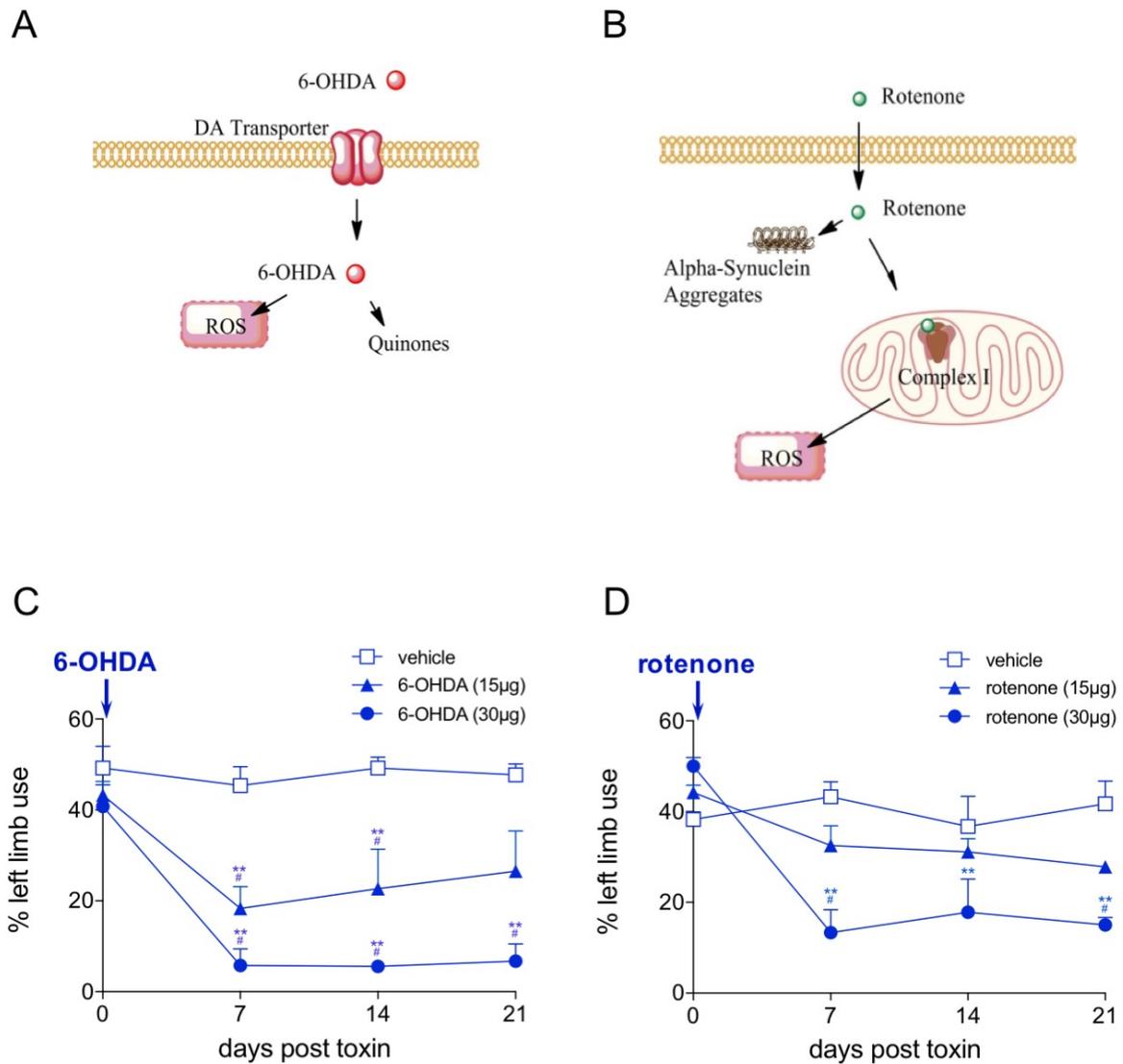
Figure 2.4. Acute 6-OHDA or rotenone injection elicits dose-dependent motor deficit in male rats. A) Schematic diagram of 6-OHDA toxicity in DA neurons. 6-OHDA crosses cell membrane via the DA transporter, and undergoes auto-oxidation to form harmful reactive oxygen species (ROS) and quinones. **B)** Rotenone is lipophilic and easily penetrates the DA cell membrane, where it inhibits mitochondrial complex 1 and induces formation of α-synuclein aggregates. **C)** Effect of unilateral nigral 6-OHDA or **D)** rotenone injection at 15ug or 30ug on motor function in the limb-use asymmetry test at pre toxin injection (day 0), 7, 14 and 21 days following injection. (n≥3/group; two-way ANOVA, # P <0.05 vs. vehicle; ** P <0.01 vs. day 0) *(Figure adapted from Cabezas et al., 2013)*

2.3.3 *Sry* expression is up-regulated in the 6-OHDA or rotenone-induced rat models of PD

To determine whether SRY is involved in the cellular events underlying DA cell loss, the regulation of *Sry* expression was assessed in the 6-OHDA and rotenone-induced models of PD. In the 6-OHDA-induced rat model of PD, nigral *Sry* mRNA expression was elevated from days 2 to 14 post 6-OHDA injection in male rats, with maximal expression at day 7 (2.89-fold, $P < 0.001$ vs. day 0, Fig. 2.5A). The increase in *Sry* mRNA expression was paralleled by elevation in expression of *Gadd45y* mRNA (a marker for DNA-damage and regulator of *Sry* expression) (Czech et al., 2014, Warr et al., 2012) (Fig. 2.5A). *Th* mRNA expression was significantly reduced from day 7 post 6-OHDA injection (0.54-fold vs. day 0, $P<0.01$, Fig. 2.5A), suggesting that *Sry* and *Gadd45y* mRNA up-regulation occurs prior to DA cell death. Similarly, acute injection of the mitochondrial and DA toxin, rotenone*,* increased *Sry* and *Gadd45y mRNA* at 7 days post-injection in male rats (2.62-fold and 1.84-fold vs. day 0, respectively, $P < 0.01$, Fig. 2.5B). Similarly, *Th* mRNA expression was significantly reduced at day 7 post rotenone injection (0.46-fold vs. day 0, $P<0.05$, Fig. 2.5B).

These results show that *Sry* is highly and persistently up-regulated in multiple *in vivo* models of PD, and that this induction precedes DA cell death. (Results in section 2.3.3 performed by Hannah Loke and used with permission).
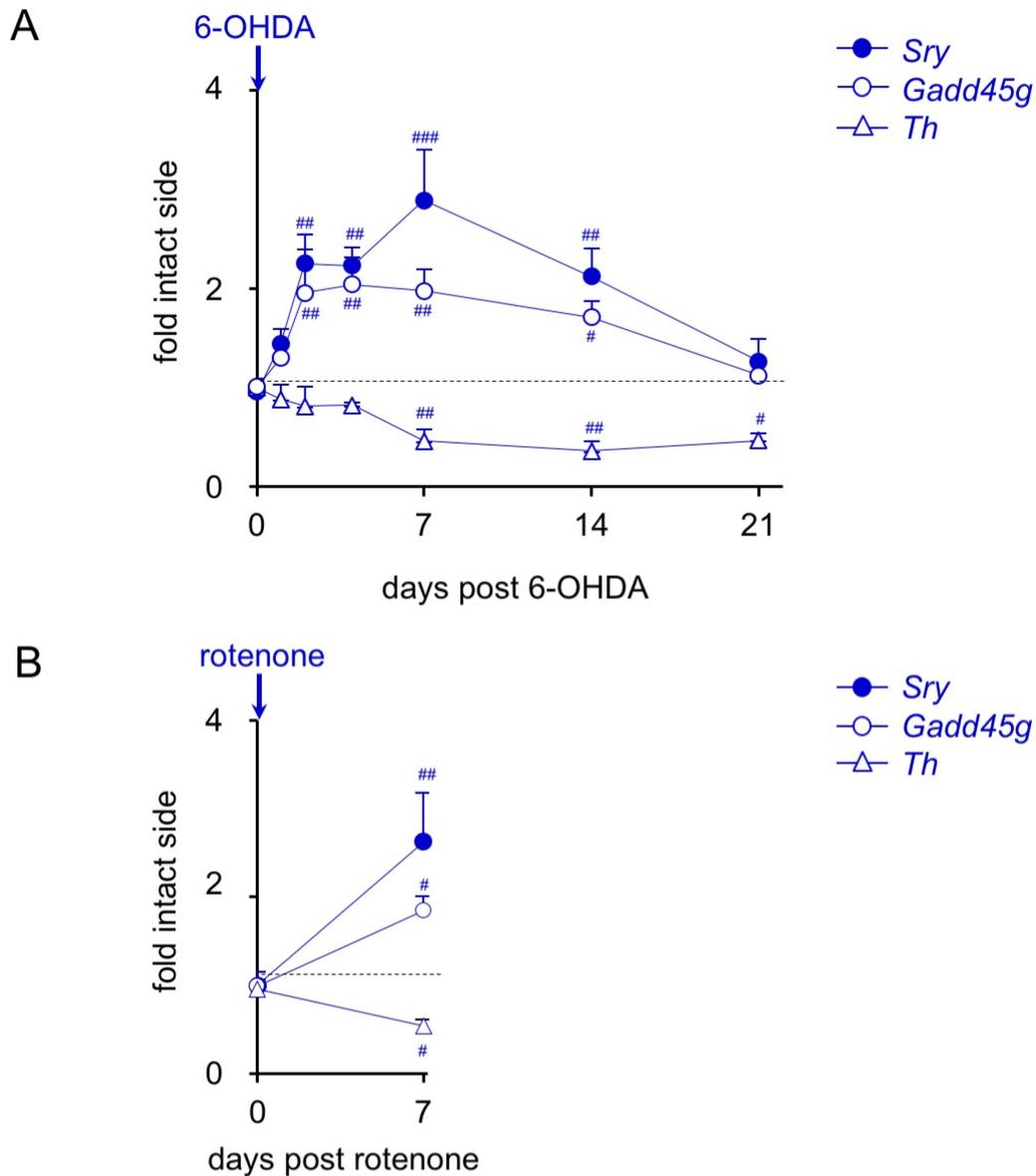
Figure 2.5. Nigral *Sry* mRNA expression is up-regulated in 6-OHDA or rotenone-induced rat models of PD. Effect of a single intranigral **A)** 6-OHDA or **B)** rotenone injection on nigral *Sry, GADD45γ*, or *TH* mRNA expression at 0 to 21 days post injection in male rats. N.B. nigral mRNA expression was not measured after 7 days post rotenone injection to conserve animal numbers (n=5/group; one-way ANOVA, # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ vs. day 0; dashed line = baseline levels) (Study performed by H.Loke; Adapted from Lee et al., 2017).

2.3.4 Nigral *Sry* ASO-infusion in male rats diminishes motor deficits and nigrostriatal degeneration in 6-OHDA or rotenone-induced rat models of PD

To determine the role of Sry up-regulation in injured male DA neurons, the effect of reducing SRY expression was assessed in the 6-OHDA or rotenone-induced rat models of PD.

To determine whether SRY is involved in the onset of nigrostriatal degeneration in male rats, the effect of ASO-infusion prior to 6-OHDA injection was assessed in male rats. Figure 2.6A shows that ASO-infusion led to the expected reduction in limb use in male rats at day 0 (71% of pre, $P< 0.05$ vs. pre, Fig. 2.6A). Following a single 6-OHDA injection, a deficit in limb use was observed in SO-infused rats (51% of day 0, $P< 0.05$ vs. day 0, Fig. 2.6A, day 7) that persisted for further 14 days. Remarkably, prior ASO-infusion prevented 6-OHDA-induced deficits in limb-use (7 days post 6-OHDA injection, Fig 2.6A), which persisted for a further 14 days. In support, amphetamine-induced rotations were markedly reduced in the ASO-infused group (0.3 ± 0.7 net rotations/min, $P<0.01$ vs SO, Fig. 2.6B) compared to the SO-infused group (3.1 ± 0.4 net rotations/min, Fig. 2.6B). Post-mortem analysis of 6-OHDA-lesioned male rats revealed that the protective effect of ASO-infusion was associated with reduction in 6-OHDA induced losses in nigral TH protein expression (155% of SO, $P< 0.05$ vs SO, Fig. 2.6C), nigral TH-positive cells (204% of SO, $P< 0.01$ vs SO, Fig. 2.6D) and striatal DA terminal density (224.1% of SO, $P< 0.01$ vs SO, Fig. 2.6E). These results indicate that *Sry* ASO-infusion prior to 6-OHDA injection diminished 6-OHDA-induced motor deficits via alleviation of 6-OHDA-induced nigrostriatal degeneration in male rats, suggesting that the 6-OHDA-induced up-regulation of *Sry* is detrimental in male DA neurons.

To eliminate the possibility that the protective effect of ASO-infusion was via inhibition of 6-OHDA uptake, the effect of ASO-infusion following a single 6-OHDA injection in male rats was assessed (Fig. 2.7A). 6-OHDA injection induced deficits in limb use and rotation tests (SO, Fig. 2.7 A and B respectively), which were markedly reduced by ASO-infusion (days 14 and 21, $P< 0.05$ vs SO, Fig. 2.7A). The protective effect of ASO-infusion was accompanied by alleviation of 6-OHDA induced losses in nigral TH protein expression (171% of SO, $P< 0.05$ vs SO, Fig. 2.7C), TH-positive cells (198% of SO, $P< 0.01$ vs SO, Fig. 2.7D), as well as striatal DA terminal density (175.8% of SO, $P< 0.01$ vs SO, Fig. 2.7E). Together, these results indicate that the ASO-infusion reduced 6-OHDA induced toxicity whether given before or after 6-OHDA injection, indicating that the protective effects were not due to inhibition of 6-OHDA uptake.

To confirm that the protective effect of *Sry* ASO-infusion was mediated by reducing nigral SRY, the effect of ASO-infusion prior to 6-OHDA injection was assessed in female rats. Figure 2.8A showed that ASO-infusion had no effect on limb-use in healthy females (Fig. 2.8A, day 0). Following 6-OHDA injection, there were no differences in 6-OHDA-induced motor deficits in limb-use (Fig. 2.8A, day 7) or rotations (Fig. 2.8B) between SO and ASO-infused female rats, which correlated with no differences in nigrostriatal degeneration (Fig. 2.8 C to E). These results indicate that the protective effect of *Sry* ASO-infusion in the 6-OHDA-induced rat model of PD is male specific.

To determine whether ASO-infusion was also protective against a toxin with different neurotoxic mechanisms to 6-OHDA, the effect of ASO-infusion prior to rotenone injection in male rats was assessed. Rotenone injection induced deficits in limb use and rotation tests (SO, Fig. 2.9A and B). Suppressing SRY synthesis led

to even greater protection in the rotenone model of PD, as ASO-infusion reversed rotenone-induced motor deficits in limb-use ($P < 0.05$ vs. SO, Fig. 2.9A) and reduced rotations ($P < 0.01$ vs. SO, Fig. 2.9B). Post-mortem analysis showed that ASO-infusion also prevented rotenone-induced losses in nigral TH protein (169.9% of SO, $P < 0.01$ vs SO, Fig. 2.9C), TH-positive cells (176.6% of SO, $P < 0.01$ vs SO, Fig. 2.9D), and striatal DA terminal density (155.2% of SO, $P < 0.01$ vs SO, Fig. 2.9E). Taken together, these results show that toxin-induced up-regulation of nigral *Sry* is detrimental, as ASO-infusion in male rats mitigate 6-OHDA or rotenone-induced nigrostriatal degeneration and motor deficits, perhaps through a common downstream mechanism(s) shared by both toxins.
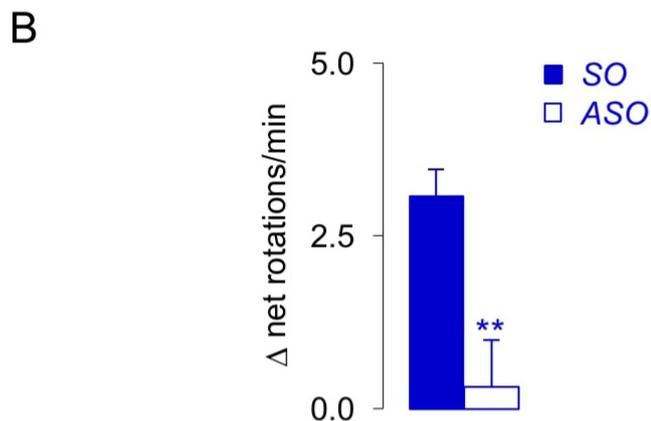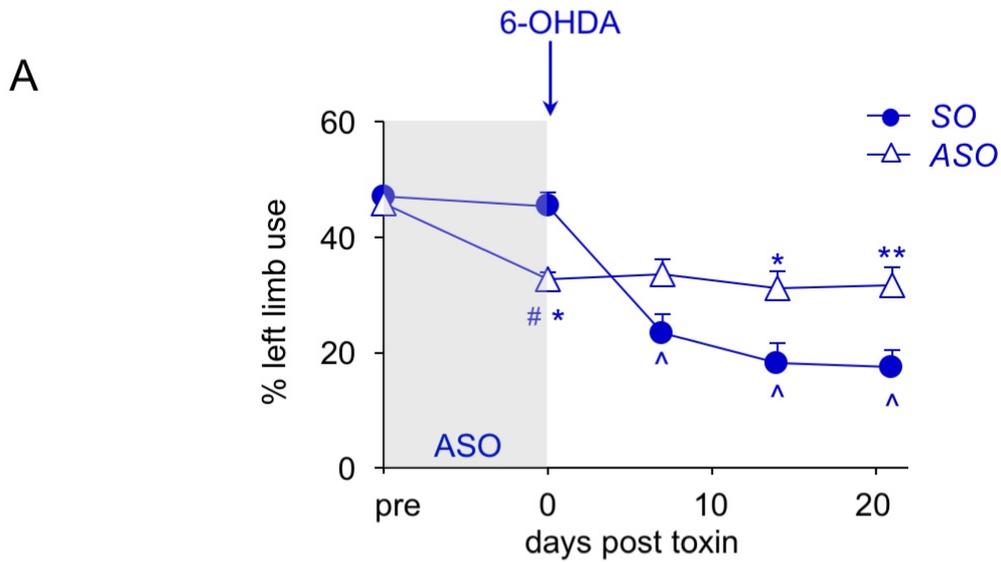
Figure 2.6. Nigral *Sry* ASO-infusion prior to 6-OHDA injection ameliorates 6-OHDA induced motor deficits and nigrostriatal degeneration in male rats. ASO (or SO) was infused prior to 6-OHDA in male rats. Motor deficits were assessed by the **A)** limb-use asymmetry or **B)** amphetamine-induced rotation test (two-way ANOVA, n≥12/group; *P<0.05, **P<0.01 to SO; #P<0.05 to pre; ^P<0.05 to day 0; pre = pre ASO-infusion).

C

TH protein

D

TH cell counts

E
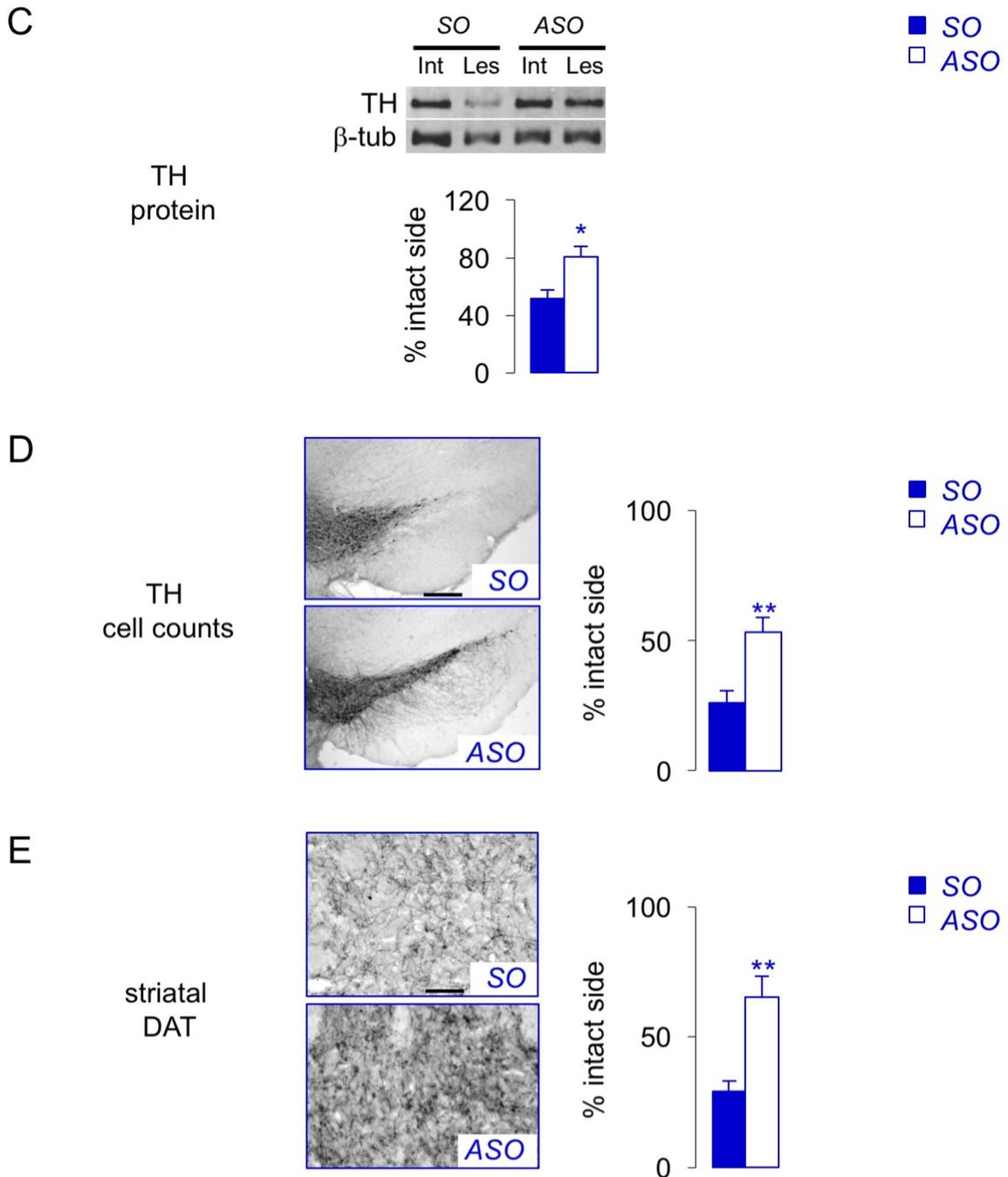
striatal DAT

Figure 2.6 (continued). Nigrostriatal degeneration was assessed by **C)** nigral TH protein expression, **D)** total nigral TH cell counts (scale=400µm) and **E)** striatal DAT terminal density (scale=40µm) as % of intact side (unpaired t-test; *P<0.05, **P<0.01 to SO).
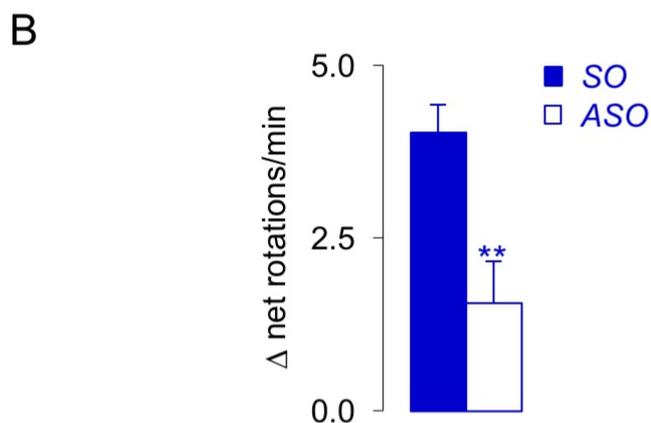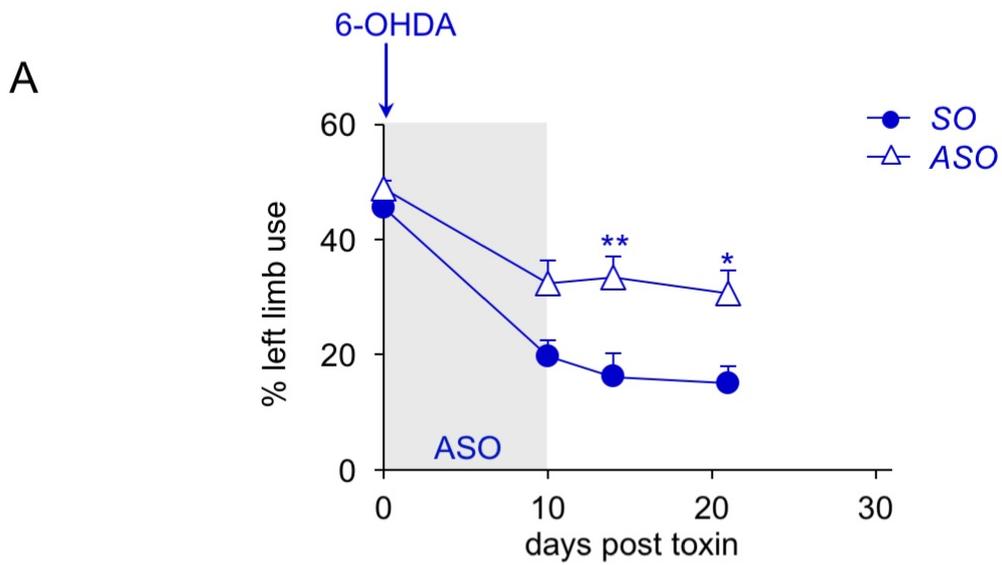
Figure 2.7. Nigral *Sry* ASO-infusion following 6-OHDA injection attenuates 6-OHDA induced motor deficits and nigrostriatal degeneration in male rats. ASO (or SO) was infused following 6-OHDA injection in male rats. Motor deficits were assessed by the **A)** limb-use asymmetry or **B)** amphetamine-induced rotation test (two-way ANOVA, n≥12/group; *P<0.05, **P<0.01 to SO; #P<0.05; pre = pre ASO-infusion).

C

SO   ASO

Int Les   Int Les

TH

β-tub

■ SO
□ ASO

TH
protein

% intact side

120

80

40

0

*

D

TH
cell counts

SO

ASO

■ SO
□ ASO

% intact side

100

50

0

**

E

striatal
DAT

SO

ASO

■ SO
□ ASO

% intact side

100

50

0

**
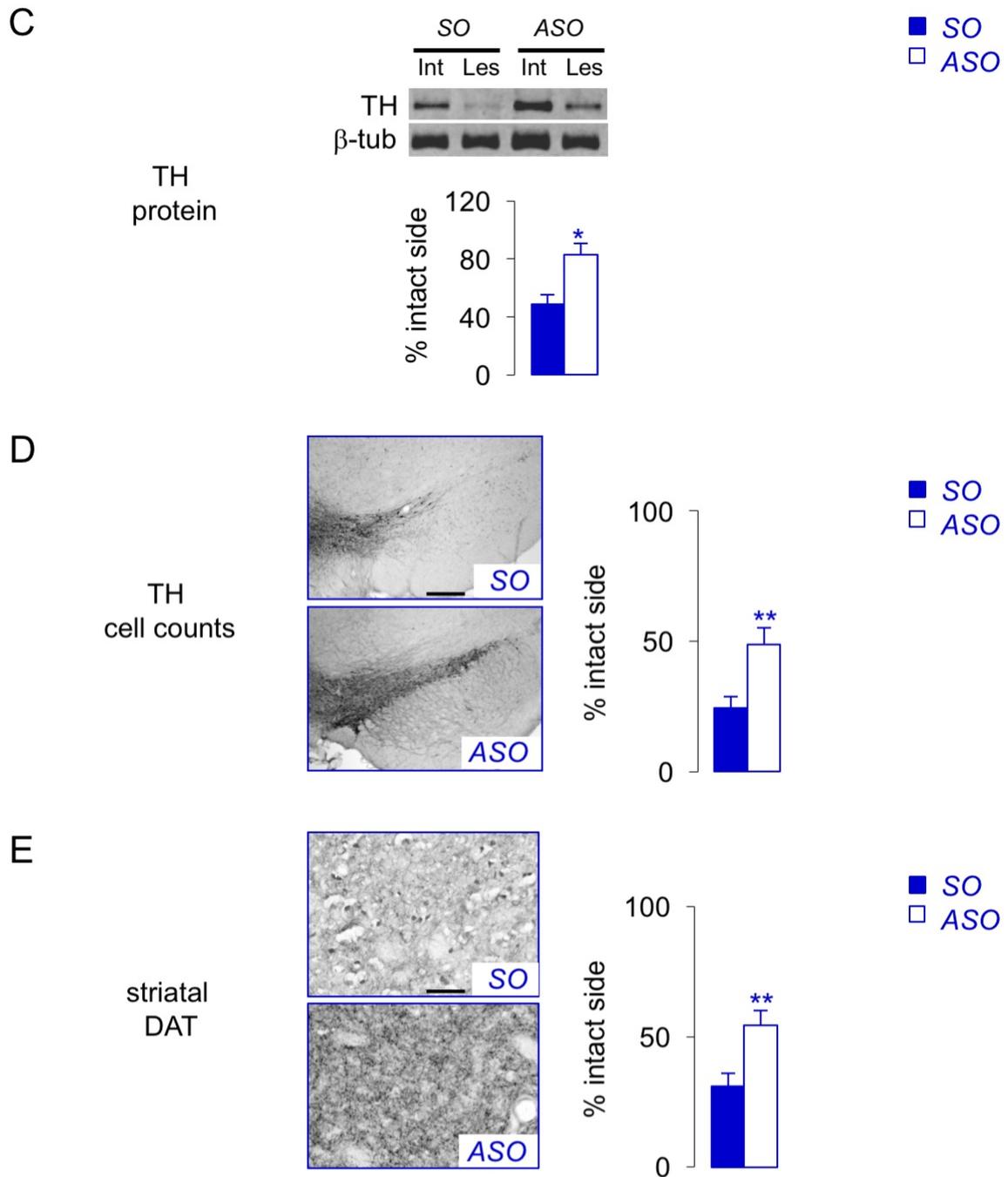
Figure 2.7 (continued). Nigrostriatal degeneration was assessed by **C)** nigral TH protein expression, **D)** total nigral TH cell counts (scale=400µm) and **E)** striatal DAT terminal density (scale=40µm) as % of intact side (unpaired t-test; *P<0.05, **P<0.01 to SO).
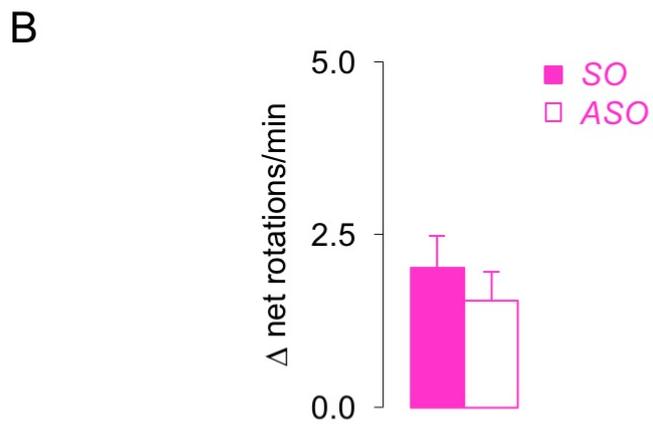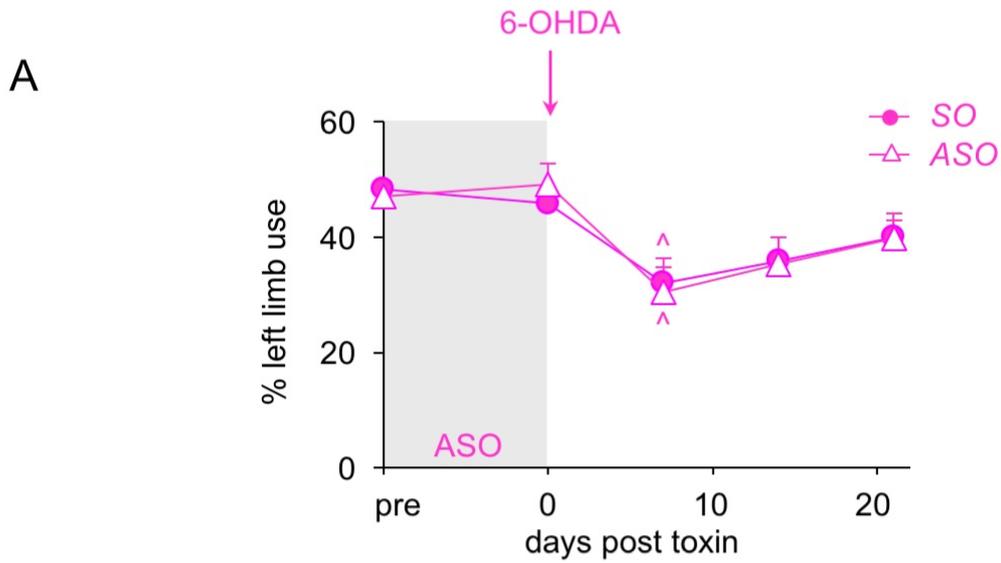
Figure 2.8. Nigral *Sry* ASO-infusion has no effect on 6-OHDA-induced motor deficits or nigrostriatal degeneration in female rats. ASO (or SO) was infused prior to 6-OHDA injection in female rats. Motor deficits were assessed by the **A)** limb-use asymmetry or **B)** amphetamine-induced rotation test (two-way ANOVA, n≥12/group; ^$P<0.05$ to day 0; pre = pre ASO-infusion).
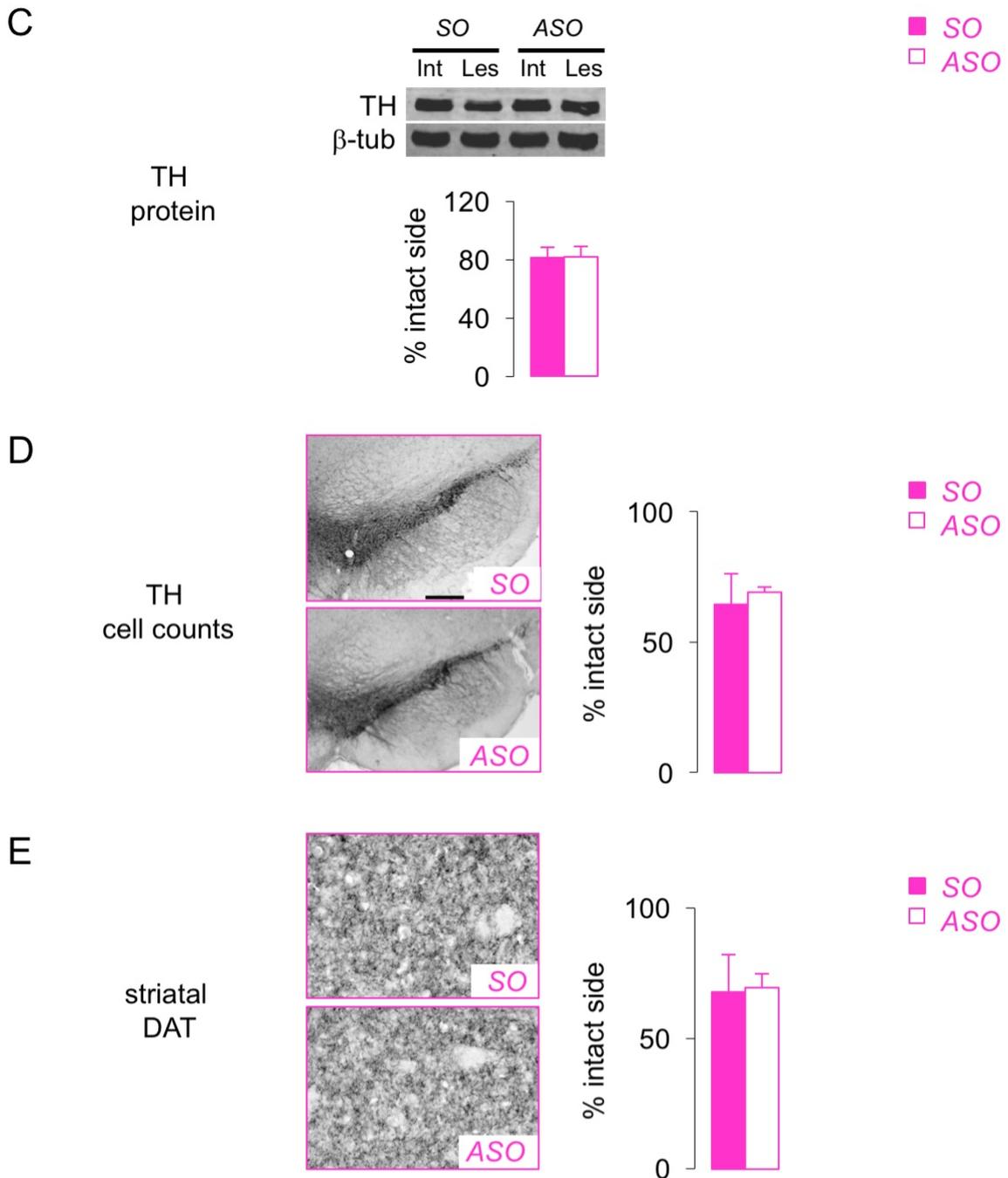
Figure 2.8 (continued). Nigrostriatal degeneration was assessed by **C)** nigral TH protein expression, **D)** total nigral TH cell counts (scale=400µm) and **E)** striatal DAT terminal density (scale=40µm) as % of intact side (unpaired t-test).
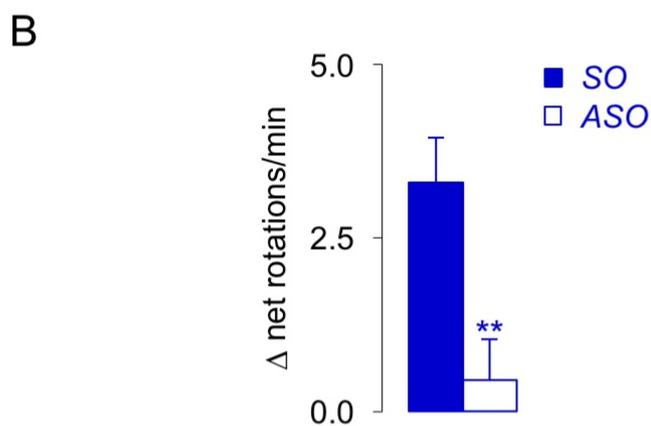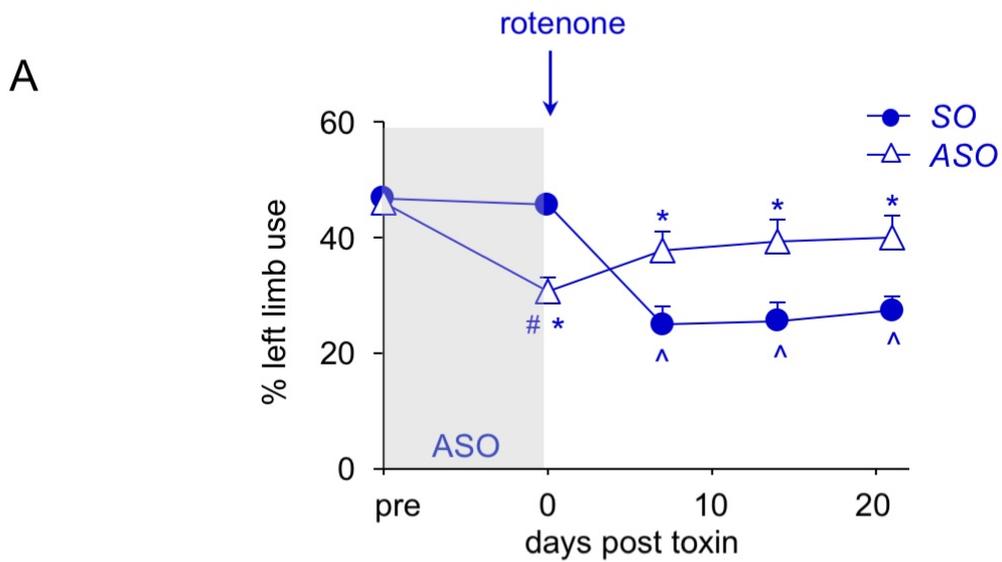
Figure 2.9. Nigral *Sry* ASO-infusion prior to rotenone injection ameliorates rotenone induced motor deficits and nigrostriatal degeneration in male rats. ASO (or SO) was infused prior to rotenone injection in male rats. Motor deficits was assessed by the **A)** limb-use asymmetry or **B)** amphetamine-induced rotation test (two-way ANOVA, n≥12/group; *P<0.05 to SO; #P<0.05; to pre; ^P<0.05 to day 0; pre = pre ASO-infusion).
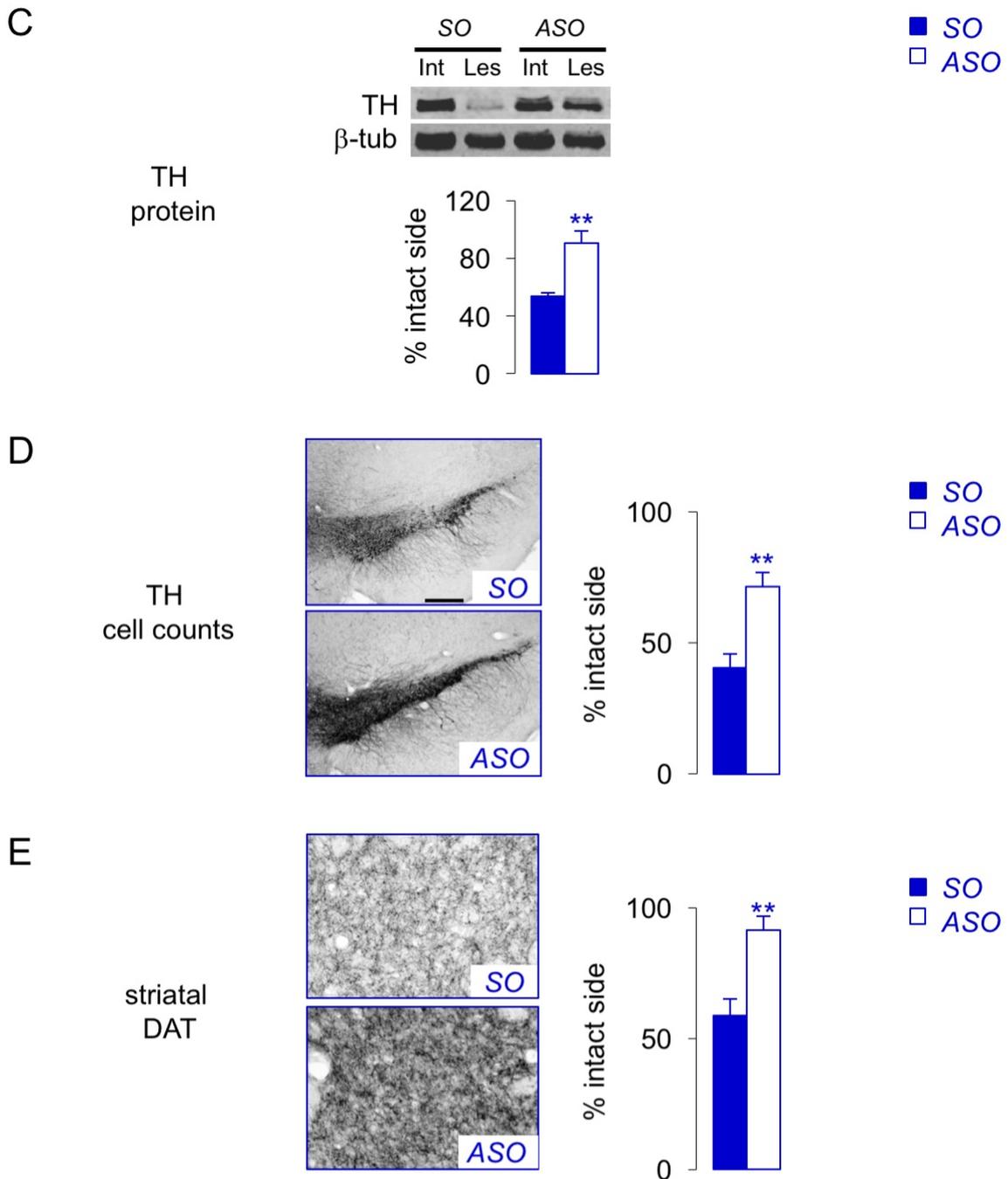
Figure 2.9 (continued). Nigrostriatal degeneration was assessed by **C)** nigral TH protein expression, **D)** total nigral TH cell counts (scale=400µm) and **E)** striatal DAT terminal density (scale=40µm) as % of intact side (unpaired t-test; **P<0.01 to SO).

2.3.5 *Sry* ASO-infusion normalizes male-biased increases in mitochondrial degradation, DNA damage and neuroinflammation in toxin-induced rat models of PD

To identify the mechanism(s) underlying the neuroprotective effect of ASO-infusion, expression of key sensors and mediators of cell death pathways such as mitochondrial dysfunction, DNA damage and neuroinflammation were assessed in 6-OHDA injected male and female rats infused with ASO or SO that were culled two days post-toxin, when markers and sensors of cell death mechanisms are highly expressed (Haas et al., 2016, Zuch et al., 2000, Smith and Cass, 2007).

Co-immunofluorescence staining for TOM-20 (a mitochondrial membrane receptor, and marker for mitochondrial integrity) (Di Maio et al., 2016), and TH revealed marked losses in the number of TOM-20 and TH co-positive neurons following 6-OHDA or rotenone injection (SO, Fig. 2.10A and B), which was reversed with ASO-infusion in male rats (138.1% and 163.9% of SO, $P<0.05$ vs. SO, Fig. 2.10A and B, respectively). Conversely, no significant difference in the number of TOM-20 and TH co-positive neurons was observed between the two groups in the 6-OHDA-injected female rats (Fig. 2.10C).

TUNEL staining (a marker of DNA damage) revealed that 6-OHDA or rotenone injection in male rats induced significant increases in the number of TUNEL-positive neurons in the SNc (SO, Fig. 2.11 A and B) which were attenuated by ASO-infusion (48.7% and 29.9% of SO, $P<0.05$ vs. SO, Fig. 2.11A and B, respectively). In contrast, there were no differences in the number of TUNEL-neurons between SO- and ASO-infusion in the 6-OHDA-lesioned female rats (Fig. 2.11C).

Similarly, marked increases in iNOS (a pro-inflammatory mediator)- positive neurons were induced by 6-OHDA or rotenone injections in male rats (SO, Fig. 2.12A and B, respectively), which were mitigated by ASO-infusion (13.6% and 7.4% of SO, *P*< 0.01 vs. SO, Fig. 2.12A and B, respectively). In contrast, there were no differences in the number of iNOS-positive neurons between the two groups in the 6-OHDA-lesioned female rats (Fig. 2.12C).

Taken together, these results indicate that *Sry* ASO-infusion exerts male-specific neuroprotection via attenuating toxin-induced increases in mitochondrial degradation, DNA damage, and neuroinflammation in acute toxin-induced rat models of PD.

2.3.6 *Sry* ASO-infusion in male rats removes the male-bias in the 6-OHDA-induced rat model of PD.

To determine whether SRY contributes to the male-bias in experimental PD models, motor behaviour, nigrostriatal DA, and PD pathogenesis marker expression were compared between 6-OHDA injected male and female rats infused with ASO or SO (Table 2.2).

Acute 6-OHDA injection led to greater deficit in limb use in SO-infused male rats (17.5 ± 3.1% left limb use, Table 2.2) compared to SO-infused females (40.0 ± 3.9, Table 2.2). This 6-OHDA-induced male bias was attenuated by ASO-infusion in 6-OHDA injected male rats (31.8 ± 3.1, Table 2.2). Similarly, 6-OHDA injection induced a male bias in nigrostriatal degeneration (Male SO 6-OHDA vs. Female SO 6-OHDA, Table 2.2) which was diminished by ASO-infusion in male rats (Male SO 6-OHDA vs. Male ASO 6-OHDA, Table 2.2). The 6-OHDA induced male-bias in motor deficit and nigrostriatal degeneration was associated with increased

expression of markers of DNA damage (TUNEL) and neuroinflammation (iNOS) in SO-infused male rats compared to females (Male SO 6-OHDA vs. Female SO 6-OHDA, Table 2.2) which was diminished by ASO-infusion in male rats (Male SO 6-OHDA vs. Male ASO 6-OHDA, Table 2.2). Together, these results demonstrate that dysregulation of nigral SRY directly contributes to the sex differences in experimental models of PD, highlighting the importance of considering the influence of both sex-specific genes and sex hormones in male-susceptibility to PD.
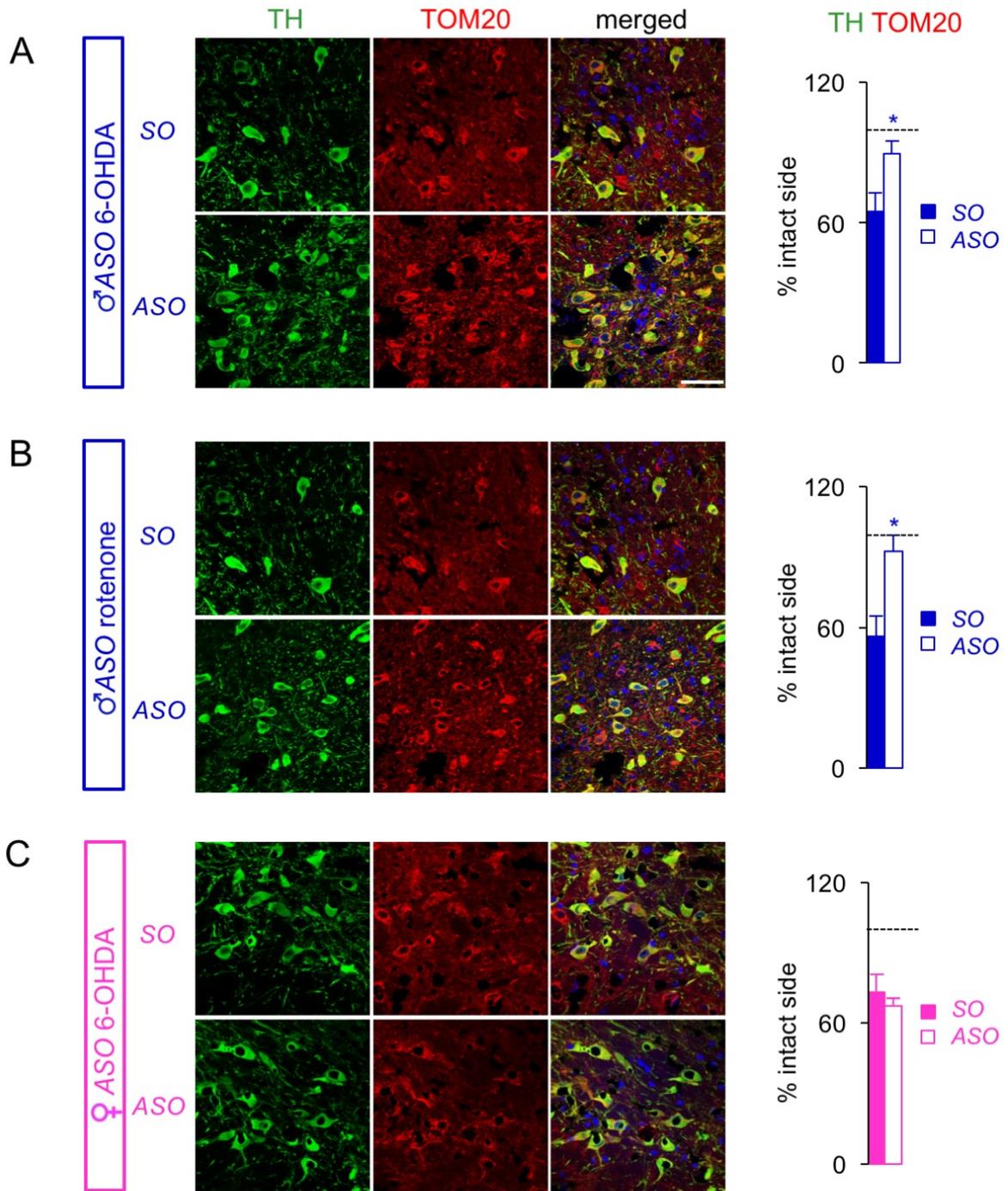
Figure 2.10. Reducing nigral SRY expression diminishes toxin-induced mitochondrial dysfunction in male rats. Effect of ASO (or SO) infusion on nigral TH- (green) or TOM-20-positive (red) neurons or TH- and TOM-20-positive (merged) neurons at two days post **A)** 6-OHDA or **B)** rotenone injection in male rats or **C)** 6-OHDA injection in female rats (unpaired t-test; n=7/group; * $P < 0.05$, ** $P < 0.01$ vs. SO, scale=40µm).

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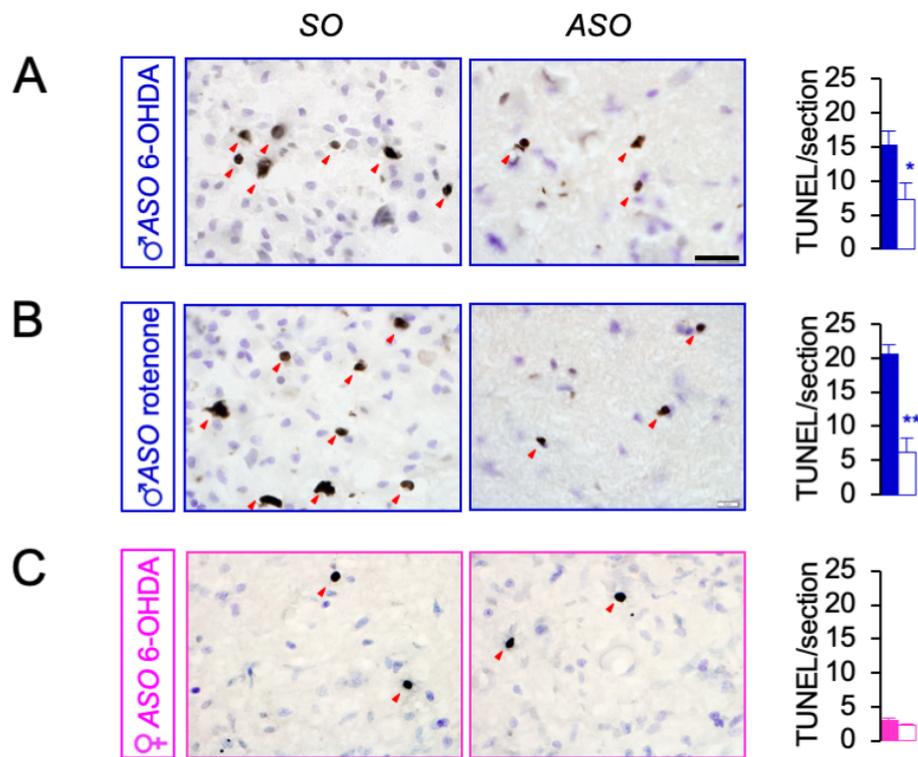
Figure 2.11. Reducing nigral SRY expression diminishes toxin-induced DNA damage in male rats. Effect of ASO (or SO) infusion on TUNEL-positive neurons (as % of intact side) at two days post **A)** 6-OHDA or **B)** rotenone injection in male rats or **C)** 6-OHDA injection in female rats. Arrows represent TUNEL-positive cells in the SNc (unpaired t-test; n=7/group; *P<0.05, ** P < 0.01 vs. SO, scale=20μm).
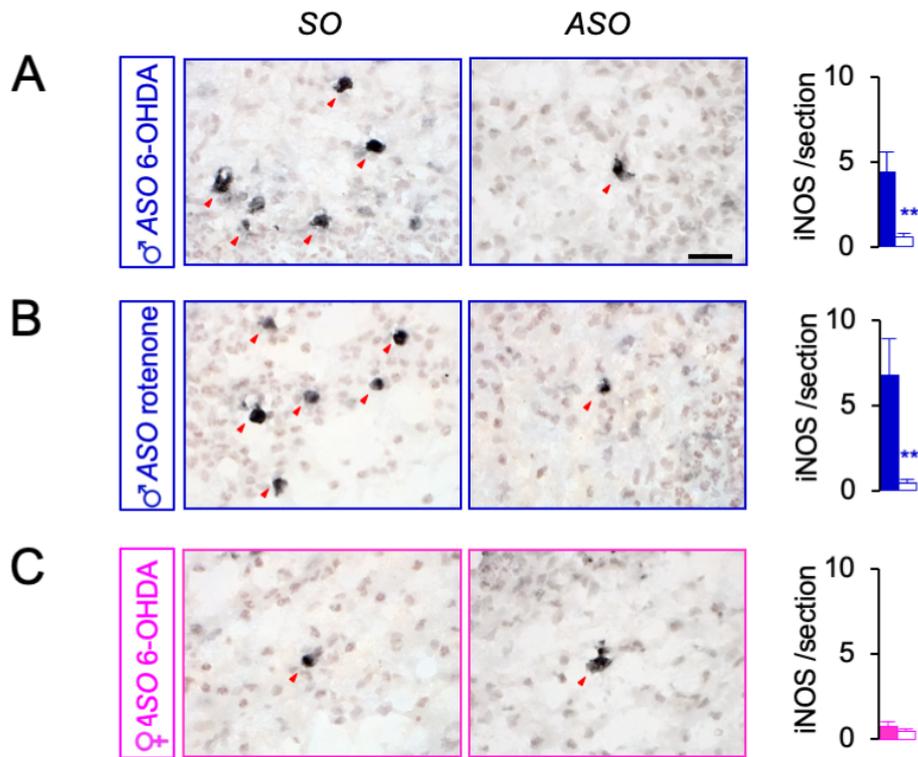
Figure 2.12. Reducing nigral SRY expression diminishes toxin-induced neuroinflammation in male rats. Effect of ASO (or SO) infusion on iNOS-positive neurons (as % of intact side) at two days post **A)** 6-OHDA or **B)** rotenone injection in male rats or **C)** 6-OHDA injection in female rats. Arrows represent iNOS-positive cells in the SNc (unpaired t-test; n=7/group; *P<0.05, ** P < 0.01 vs. SO, scale=20μm).

Parameter	Male SO 6-OHDA	Female SO 6-OHDA	Male ASO 6-OHDA	Female ASO 6-OHDA
Limb use (% left limb use at 21d post 6-OHDA)	17.5 ± 3.1	40.0 ± 3.9 ***	31.8 ± 3.1**	39.9 ± 1.8***
Rotations (Δ net rotations / min at 20d post 6-OHDA)	3.1 ± 0.4	2.0 ± 0.3	0.3 ± 0.7 **	1.5 ± 0.4
TH protein (% intact side at 21d post 6-OHDA)	26.1 ± 4.7	64.5 ± 11.6***	53.2 ± 5.7 **	69.2 ± 1.9***
TH cell counts (% intact side at 21d post 6-OHDA)	51.7 ± 5.8	81.6 ± 6.8 *	80.4 ± 7.2**	81.9 ± 7.1*
Striatal DAT density (% intact side at 21d post 6-OHDA)	29.2 ± 4.0	65.4 ± 7.8 *	67.8 ± 14.3 *	69.5 ± 5.4 *
TH/TOM-20 +ve neurons (% intact side at 2d post 6-OHDA)	64.8 ± 7.9	73.1 ± 7.7	89.5 ± 5.4 *	67.4 ± 3.1
TUNEL+ve neurons/section (% intact side at 2d post 6-OHDA)	15.2 ± 2.1	3.0 ± 0.4 ***	7.4 ± 2.3 *	2.3 ± 0.2 ***
iNOS +ve neurons/section (% intact side at 2d post 6-OHDA)	4.4 ± 1.1	0.7 ± 0.2 **	0.6 ± 0.2 **	0.7 ± 0.1 **

Table 2.2. Comparisons of motor behaviour, nigrostriatal DA, or PD pathogenesis marker expression between male and female rats infused with ASO (or SO) prior to 6-OHDA injection (one-way ANOVA; n=8 to18 / group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. Male SO 6-OHDA).

2.4 Discussion

Results from this chapter demonstrate that SRY directs a novel genetic mechanism for nigral DA cell loss in males. Firstly, results showed that reducing nigral SRY expression via *Sry* ASO-infusion reduces motor function in healthy male rats. Secondly, nigral *Sry* expression was highly and persistently up-regulated in multiple animal models of PD in male rats. Remarkably, reducing nigral SRY expression diminished or prevented motor deficits and nigrostriatal degeneration in the 6-OHDA and rotenone-induced rat models of PD in male rats. Furthermore, reducing SRY expression attenuated toxin-induced DA toxicity by reducing mitochondrial degradation, DNA damage, and neuroinflammation in male rats. Importantly, the protective effect of ASO-infusion in acute toxin-induced rat models of PD was absent in female rats (which do not express *Sry*), demonstrating for the first-time neuro-susceptibility mediated by a sex-specific gene.

Nigral SRY directly contributes to the sex differences in experimental PD

The male-bias observed in clinical PD is reproduced in experimental PD models, as male rodents and primates are more susceptible to DA toxin-induced nigrostriatal degeneration than their female counterparts (Murray et al., 2003, Ookubo et al., 2009). Similarly, males exhibit increased expression of markers of oxidative stress (Misiak, 2010), apoptosis (Rodriguez-Navarro et al., 2008), and neuroinflammation (Joniec et al., 2009, Mitra et al., 2015, Villa et al., 2016) compared to females in experimental models of PD and neurodegeneration. Traditionally, the inherent male-bias in PD was thought to arise solely from the neuroprotective actions of oestrogen in females. For instance, onset of menopause or withdrawal of oestrogen replacement therapy worsen symptoms in female PD

patients(Quinn and Marsden, 1986, Sandyk, 1989), whilst oestrogen treatment improves PD symptoms in post-menopausal women with PD (Benedetti et al., 2001). In the current study however, reducing nigral SRY expression in male rats attenuated or blocked the male-bias in motor deficits, nigrostriatal degeneration, as well as apoptosis and neuroinflammation in the 6-OHDA-induced rat model of PD (Table 2.2). Together, these results demonstrate that nigral SRY dysregulation also contributes to male-bias in experimental PD, independent of gonadal hormone influences.

Divergent role for Sry in the healthy and injured male SNc

The results from the current chapter significantly expands our understanding of Sry in the heathy and diseased male brain. In healthy male rats, reducing nigral SRY expression, via repeated ASO-infusion, transiently reduced nigrostriatal DA biosynthesis and consequently motor function (Figure 2.13, Healthy), which is in line with previous studies from our group (Czech et al., 2012, Dewing et al., 2006). However, in the 6-OHDA or rotenone-injured SNc, transcription of *GADD45γ* is up-regulated perhaps in response to DNA damage, stimulating the p38-MAPK pathway (Czech et al., 2014). In males, this leads to an increase in *Sry* transcription, which is accompanied by increased oxidative and mitochondrial dysfunction and neuroinflammation (Figure 2.13, Injured). Moreover, reducing nigral SRY expression in 6-OHDA or rotenone lesioned male rats diminished motor deficits and nigrostriatal degeneration (Figure 2.13, Injured + ASO), indicating that *Sry* up-regulation has a detrimental role in injured male DA neurons. Thus, the contrasting effect of *Sry* ASO-infusion in healthy and toxin-induced rat models of PD indicates a double-edged role for Sry in the healthy and injured male SNc.
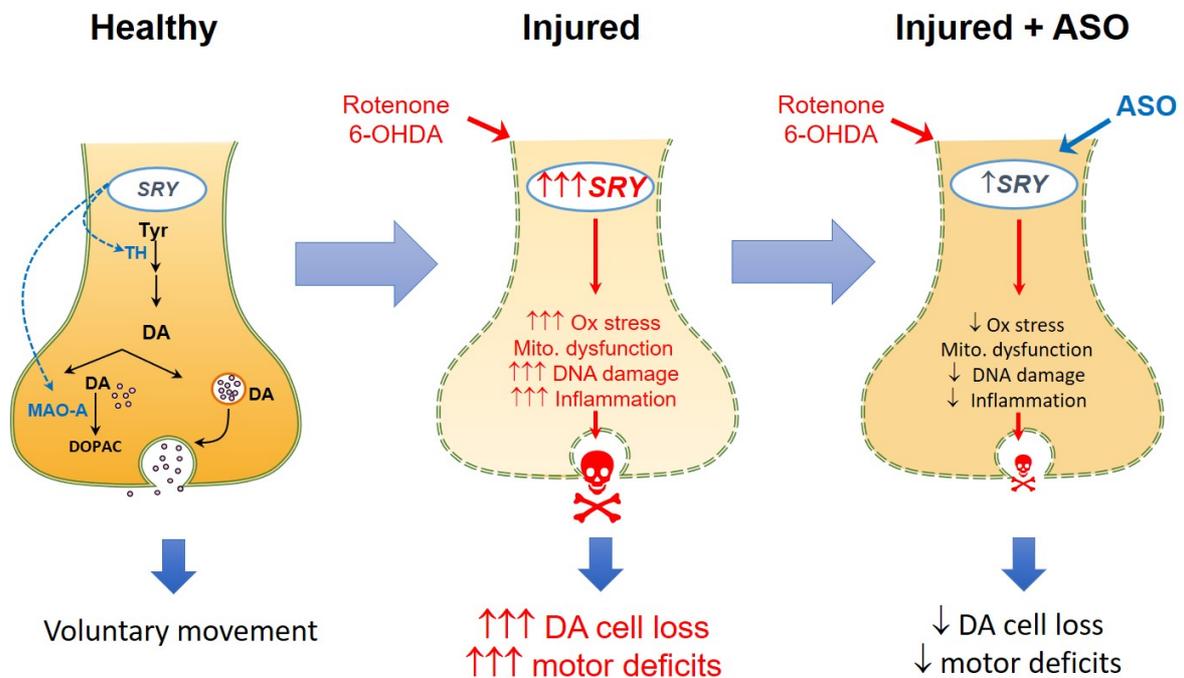
Figure 2.13. The proposed role for Sry in healthy and injured nigral male DA neurons. SRY positively regulates DA biosynthesis in male DA neurons, and consequently regulates voluntary movement in males **(Healthy)**. Nigral Sry is up-regulated in response to acute toxin treatment in the male SNc. Toxin treatment is associated with increases in DA cell death mechanisms, which increase DA cell death and motor deficits in male rats **(Injured)**. Reducing nigral SRY via ASO-infusion alleviates toxin-induced DA cell death and motor deficits, via normalising increases in mitochondrial dysfunction, DNA damage, and neuroinflammation **(Injured + ASO)**.

Mechanism(s) underlying the detrimental effect of Sry up-regulation?

Whilst my chapter has linked potential PD pathogenesis pathways with Sry up-regulation in male PD, the precise mechanism(s) by which *Sry* up-regulation exacerbates DA cell loss remain unclear. In the embryonic gonads, nuclear SRY transcriptionally activates Sox9 to initiate the development of testes (Sekido and Lovell-Badge, 2008). In contrast, the lack of Sox9 expression in the male SNc (Pompolo and Harley, 2001) and the presence of SRY protein in both nucleus and cytoplasm of neurons (Czech et al., 2012) suggest additional role(s) for SRY in the male brain. Indeed, in human and rodent DA neurons, SRY transcriptionally activates *Th* (Milsted et al., 2004, Czech et al., 2012) and *Maoa* (Wu et al., 2009) and regulates the expression of DA machinery genes including *Drd2* (involved in auto-regulatory feedback to control responsiveness to DA), *Aadc* (enzyme required for the conversion of L-DOPA into dopamine), and *Dbh* (enzyme that catalyses the conversion of DA to noradrenaline) which suggests that up-regulation of *Sry* may dysregulate DA machinery genes. Indeed, nigral *Th* expression is increased in response to 6-OHDA-induced DA cell injury in rats, and is associated with increased DA synthesis and turnover (Zigmond et al., 1984). Similarly, in post-mortem SNc tissue from PD patients, TH homospecific activity (activity per enzyme protein) is increased 3-fold in PD compared to healthy controls, which suggests compensatory increase in TH activity in response to DA cell injury (Mogi et al., 1988). Given that increased DA turnover increases oxidative stress and consequently loss of DA neurons (Hastings et al., 1996), and that baseline striatal DOPAC production and its oxidative stress-associated hydroxyl radical by-product is lower in female mice (Bhatt and Dluzen, 2005), dysregulation of nigral SRY may drive increased

expression of TH and consequently DA turnover, to exacerbate DA cell loss in males.

Alternatively, the toxin-induced elevation of nigral *Sry* may lead to regulation of a much broader range of binding partners, such as genes involved in PD pathogenesis-associated pathways. In the mouse E11.5 gonad, Sry interacts with and binds to Poly (ADP-ribose) polymerase 1 (Parp-1), a member of the Parp protein superfamily involved in functions that include DNA damage repair, and apoptosis (Li et al., 2006) and a known transcription factor of the inflammatory response regulator NF-κB (Kameoka et al., 2000). Given the pro-inflammatory properties of NF-κB, elevation of nigral SRY may increase a Parp-1 mediated excess production of NF-κB in the SNc, which would contribute to increased neuroinflammation. Furthermore, chromatin immunoprecipitation and whole-genome promoter tiling microarray (ChIP-chip) analysis on E11.5 mouse gonadal cells revealed that SRY binds to promoters of various target genes involved in cellular pathways such as oxidative stress (glutathione peroxidase 4, 6, nitric oxide synthase 3), inflammation (interleukin, tumor necrosis factor superfamilies), and mitochondrial function (mitochondrial membrane proteins 7, 13, 40) (Li et al., 2014). These findings indicate that further studies that assess interaction(s) between nigral SRY and pathways involved in oxidative stress, energy metabolism and inflammation may reveal the mechanism(s) underlying the toxic up-regulation of SRY in male PD.

Future studies

To identify the underlying downstream targets that are activated by SRY during nigrostriatal degeneration in male DA neurons, further investigations using genome-

wide approaches, such as RNA and Sry chromatin-immunoprecipitation sequencing, are required. In particular, the contrasting role of *Sry* in the healthy and injured male SNc may be better understood by comparing gene expression profiles of SRY-positive DA neurons from normal and Parkinsonian male rats, to identify target genes and DNA binding partners of SRY. Results from the current study indicate the importance of considering the detrimental role of SRY in males, as well as the neuroprotective effects of oestrogen in females, when investigating the mechanisms underlying sex differences in PD. However, further work is needed to better understand the interactions between the hormonal and genetic influences in the healthy and diseased DA system. For instance, manipulating sex hormone levels via gonadectomy or oestrogen treatment in *Sry* ASO-infused male rats, or alternatively in SRY over-expressing female rats, could provide further insight into the degree and nature of interactions between SRY and oestrogen that contribute to the male bias in PD. Overall, the characterisation of molecular mechanisms responsible for differences between the male and female DA system will give the basic biological tools necessary to understand the occurrence of sex differences observed in PD.

Whilst the current chapter has revealed iNOS expression in the SNc, it is unclear whether iNOS immunoreactivity is DA neuron-specific. Co-immunofluorescence studies that assess co-localization of iNOS and cell-specific markers such as TH or NeuN (neuronal), 1BA1 (microglia) and CD-68 (macrophages) are required to provide better insight into the localisation of iNOS immunoreactivity in the SNc.

Studies in this chapter provide conceptual data to demonstrate a therapeutic potential for nigral SRY inhibition in male PD. However, the short time-frame

associated with acute toxin models is not reflective of the human condition, and the compressed stages of deficits preclude the use of these models for assessing the most effective timeframe of SRY knockdown. Therefore, further work is needed to validate whether SRY inhibition can slow or halt the progression of motor deficits and nigral degeneration in models that allow for clinically relevant screening of novel therapeutic strategies.

Conclusion

In conclusion, this chapter provides compelling evidence that dysregulation of the Y-chromosome gene, *Sry*, directs a novel male-specific mechanism of DA cell death. In addition to the established protective effect of sex hormones in females, the detrimental effect of *Sry* up-regulation in males may also contribute to the male-bias in PD. This supports the notion that the cause and progression of PD is mechanistically different between males and females (Simunovic et al., 2010, Cantuti-Castelvetri et al., 2007). Results from this chapter are the first evidence of a male-specific therapeutic target for a neurodegenerative disorder, and potentially other male-biased disorders, such as autism and ADHD (Loke et al., 2015). Since normalization of SRY expression is important for the protection of male DA neurons against nigral injury, the development of human SRY inhibitors could lead to novel disease-modifying therapeutics for PD in males.

Chapter 3:
Regulation and Function of *Sry* in the chronic intraperitoneal rotenone-induced rat model of Parkinson's disease

3.1 Introduction

PD is characterized by the progressive degeneration of nigrostriatal DA neurons and loss of striatal DA content (Lang and Lozano, 1998, Marsden, 1990), leading to the appearance of motor symptoms such as bradykinesia, muscle rigidity and tremors. Motor symptoms first appear when striatal DA drops to 20–30% of physiological levels, and when more than 50% nigral DA neurons are lost (Lang and Lozano, 1998, Marsden, 1990). The time-lapse between initial DAergic degeneration and occurrence of motor symptoms is likely attributable to the development of compensatory mechanisms at the striatal level (Carta et al., 2013). To date, DA replacement therapy in the form of its precursor, levodopa, remains the primary mode of symptomatic treatment (Birkmayer and Hornykiewicz, 1960, Cenci, 2014). However, its long-term use is associated with debilitating side-effects such as dyskinesias and on-off fluctuations (Cenci, 2014, Jenner, 2008). Whilst putative neuroprotective compounds have been described in pre-clinical studies, no disease modifying, neuroprotective therapy has yet been identified capable of slowing or stop the progression of PD. However, all attempts to develop effective disease modifying therapy to date have performed poorly in clinical trials (Kalia et al., 2015, Lang and Espay, 2018), which may be due to the lack of an appropriate animal models suitable for screening neuroprotective candidates.

The neurotoxins 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been widely used to model PD in rodents and primates (Bove et al., 2005, Blum et al., 2001, Sriram et al., 1997, Schapira et al., 1990a), as these models exhibit robust nigrostriatal degeneration and clear motor deficits. However, the DA cell loss seen in these models is rapid and not progressive in nature, and do not replicate α-synuclein accumulation or the peripheral nervous system

neuropathology exhibited in clinical PD (Schober, 2004, Dauer and Przedborski, 2003, Halliday et al., 2009). On the other hand, genetic mouse models targeting PD-associated genes such as α-synuclein (Masliah et al., 2000, Giasson et al., 2002, Kirik et al., 2002), LRRK2 (Chen et al., 2012, Ramonet et al., 2011), PINK1 (Gautier et al., 2008), parkin (Goldberg et al., 2003), and DJ-1(Rousseaux et al., 2012) mutations have been developed. Whilst these genetic models do display the progressive accumulation of α-synuclein immunoreactive inclusions, they are often lacking a robust loss of DA neurons or development of locomotor deficits (Blesa et al., 2012). Taken together, the short time-frame associated with current models is not reflective of the human condition. Moreover, the compressed stages of deficits preclude the use of these models for furthering our understanding of the pathogenic mechanisms that underlie neurodegeneration in PD, which requires a greater temporal delineation of individual events. Hence, there is an unmet need for an ideal model of PD that i) recapitulates the slow, progressive behavioural and neurodegenerative deficits; ii) closely reproduces the complex pathogenesis mechanisms; and iii) reflects the widespread motor and non-motor symptoms exhibited in clinical PD (Bezard et al., 2013, Blesa and Przedborski, 2014, Dawson et al., 2010).

The chronic rotenone model of PD is one of the better animal models of PD, as it encapsulates multiple key behavioural and pathophysiological features of clinical PD (Greenamyre et al., 2010). Initial studies showed that intravenous rotenone delivery induced systemic inhibition of mitochondrial complex I which led to bilateral degeneration of DA neurons and motor deficits (Betarbet et al., 2000). Moreover, rotenone induced the development of intracellular α-synuclein inclusions, overcoming a key limitation of current neurotoxin-based models (Betarbet et al.,

128

2000). Asides from systemic mitochondrial dysfunction, rotenone also reproduces PD-relevant pathogenic mechanisms such as microglial activation, oxidative damage, and iron accumulation (Greenamyre et al., 2010, Betarbet et al., 2006). Notably, rotenone treatment reproduces key non-motor symptoms such as gastrointestinal (GI) disturbances, neuronal loss, and LB pathology in the GI tract (Drolet et al., 2009, Murakami et al., 2014b), oxidative damage to the olfactory bulb (Sherer et al., 2003a) and olfactory impairments (Morais et al., 2018) in rodents. However, limitations to the model included high mortality, lack of reproducibility, and substantial variability in magnitude and location of lesions, depending on the route of rotenone administration (Greenamyre et al., 2010, Biehlmaier et al., 2007, Cicchetti et al., 2009). These limitations were overcome by Cannon et al. (2009) who demonstrated that daily intraperitoneal rotenone injections in rats induced consistent, reproducible lesions that led to DA cell loss, progressive motor deficits and α-synuclein aggregations whilst maintaining a low mortality rate.

Recently, Van Laar and colleagues reported a detailed histological and behavioural description of a refined rotenone model of PD based on low doses of intraperitoneal rotenone in rats (Van Laar et al., 2016). This model produces a dynamic timeline of events where immediate, mild motor deficits and subsequent recovery were followed by progressive 50-60% impairment in motor function, 50% DA cell loss, and α-synuclein accumulation. Thus, compared to other current models, their refined model better resembles the slow, progressive neurodegenerative process characteristic of PD. Moreover, the slow progression of deficits provides a valuable tool to temporally delineate the different stages of PD, which could provide a better understanding of the pathogenesis of PD, but also allow for clinically relevant screening of novel therapeutic strategies.

Whilst the studies in chapter 2 provided conceptual data to demonstrate a therapeutic potential for nigral SRY inhibition in male PD, the rapid degeneration of motor function and dopamine neurons that occurs over 2 to 3 weeks in these models do not reflect the progressive nature of clinical PD and limits effective screening of neuroprotective targets at clinically relevant time-points. Thus, the main objective of chapter 3 was to determine whether SRY inhibition can slow or halt the progression of motor deficits and nigral degeneration at clinically relevant time-points in the chronic rotenone-induced rat model of PD.

Given that:

- nigral *Sry* is up-regulated in *in vitro* and *in vivo* models of PD.

- up-regulation of *Sry* is detrimental in acute rat models of PD (Chapter 2).

I hypothesise that:

- nigral *Sry* expression is bilaterally up-regulated following chronic intraperitoneal rotenone treatment in male rats.

- reducing nigral *Sry* expression protects against the onset and/or progression of chronic rotenone-induced motor deficits and nigral degeneration in male rats.

To test this hypothesis, the main aims of Chapter 3 are:

1. to determine the regulation of nigral *Sry* expression in the chronic rotenone rat model of PD

2. to assess the effect of reduced nigral SRY expression on the onset of motor deficits and DA cell loss in the chronic rotenone rat model of PD

3. to assess the effect of reduced nigral SRY expression on the progression of motor deficits and DA cell loss in the chronic rotenone rat model of PD

3.2 Materials and Methods

Animals

All methods conformed to the Australian NHMRC published code of practice for the use of animals in research and were approved by the Monash University Animal Ethics Committee (MMCB15/18). Adult Long-Evans male rats weighing between 280 and 350 g were used. Animals were housed in a 12h light: dark cycle room and had access to food and water *ad libitum.*

Stereotaxic implantation of bilateral intranigral cannula

A bilateral guide cannula (22 gauge, Plastics One) placed above the left and right SNc was implanted at 5.3 mm posterior, ±1.9mm lateral from bregma, and 6 mm ventral to the surface of dura (Fig. 3.1A). The guide cannula was secured to the skull with stainless-steel screws and dental cement. Dummy cannulae that protruded <0.5 mm beyond the opening were placed in the guide cannulae.

Repeated intranigral SRY antisense oligonucleotide (ASO) infusions

SRY expression in both left and right SNc was reduced by bilateral intranigral infusions of Sry antisense oligonucleotides (ASO; Fig. 3.1A). The Sry ASO used was a cocktail of 3 distinct ASO directed against rat *Sry* mRNA added in equal proportions, whilst the control sense oligonucleotide (SO) cocktail corresponded to the 3 complement sequences of the three ASO (Table 2.1, Chapter 2). ASO (and SOs) were HPLC-purified (Invitrogen) and dissolved in artificial cerebrospinal fluid (aCSF) to a final concentration of 2μg/μL. ASO (or SO) were infused at a rate of 0.5μL/min directly into the left and right SNc, via the bilateral intranigral cannula for 2 min to deliver a total amount of 2μg of ASO (or SO) per SNc. Infusion was followed

by a 2 min equilibration period, during which the needle remained in place. All rats were repeatedly infused with ASO or SO daily for either 7 or 14 consecutive days.
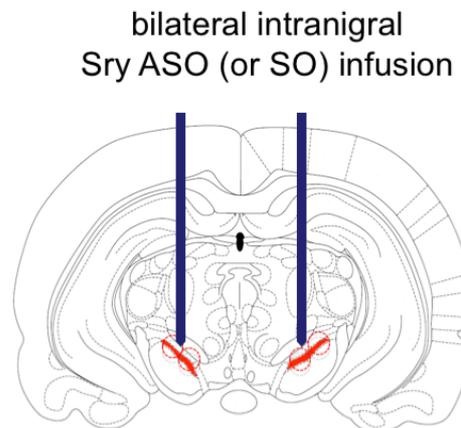
Figure 3.1. Coronal section of the rat midbrain showing placement of the guide cannula above the left and right SNc (red) for repeated bilateral SRY antisense oligonucleotide (ASO) or control sense oligonucleotide (SO) infusions.

Characterisation of the chronic rotenone rat model of PD

Preparation of rotenone

Rotenone solution was first prepared as a 50x stock in 100% dimethylsulfoxide (DMSO) and diluted in medium-chain triglyceride, Miglyol 812 N (Cremer Oleo Gmbh & Co. KG, Hamburg, Germany) to obtain a final concentration of 2.75 or 3.0 mg/mL rotenone in 98% Miglyol 812 N, 2% DMSO. Vortexing the solution creates a stable emulsion of the DMSO containing rotenone and Miglyol 812N. The solution was made fresh 2-3 times/week and stored protected from light and inverted and vortex several times before each injection to eliminate the possibility of settling. The solution was administered at 1 mL/kg and control animals received the vehicle (98% Miglyol 812 N, 2% DMSO) only.

Repeated administration of intraperitoneal rotenone in rats

Bilateral parkinsonism was elicited in rats by repeated daily administration of rotenone at either 3.0 or 2.75 mg/kg/day (dissolved in 98% Miglyol 812 N, 2% DMSO; intraperitoneal; as described by Cannon et al., 2009) to determine the optimal dose that produces loss in motor function and minimal adverse effects (Fig. 3.2). Male rats (n = 4 /group) received repeated daily administration of rotenone or vehicle (98% Miglyol 812 N, 2% DMSO, intraperitoneal) once daily for 14 days. Motor function was assessed by the rearing test and open-field test at days 0, 3, 7, 10 and 14 of rotenone treatment (see Fig. 3.2).
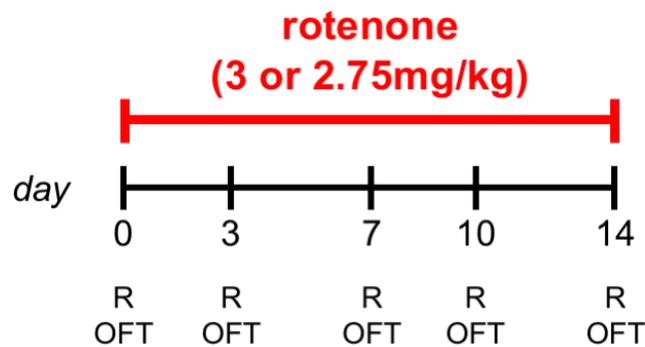
Figure 3.2. Male rats received repeated daily administration of rotenone or vehicle (3.0 or 2.75 mg/kg/day) for 14 days. Motor behaviour was tested by the rearing test (R) and open field test (OFT) at days 0, 3, 7, 10 and 14 of rotenone treatment.

Regulation of nigral Sry expression in the chronic rotenone-induced rat model of PD

To determine the regulation of *Sry* expression in the chronic rotenone-induced rat model of PD, nigral *Sry* mRNA expression was measured at various time points during rotenone administration. In brief, male rats received repeated daily administration of rotenone (2.75 mg/kg/day, intraperitoneal) and killed at various time points of rotenone injections (days 0, 3, 7, 10 or 14) for measurement of nigral *Sry* mRNA and nigral TH immunostaining (see Figure 3.3)
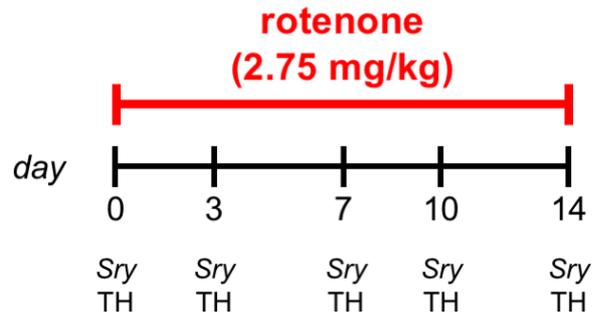
Figure 3.3. Male rats received repeated daily administration of rotenone (2.75 mg/kg/day) for 14 days. Nigral *Sry* mRNA expression and nigral TH cell counts were assessed at days 0, 3, 7, 10 and 14 of rotenone treatment.

Effect of reducing nigral SRY expression on motor deficits and nigral degeneration in the chronic rotenone-induced rat model of PD

To determine the role of Sry during various stages of nigral degeneration in the chronic rotenone rat model of PD, the effect of intranigral Sry ASO-infusion was assessed at various times prior to and following rotenone treatment in male rats.

Effect of reducing nigral SRY expression on the onset of rotenone-induced motor deficits and DA cell loss in male rats

To determine the effect of reducing nigral SRY expression prior to rotenone-induced degeneration, male rats received daily bilateral *Sry* ASO (or SO) infusions into the SNc for 7 days, prior to repeated daily administration of rotenone for 14 days (2.75mg/kg; intraperitoneal). Motor behaviour was assessed at day 0, at day 7 of ASO-infusion, at day 7 and 14 of rotenone injection and at 7 days following the last rotenone injection via the rearing and open field tests. Animals were culled at day 28 for nigral protein measurements (Fig. 3.4A).

To determine the effect of reducing nigral SRY expression during early stages of rotenone-induced nigral degeneration, rats received daily administration

of rotenone for 7 days (2.75mg/kg; intraperitoneal), which was followed by daily intranigral Sry ASO (or SO) infusions for 7 days at one day following the last rotenone administration (Fig. 3.4B). Motor behaviour was assessed at day 0, day 7 of rotenone administration, day 7 of ASO-infusion and 7 and 14 days following the last ASO-infusion using the rearing and open field tests. All animals were culled at day 28 for nigral mRNA and protein measurements (Fig. 3.4B).
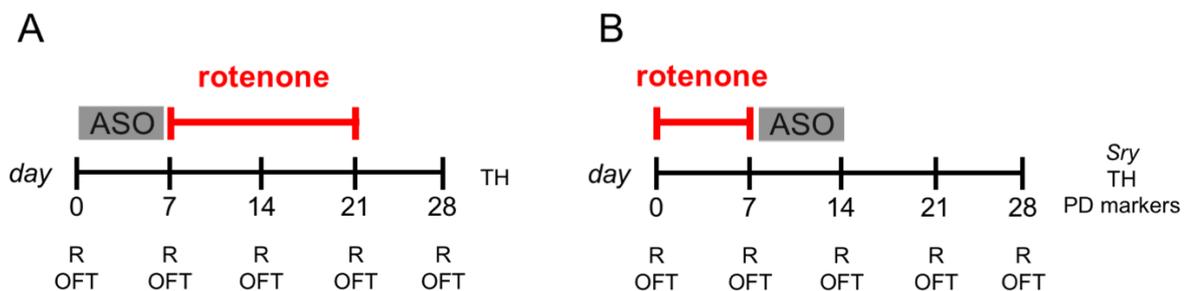
Figure 3.4. Timeline of experiments for Aim 2. SRY ASO (or SO) was infused bilaterally into the SNc (once daily for 7 days) **A)** prior to rotenone treatment or **B)** at 1 day following rotenone treatment in male rats. Motor behaviour was assessed by the rearing test and open field test (R= rearing test, OFT= open-field test). Rats were culled on day 28 and post-mortem samples were assessed for nigral mRNA and protein measurements.

Effect of reducing nigral SRY *expression on the progression of rotenone-induced motor deficits and DA cell loss in male rats*

To determine the effect of reducing nigral SRY expression following the progression of rotenone-induced nigral degeneration, the effect of Sry ASO-infusion was assessed at 4 or 5 weeks following the last rotenone administration in male rats (Fig. 3.5A and 3.5B). The ASO infusion regimen was initiated once the progression of rotenone-induced motor symptoms was evident, which provides a greater level of clinical relevance for PD patients.

In brief, one group of rats received daily administration of rotenone for 7 days (2.75mg/kg; intraperitoneal), which was followed by repeated intranigral Sry ASO (or SO) infusions for 14 days at 5 weeks following the last rotenone administration (Fig. 3.5A). Another group of rats received daily administration of rotenone for 14 days (2.75mg/kg; intraperitoneal), which was followed by repeated intranigral Sry ASO (or SO) infusions for 14 days at 4 weeks following the last rotenone administration (Fig. 3.5B). Motor behaviour was assessed weekly by the rearing test and bi-weekly by the open field test for 12 weeks - i.e. from the start of rotenone administration to 4 weeks following the last ASO (or SO) infusion. All animals were culled at week 12 for nigral mRNA and protein measurements.
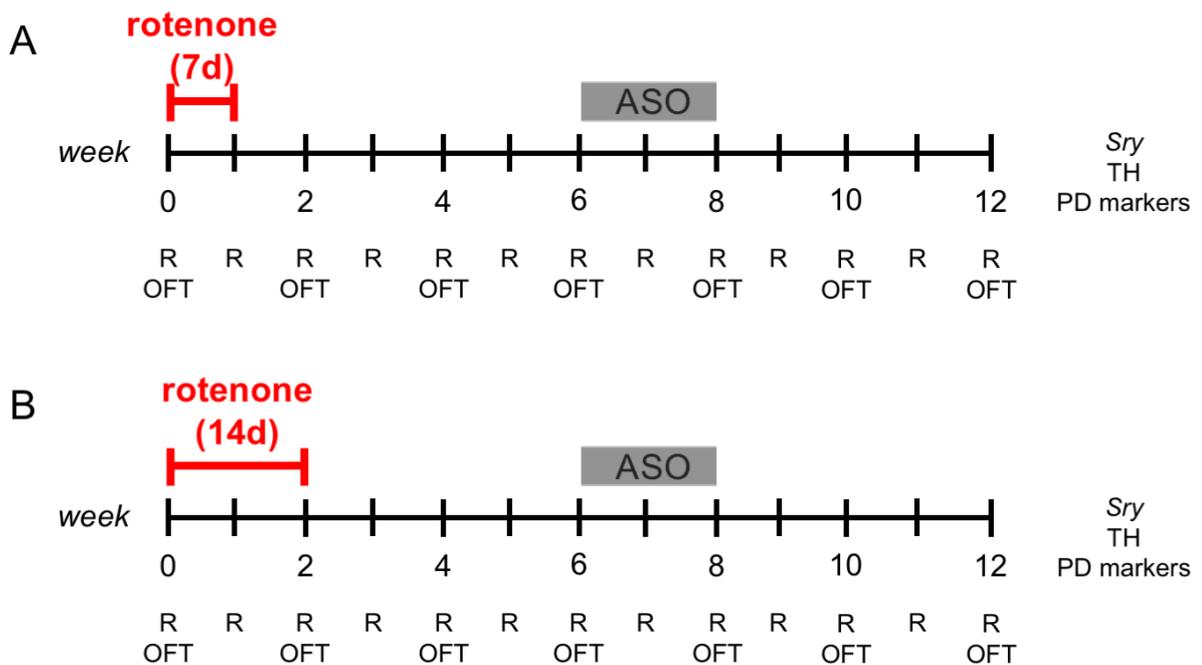
Figure 3.5. Timeline of experiments for Aim 3. SRY ASO (or SO) was infused **A)** at 5 weeks following 7 days of rotenone treatment or **B)** at 4 weeks following 14 days of rotenone treatment. Motor behaviour was assessed weekly via the rearing test and bi-weekly via the open field test for 12 weeks (R= rearing test, OFT= open-field test). Rats were culled at week 12 and post-mortem samples were assessed for nigral mRNA and protein measurements.

Motor behaviour

Motor behaviour tests used in chapter 2 (limb-use asymmetry, amphetamine-induced rotation test) assessed unilateral effects of intranigral toxin injection and ASO infusion on motor function. However, the chronic intraperitoneal rotenone rat model of PD exhibits bilateral motor deficits and therefore the rearing and open field tests were employed in chapter 3 to assess bilateral deficits (or gain) in motor function.

Rearing test

The rearing test assessed spontaneous rearing during vertical exploration in rats, where motor impairment is indicated by a reduction in rears performed. In brief, the rat was placed in a clear plexiglass cylinder (height = 30 cm, diameter = 20 cm) for 5 minutes (Fig. 3.6A) and the total number of rears was quantified. To be classified as a rear, the animal had to raise forelimbs above shoulder level and make contact with the cylinder wall with either one or both forelimbs. Removal of both forelimbs from the cylinder wall and contact with the table surface was required before another rear was scored. The data was expressed as total rears for the 5-minute test period.

Open field test

The open field test measured exploratory behaviour and general locomotor activity in rodents. This test was conducted in a 60cm x 60cm x 50cm open field area. The rat was placed individually into the arena for a 10 minute testing period (Fig. 3.6B). A video camera above the arena recorded the locomotion of the rats, and various parameters of movement (i.e. average velocity, time spent stationary) during the 10-minute period were measured by the TopScan (V.7.3.1) program.
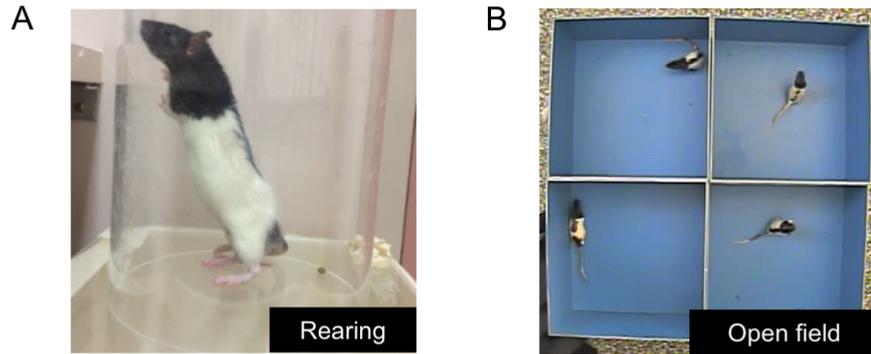
Figure 3.6. Photo images of **A)** the rearing test and **B)** open field test.

Tissue processing and histology

Rat brains were either intracardially perfused and processed for immunohistochemistry or isolated fresh and processed for mRNA measurements. Coronal sections were cut serially through the SNc (Fig. 3.1) and stored at -80°C. Between each series, a 200µm slab was collected to isolate SNc tissue to measure nigral Sry mRNA.

TH immunohistochemistry

TH immunohistochemistry was performed by incubating 5 regularly spaced 10µm-thick sections covering the whole SNc in sheep anti-TH primary antibody (1:2000, Pelfreez P60101, overnight at 4°C), followed by a 2hr incubation in biotinylated secondary antibody (goat, anti-sheep IgG, 1:1000, Vector Labs, USA) and reacted with cobalt and nickel-intensified diaminobenzidine (DAB, Sigma-Aldrich). DAB-immunostained sections were counterstained with neutral red to visualise nuclei. DAB-immunostained sections were analysed by bright-field microscopy, using an Olympus microscope equipped with Olympus cellSens image analysis software (v.1.7.1). TH-immunoreactive and neutral-red positive cell bodies were quantified by

manual whole-section counts on regularly spaced bilateral sections covering the whole SNc.

TOM-20 and TH co-immunofluorescence

Mitochondrial dysfunction was assessed by TOM-20 and TH co-immunofluorescence staining, and performed by incubating 5 regularly spaced 10µm-thick sections covering the whole SNc in sheep anti-TH and rabbit anti-TOM-20 primary antibody (anti-TH, 1:1000, Pelfreez P60101; anti-TOM-20, 1:200, Santa Cruz SC-17764, overnight at 4°C), followed by a 2hr incubation in donkey anti-rabbit 594 and donkey anti-sheep 488 (1:500, 1hr at RT, Invitrogen) and DAPI (1:1000, Invitrogen). Immunofluorescence staining was visualised by confocal microscopy (Nikon C1 macro laser confocal, Shinagawa-ku, Tokyo, Japan) equipped with NIS-Elements image software. For quantification of TH, TOM-20 and DAPI-positive labelling, confocal images at 20x magnification of regularly spaced sections covering the whole SNc were analysed using IMARISx64 software (v7.6.5). Fluorescent intensity values of TH and TOM-20 above background threshold were given for all DAPI positive cells, and were bilaterally quantified for co-immunostaining.

TUNEL and iNOS staining

DNA damage was assessed by TUNEL staining, and performed by incubating 5 regularly spaced 10µm-thick sections covering the whole SNc in TdT enzyme and nucleotide mixture (1hr at 37°C, In Situ Cell Death Detection Kit, Roche), followed by incubation in anti-fluorescein antibody-peroxidase POD (30min at 37°C, Roche) reacted with cobalt and nickel-intensified diaminobenzidine (DAB, Sigma-Aldrich).

Sections were counterstained with cresyl violet (Sigma-Aldrich Inc) to visualise nuclei.

Neuroinflammation was assessed by iNOS immunohistochemistry and performed by incubating 5 regularly spaced 10μm-thick sections covering the whole SNc in mouse anti-iNOS primary antibody (1:200, Santa Cruz SC-7271, overnight at 4°C), followed by 2hr incubation in biotinylated secondary antibody (rat, anti-mouse IgG, 1:1000, Vector Labs, USA) and reacted with cobalt and nickel-intensified diaminobenzidine (DAB, Sigma-Aldrich). DAB-immunostained sections were counterstained with neutral red to visualise nuclei. TUNEL-positive or iNOS-positive SNc sections were analysed by bright-field microscopy (Olympus BX53F, Japan) equipped with Olympus cellSens image analysis software (v.1.7.1) and quantified by manual whole-section counts on 5 regularly spaced bilateral sections covering the whole SNc. Neurons with deep black nuclei were identified as TUNEL-positive or iNOS-positive neurons.

Nigral Sry mRNA expression

The extent of SRY knockdown following SRY ASO-infusion was assessed by qPCR. In brief, total RNA (100-300ng) was isolated using TRI-Reagent (Sigma Aldrich) was reverse-transcribed into cDNA (Go Script, Promega) and equal amount of cDNA template was added to SensiMixTM SYBR Hi-ROX Mastermix (Bioline) using primers listed in Supplementary table 1 (Appendix). The relative level of mRNA was interpolated from a standard curve prepared by serially diluting the cDNA reaction. All quantitative PCR reactions were conducted in triplicates. Final values represent fold change of *Sry* expression relative to the housekeeping genes TATA-Box Binding Protein (Tbp, rat).

Statistical Analysis

All values are expressed as the mean ± S.E.M. All data was analysed using tools within Graphpad Prism 7. Motor behaviour studies of the treatment groups across the days of testing was analysed by two-way analysis of variance (ANOVA) and post hoc Bonferroni test for Figures 3.7 to 3.10, and two-tailed unpaired Student t-test for behaviour studies in Figures 3.11 to 3.13. Histological and biochemical studies were analysed using by one-way analysis of variance (ANOVA). The exact P-values of the statistical tests are given in the figure legends. Probability level of 5% ($P < 0.05$) was considered significant for all statistical tests.

3.3 Results

Chronic rotenone treatment in male rats induces progressive loss in motor function in the rearing and open field test

To validate the chronic rotenone-induced rat model of PD, the effect of repeated daily administration of rotenone at two different doses (3 and 2.75mg/kg) on motor function was assessed in male rats.

Repeated daily injections of 3mg/kg rotenone in male rats induced a 59% reduction in total rears at day 3 of injection (41% of day 0; Fig. 3.7A, left), with only 10 to 20% further reductions at days 7, 10 and 14 (Fig. 3.7A, left). Similarly, 3mg/kg rotenone treatment induced a 67% reduction in distance travelled (33% of day 0; Fig. 3.7B, left) and 68% reduction in velocity (32% of day 0; Fig. 3.7C, left) at day 3, with only 6 to 8% further reductions at days 7, 10 and 14 (Fig. 3.7B and 3.7C, left) in male rats. Conversely, 3mg/kg rotenone increased time stationary by 169%, 288%, 279%, and 147% at days 3, 7, 10 and 14 of injection (Fig. 3.7D, left).

Repeated rotenone injections at 2.75mg/kg in male rats led to a much slower and gradual reduction in total rears (60%, 48%, 40%, 16% of day 0, Fig. 3.7A, right), distance travelled (61%, 41%, 25%, 34% of day 0; Fig. 3.7B, right), and velocity (62%, 40%, 17%, and 31 % of day 0; Fig. 3.7C, right) over the 14 days. Similarly, 2.7mg/kg rotenone progressively increased time stationary (168%, 273%, 313%, 319% of day 0; Fig. 3.7D, right) over the 14 days.

Given the slower and progressive nature of the motor deficits induced by the 2.75mg/kg dose-regimen when compared to the 3mg/kg, the dose of 2.75mg/kg rotenone was chosen for the remainder of the studies in this chapter.
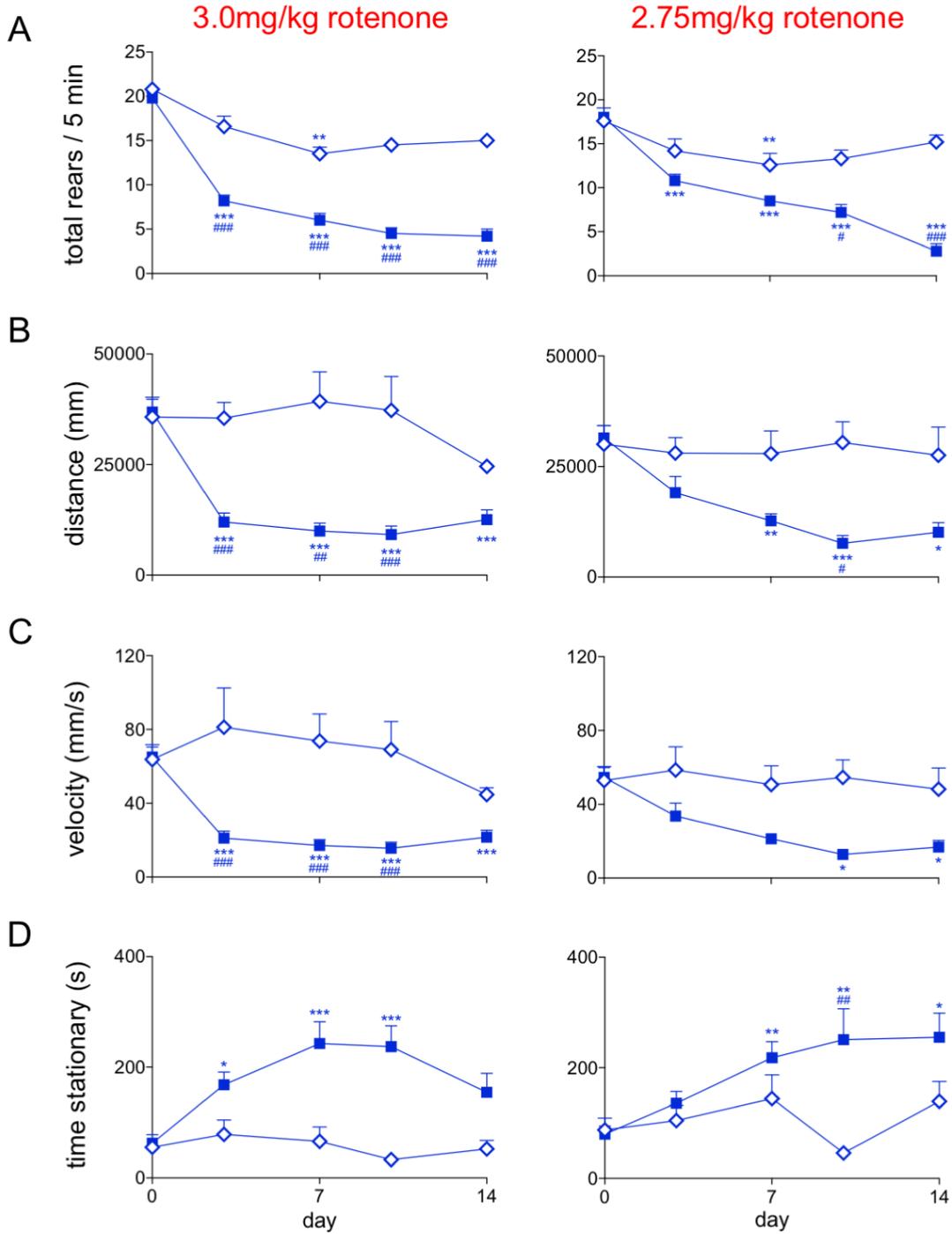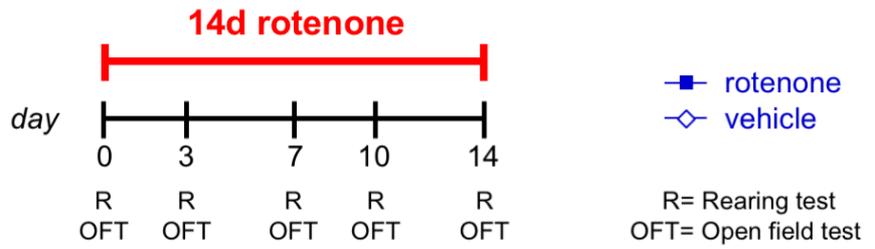
Figure 3.7. Effect of repeated intraperitoneal rotenone treatment (3.0 or 2.75 mg/kg) on motor function in male rats. Male rats received daily intraperitoneal administration of rotenone (3.0 or 2.75 mg/kg) or vehicle for 14 days. Motor function was assessed at days 0, 3, 7, 10 and 14 of rotenone treatment by the rearing and open field test. The effect of 3.0 mg/kg (left) or 2.75 mg/kg (right) rotenone treatment on motor function was assessed in the **A)** rearing test (total rears / 5 min) and in the **B-D)** open field test by the **B)** distance travelled, **C)** average velocity, and **D)** time stationary over 10 mins (n≥4/group; two-way ANOVA, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to day 0; ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$ compared to vehicle).

Nigral *Sry* expression is up-regulated in the chronic rotenone-induced rat model of PD

To determine the regulation of nigral *Sry* expression in the chronic rotenone-induced rat model of PD, nigral *Sry* mRNA expression was assessed at various time points of daily rotenone administration in male rats.

Figure 3.8 demonstrates that repeated daily rotenone injections (2.75mg/kg) induced gradual increases in nigral *Sry* mRNA expression with maximal increase observed at day 10 of injection (512% of day 0, $P < 0.001$ vs day 0, Fig. 3.8B). In parallel, rotenone injections induced a gradual loss of nigral TH-positive cells over the 14 days of treatment, with maximal loss observed at day 14 of injection (64% of day 0, $P < 0.05$ vs day 0, Fig. 3.8D).

Together, these results show that nigral *Sry* mRNA expression is highly up-regulated and that the up-regulation of *Sry* precedes DA cell loss in the chronic rotenone rat model of PD in male rats, which is in line with the findings from the acute rat models of PD in chapter 2.
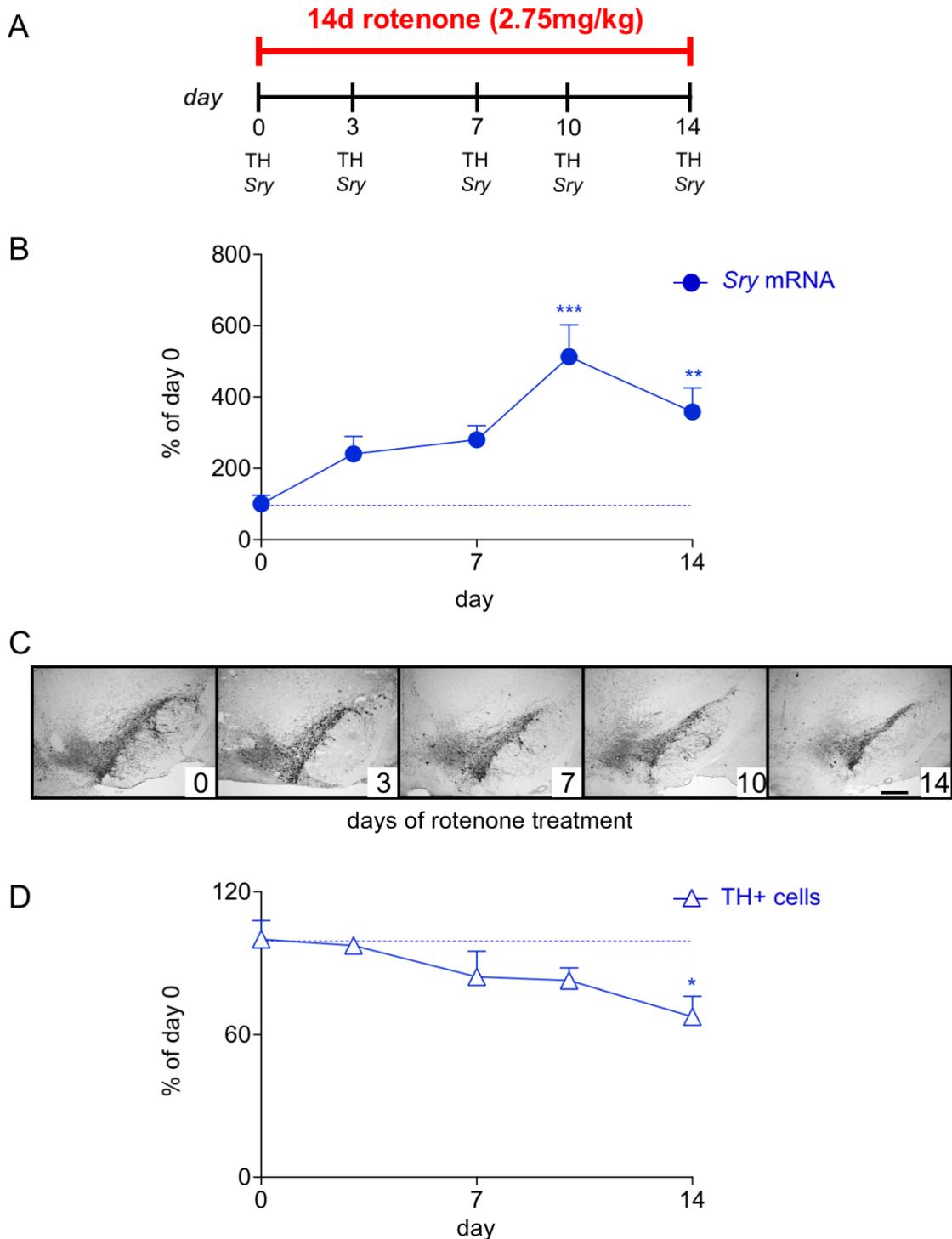
Figure 3.8. Effect of repeated intraperitoneal rotenone treatment (2.75mg/kg daily for 14 days) on nigral *Sry* mRNA expression and TH-positive cells in male rats. **A)** Male rats were culled at various time points of rotenone treatment (days 0, 3, 7, 10 or 14) and were processed for **B)** nigral *Sry* mRNA expression and **C-D)** nigral TH-positive cell counts (n= 5/group; one-way ANOVA, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs. SO; scale=400μm; dashed line = baseline)

Effect of reducing nigral SRY expression during the onset of rotenone-induced motor deficits and nigral cell loss

To determine whether reducing nigral SRY expression can slow or prevent the onset of rotenone-induced motor deficits and nigral degeneration in male rats, the effect of intranigral *Sry* ASO-infusion prior to rotenone treatment (Fig. 3.9) or at 1 day following rotenone treatment (Fig. 3.10) was assessed in male rats. A group of untreated male rats (n=6) at the same age (14 weeks old) were included as controls.

Figure 3.9 shows that intranigral *Sry* ASO-infusion for 7 days in male rats reduced the total number of rears (56% of SO, $P < 0.05$ vs SO, Fig. 3.9A, day 7), without significantly affecting velocity or time stationary (Fig. 3.9B and C, day 7). Subsequent rotenone injections for 14 days led to reductions in total rears ($P < 0.001$ vs day 7, Fig. 3.9A, day 21) and velocity ($P < 0.01$ vs day 7, Fig. 3.9B, day 21) and increased time stationary ($P < 0.001$ vs day 7, Fig. 3.9C, day 21) in SO-infused rats. Interestingly, partial recovery in motor function was observed at 7 days following the last rotenone injection in the SO-infused group (SO, Fig. 3.9A to C, day 28). Prior ASO-infusion did not prevent rotenone-induced deficits in total rears or velocity (Fig. 3.9A and B, day 14 to 28), but prevented rotenone-induced increases in time stationary at day 21 (Fig. 3.9C, day 21).

Post-mortem analysis of SNc sections at day 28 revealed small but significant losses in nigral TH-positive cells for both SO (70% of control, $P< 0.0001$ vs control, Fig. 3.9D) and ASO-infused male rats (78% of control, $P< 0.001$ vs control, Fig. 3.9D) with respect to controls. However, there was no significant difference in the number of TH-positive cells between SO and ASO-infused rats (Fig. 3.9D).
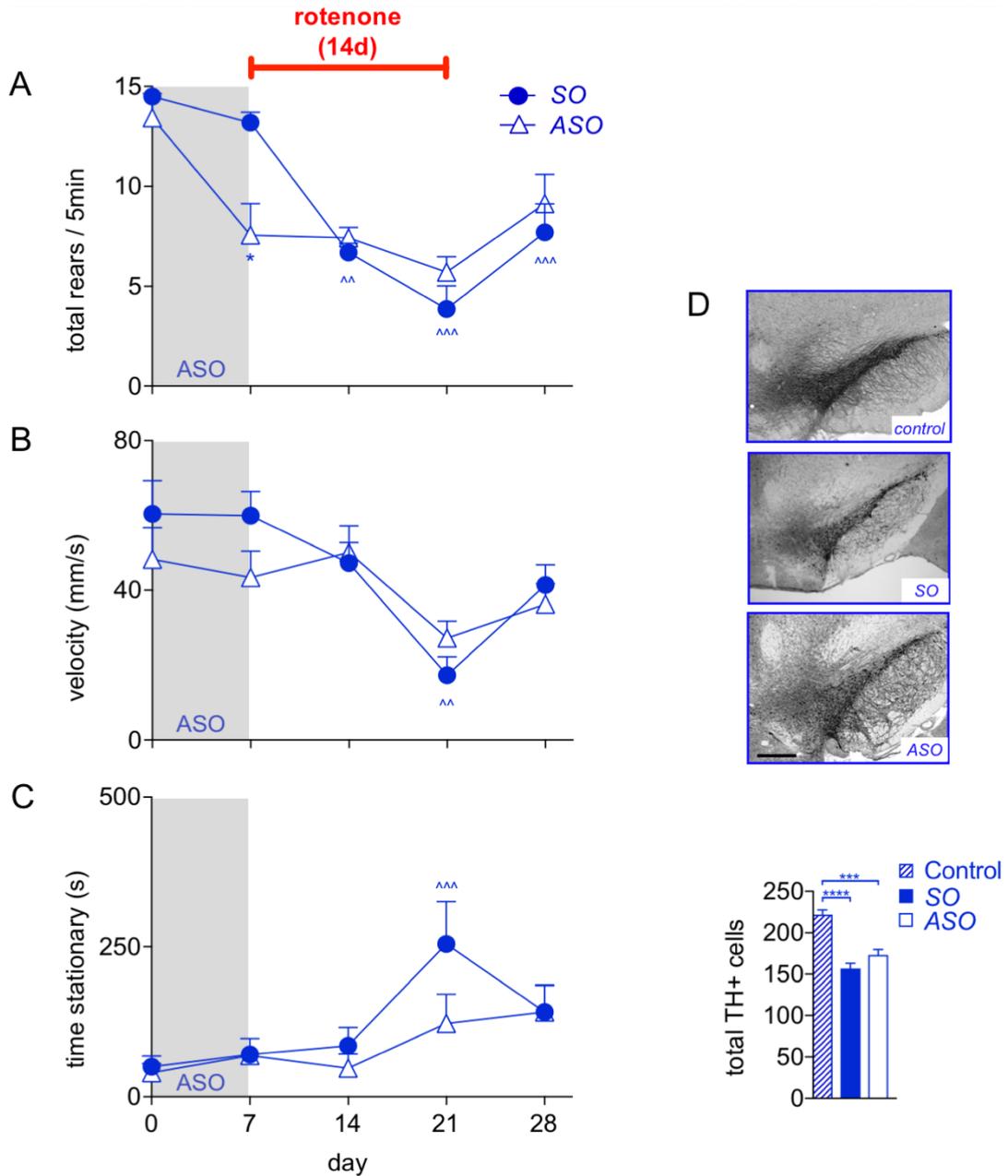
Figure 3.9. Effect of nigral *Sry* ASO-infusion prior to rotenone treatment on motor deficits and nigral DA cell loss in male rats. ASO (or SO) was infused bilaterally into the SNc for 7 days, followed by daily rotenone administration for 14 days in male rats. Motor function was assessed by the **A)** rearing test or the open-field test to measure **B)** velocity and **C)** time stationary. (n=7/group; two-way ANOVA, * $P < 0.05$ vs SO; ^ $P < 0.05$, ^^ $P < 0.01$, ^^^ $P < 0.001$ vs day 7). Nigral degeneration was assessed by **D)** nigral TH-positive cell counts (n=7/group; one-way ANOVA, **** $P < 0.0001$, *** $P < 0.001$ vs control; scale=400µm).

Figure 3.10 shows that rotenone injections for 7 days in male rats, prior to SO- or ASO-infusion, led to a significant reduction in total rears ($P < 0.01$ vs day 0, Fig. 3.10A, day 7), but not velocity or time stationary (Fig. 3.10B and C, day 7). The rotenone-induced deficits in total rears persisted for the next 21 days during and following the SO-infusion (Fig. 3.10A, day 7 to 28). In contrast, ASO-infusion at 1 day following the last rotenone injection increased total rears at days 21 and 28 when compared to the SO-infused group ($P < 0.01$ vs SO, day 21 and 28, Fig. 3.10A).

Post-mortem analysis of SNc sections at day 28 revealed significant losses in nigral TH-positive cells in both the SO-infused (60% of control, $P < 0.0001$ vs control, Fig. 3.10D) and ASO-infused group (65% of control $P < 0.0001$ vs control, Fig. 3.10D) when compared to the control group. However, there was no significant difference in the number of TH-positive cells between SO and ASO-infused groups (Fig. 3.10D). Co-immunofluorescence staining for TOM-20 and TH revealed a significant loss in the number of TOM-20 and TH co-positive neurons in the SO-infused group (77% of control, $P < 0.01$ vs control, Fig. 3.10E), but not in the ASO-infused group, when compared to the control group. Nigral TUNEL staining revealed small, but insignificant, increases in TUNEL-positive neurons in both the SO and ASO-infused groups when compared to the control group (Fig. 3.10F). Nigral iNOS immunostaining revealed significant increases in the number of iNOS-positive neurons in both the SO- and ASO-infused group when compared to controls ($P < 0.05$ vs control, Fig. 3.10G), although there was no significant difference between the SO and ASO-infused rats (Fig. 3.10G).

Together, these studies indicate that *Sry* ASO-infusion in male rats, before or during the onset of rotenone-induced motor deficits, exerted modest protective effects on motor function, but did not affect nigral DA cell loss or cellular events underlying nigral DA cell loss.
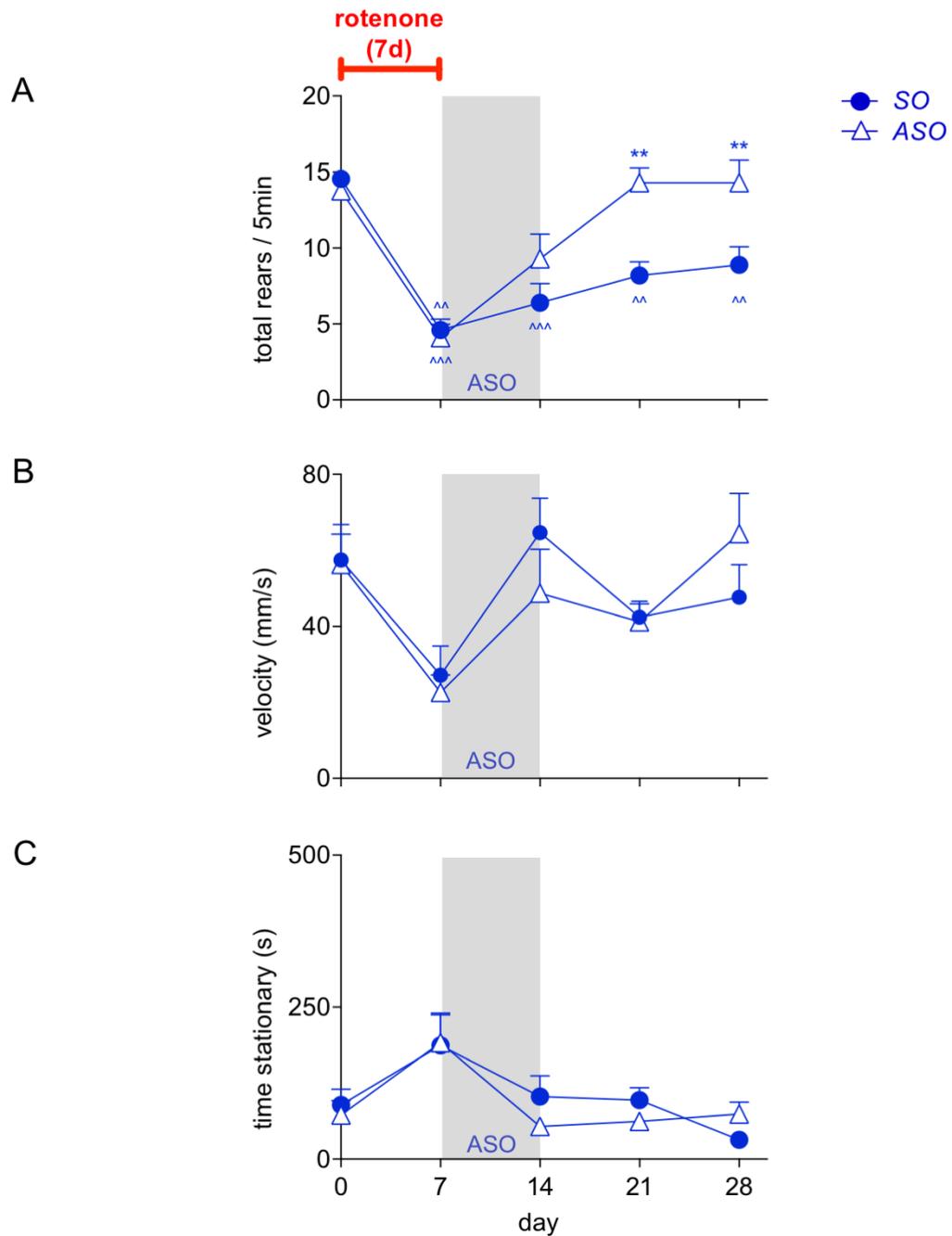
Figure 3.10. Effect of nigral *Sry* ASO-infusion at 1 day following rotenone treatment on motor deficits and nigral DA cell loss in male rats. Rotenone was administered daily for 7 days, followed by bilateral ASO (or SO) infusion into the SNc for 7 days in male rats. Motor function was assessed by the **A)** rearing test or the open-field test to measure **B)** velocity and **C)** time stationary (n=10/group; two-way ANOVA, ** $P < 0.01$ vs SO; ^^ $P < 0.01$, ^^^ $P < 0.001$ vs day 0 i.e. prior to rotenone).
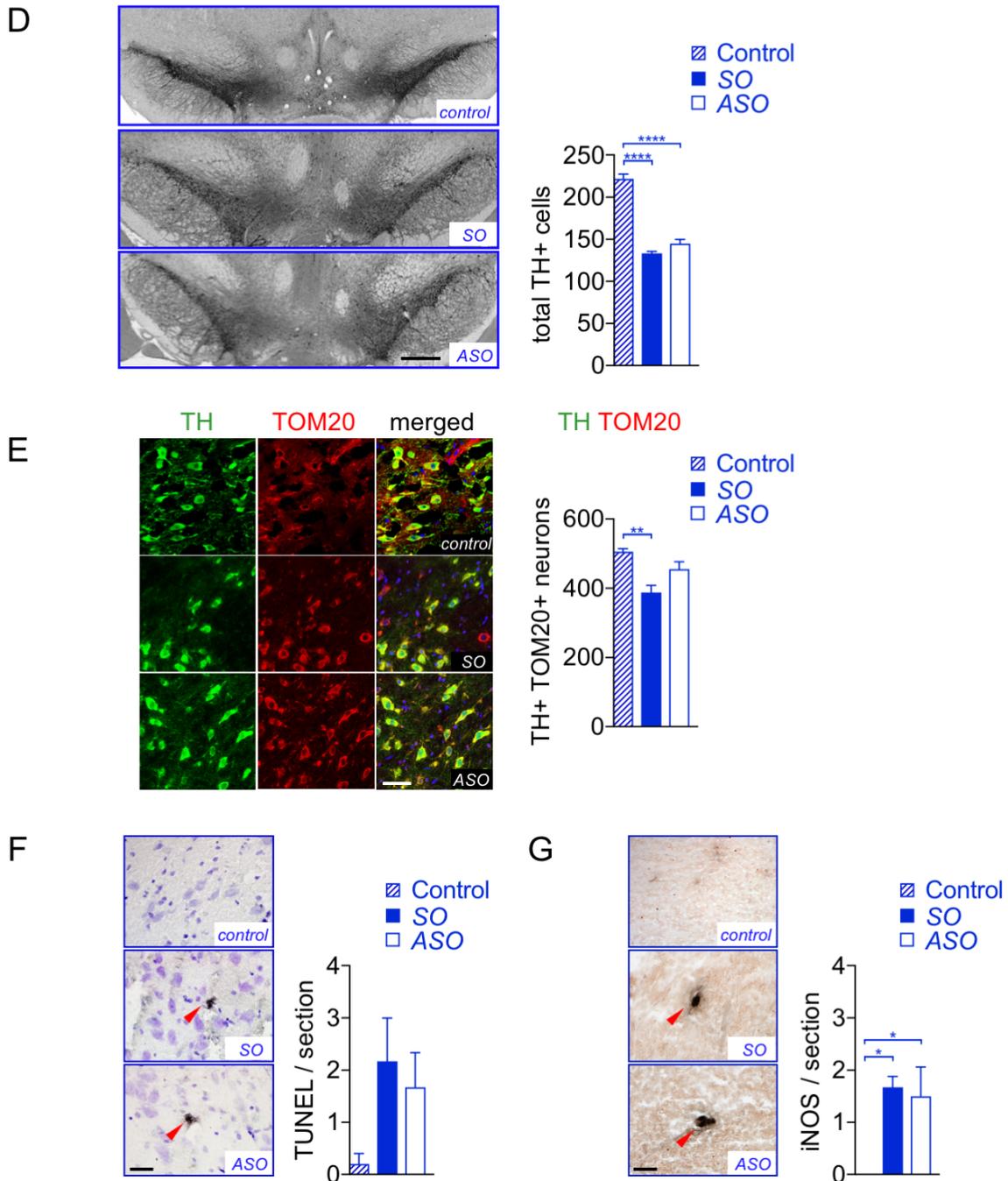
Figure 3.10 (continued). Nigral degeneration was assessed by **D)** nigral TH-positive cell counts (n=10/group; one-way ANOVA, **** $P < 0.0001$ vs control; scale=400µm). Effect of ASO (or SO) infusion on **E)** nigral TH- and TOM-20-positive (merged) neurons, **F)** TUNEL-positive neurons, and **G)** iNOS-positive neurons (n=6/group; one-way ANOVA, * $P < 0.05$, ** $P < 0.01$ vs control; scale=20µm).

Effect of reducing nigral SRY expression during the progression of rotenone-induced motor deficits and nigral cell loss

To determine whether reducing nigral SRY expression can slow or halt the progression of nigral degeneration in male rats, the effect of intranigral *Sry* ASO-infusion was assessed at 4 to 5 weeks following the last rotenone treatment administration in male rats. Rats received either 7, 10 or 14 days of rotenone treatment. A group of untreated male rats (n=6) at the same age (23-24 weeks old) were included as controls for the study.

Figure 3.11 shows that rotenone treatment for 7 days in male rats led to a significant reduction in total rears ($P < 0.001$ vs week 0, Fig. 3.11A, week 1). A partial recovery in motor function was observed between week 1 to 3 (Fig. 3.11A to C, week 1 to 3), followed by a gradual decline in total rears and velocity over the next 9 weeks for both groups (Fig. 3.11A to C, week 4 to 12). However, multiple comparison tests revealed there were no significant differences in total rears, velocity or time stationary between SO and ASO-infused male rats (Fig. 3.11A to C, week 12). There was no significant interaction between time $\times$ treatment for total rears, velocity or time stationary, although a main effect of time was observed for total rears ($F_{12, 208} = 39$, $P < 0.0001$) and velocity ($F_{6, 112} = 16.09$, $P < 0.0001$). Post-mortem analysis of SNc sections at week 12 revealed significant losses in nigral TH-positive cells in both SO-infused (50% control, $P < 0.0001$ vs control, Fig. 3.11D) and ASO-infused rats (52% control, $P < 0.0001$ vs control, Fig. 3.11D) when compared to control, although there was no significant difference between the SO and ASO groups (Fig. 3.11D). TOM-20 and TH immunostaining revealed a small reduction in the number of TOM-20 / TH co-positive neurons in SO-infused rats (84% of control, $P < 0.05$ vs control, Fig. 3.11E) which was not observed in

ASO-infused rats ($P > 0.05$ vs control, Fig. 3.11E). However, there was no significant difference in the number of TOM-20 and TH-co-positive neurons between the SO and ASO group ($P = 0.052$ vs ASO, Fig. 3.11E). Nigral TUNEL or iNOS staining revealed that rotenone treatment for 7 days did not significantly increase the number of TUNEL or iNOS-positive neurons in the SO or ASO-infused rats when compare to control (Fig. 3.11F). Thus, ASO-infusion following 7-day rotenone treatment did not slow or halt the progressive loss in motor function and nigral degeneration in male rats.

Supplementary figure 1 shows that rotenone treatment for 10 days led to a similar behavioural profile as observed with 7 days rotenone treatment. Intranigral ASO-infusion at 4 weeks following the last rotenone treatment did not significantly affect velocity or time stationary compared to the SO-infused group (Supplementary Fig. 1B and C, day 81). Whilst ASO-infusion led to increased rears on days 67, 74 and 81, this was not significantly different when compared to the SO group (Supplementary Fig. 1A, day 67 to day 81). Rotenone treatment for 10 days led to significant losses in nigral TH-positive cells (Supplementary Fig. 1D) and TOM-20 and TH co-positive neurons (Supplementary Fig. 1E) in both SO and ASO-infused rats, with no significant difference between the two groups (Supplementary Fig. 1D and E). Moreover, rotenone treatment did not increase the number of TUNEL-positive (Supplementary Fig. 1F) or iNOS-positive neurons (Supplementary Fig. 1G). Thus, ASO-infusion following 10-day rotenone treatment did not significantly affect progressive loss in motor function and nigral degeneration in male rats.
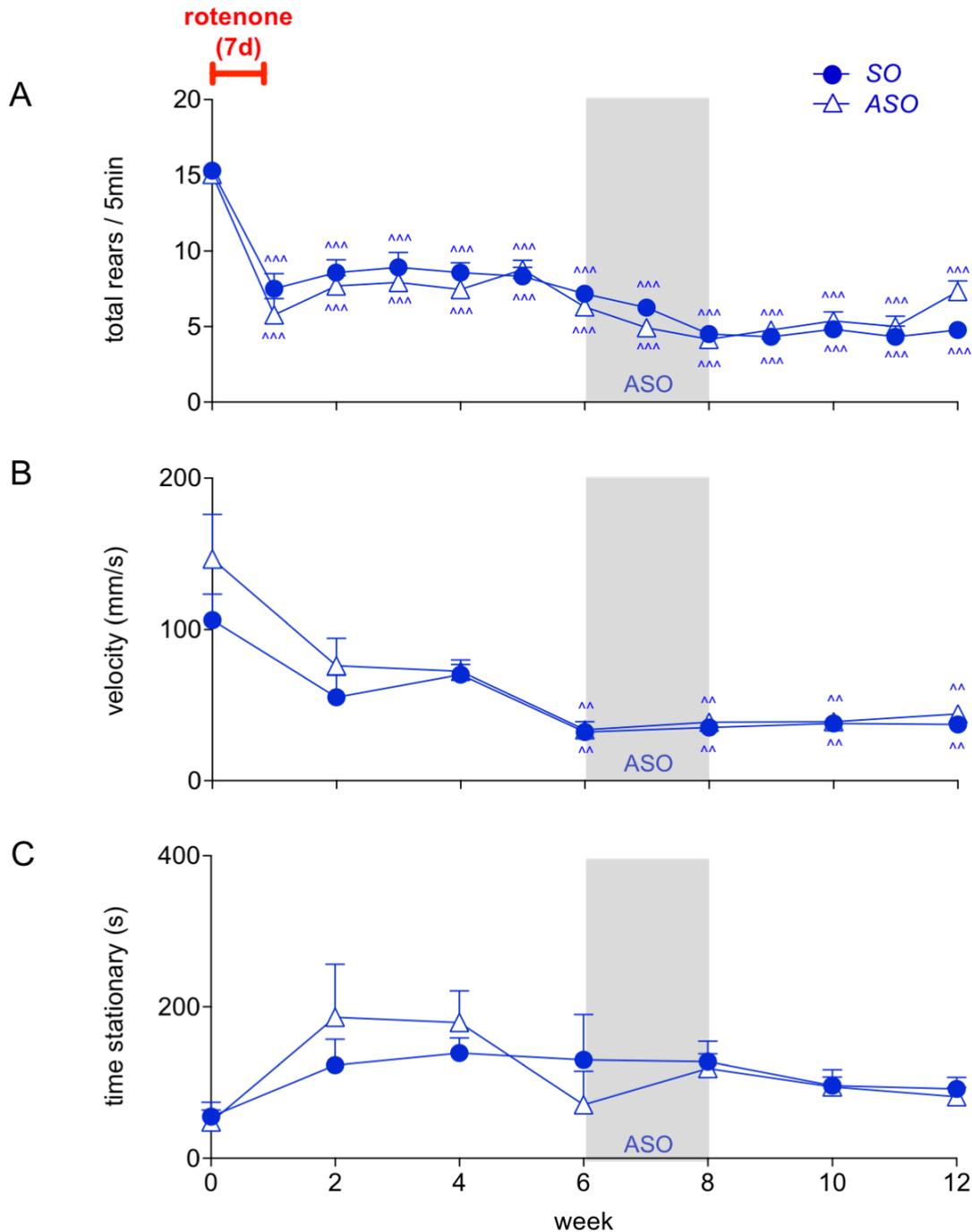
Figure 3.11. Effect of nigral *Sry* ASO-infusion following 7 days rotenone treatment on progression of rotenone-induced motor deficits and nigral DA cell loss in male rats. ASO (or SO) was infused bilaterally into the SNc for 14 days, 5 weeks following repeated daily rotenone administration for 7 days in male rats. Motor function was assessed by the **A)** rearing test or the **(B-C)** open-field test to measure **B)** velocity and **C)** time stationary. (n=9/group; two-way ANOVA, ^^ P < 0.01, ^^^ P < 0.001 vs day 0).
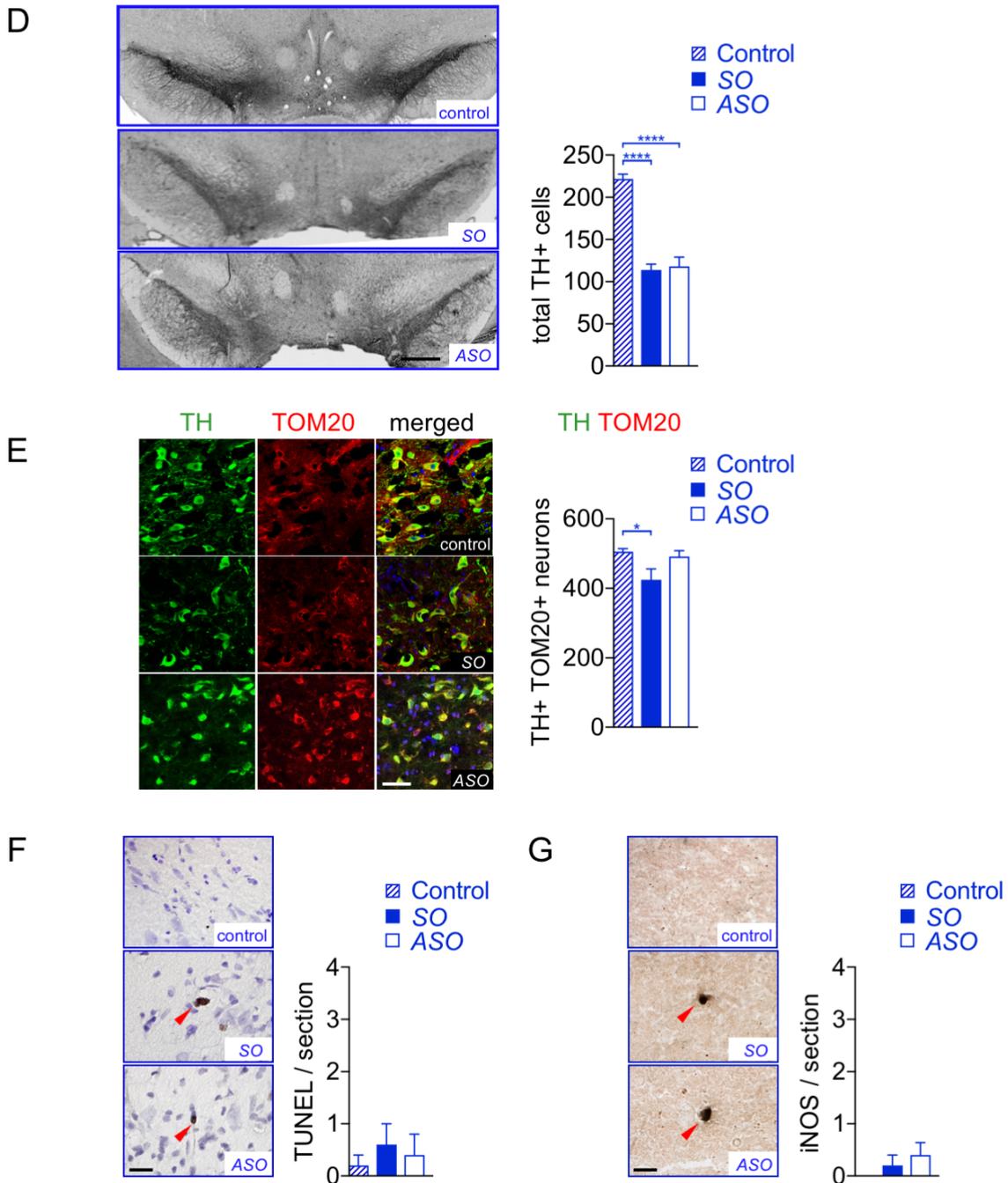
Figure 3.11 (continued). Nigral degeneration was assessed by **D)** nigral TH-positive cell counts (n=9/group; one-way ANOVA, **** P < 0.0001; scale=400μm). Effect of ASO (or SO) infusion on **E)** nigral TH- and TOM-20-positive (merged) neurons, **F)** TUNEL-positive neurons, and **G)** iNOS-positive neurons (n≥5/group; one-way ANOVA, * P < 0.05 vs control; scale=20μm).

Figure 3.12 shows that rotenone treatment for 14 days in male rats led to significant reductions in total rears and velocity ($P < 0.001$ vs week 0, Fig. 3.12A to B, week 2), followed by a partial recovery in total rears and velocity between week 2 to 4 for both SO and ASO groups (Fig. 3.12A to C, week 2 to 4). Intranigral SO infusion did not affect the progressive decline in motor function from week 6 to 12 (Fig. 3.12A to C, week 6 to 12). In contrast, ASO-infusion reversed the rotenone-induced decline in total rears at week 11 and 12 when compared to the SO group ($P < 0.01$ and $P < 0.001$ vs SO, week 11 and 12, Fig. 3.12A). A significant interaction between time x treatment was observed for total rears ($F_{12, 367} = 4.026$, $P < 0.0001$) but not for velocity or time stationary, whilst a main effect of time was observed for total rears ($F_{12, 367} = 34.89$, $P < 0.0001$), velocity ($F_{6, 138} = 21.05$, $P < 0.0001$) and time stationary ($F_{6, 198} = 5.207$, $P < 0.0001$).

Post-mortem analysis of SNc sections revealed a significant loss in nigral TH-positive cells in SO-infused rats (40% of control, $P < 0.0001$ vs control, Fig. 3.12D), which was partially restored in ASO-infused rats ($P < 0.01$ vs SO, 62% of control, $P < 0.0001$ vs control, Fig. 3.12D). Co-immunofluorescence staining for TOM-20 and TH revealed a significant reduction in the number of TOM-20 and TH co-positive neurons in SO-infused rats (64% of control, $P < 0.05$ vs control, Fig. 3.12E), which was reversed in ASO-infused rats ($P < 0.05$ vs SO, Fig. 3.12E). Nigral TUNEL staining revealed a significant increase in TUNEL-positive neurons in SO-infused rats ($P < 0.05$ vs control, Fig. 3.12F), which was reversed in ASO-infused rats ($P < 0.05$ vs SO, Fig. 3.12F). Similarly, iNOS immunostaining revealed a significant increase in the number of iNOS-positive neurons in the SO-infused group when compared to controls ($P < 0.05$ vs control, Fig. 3.12G), which was reversed in ASO-infused rats ($P < 0.05$ vs SO, Fig. 3.12G).

Together, these results demonstrate that *Sry* ASO-infusion following 14-day rotenone treatment in male rats reduces the progression of rotenone-induced motor deficits and nigral DA cell loss, which was associated with blockade of rotenone-induced increases in mitochondrial degradation, DNA damage, and neuroinflammation.
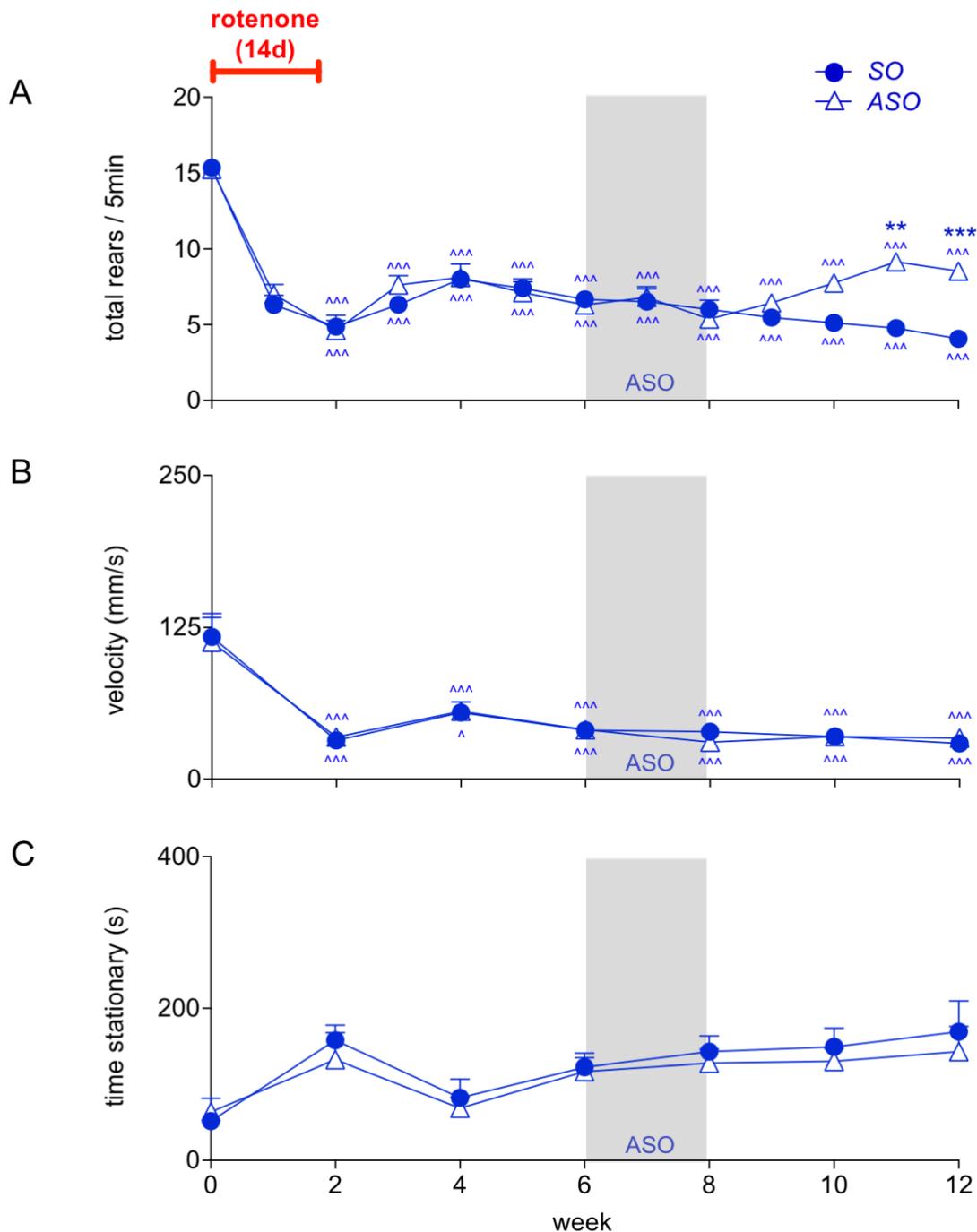
Figure 3.12. Nigral *Sry* ASO-infusion following 14 days rotenone treatment halts progression of rotenone-induced motor deficits and nigral degeneration in male rats. ASO (or SO) was infused bilaterally into the SNc for 14 days, 5 weeks following repeated daily rotenone administration for 14 days in male rats. Motor function was assessed by the **A)** rearing test or the **(B-C)** open-field test to measure **B)** velocity and **C)** time stationary. (n≥13/group; two-way ANOVA, ** $P < 0.01$, *** $P < 0.001$ vs SO, ^^^ $P < 0.001$ vs week 0).
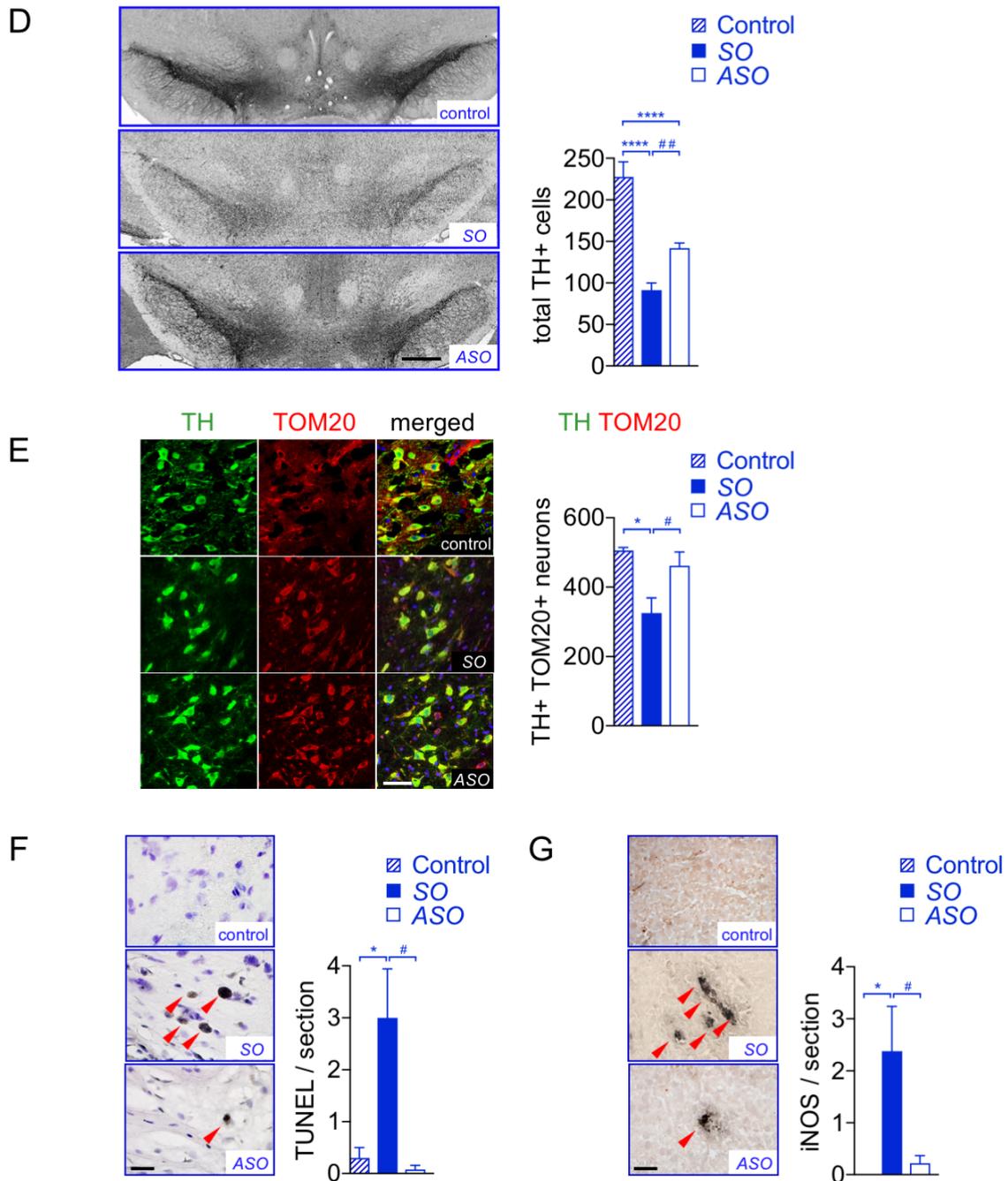
Figure 3.12 (continued). Nigral degeneration was assessed by **D)** nigral TH-positive cell counts (n=10/group; one-way ANOVA, **** $P < 0.0001$, ## $P < 0.01$ vs SO; scale=400µm. Effect of ASO (or SO) infusion on **E)** nigral TH- and TOM-20-positive (merged) neurons, **F)** TUNEL-positive neurons, and **G)** iNOS-positive neurons (n≥8/group; one-way ANOVA, * $P < 0.05$ vs control, # $P < 0.05$ vs SO; scale=20 µm).

160

3.4 Discussion

Previous work from our group has shown that dysregulation of SRY expression may contribute to the male susceptibility to PD (Dewing et al., 2006, Czech et al., 2012, Czech et al., 2014). Moreover, results from chapter 2 of my thesis provide strong evidence that reducing nigral SRY expression exerts male-specific neuroprotective actions in acute 6-OHDA and rotenone-induced rat models of PD. Whilst these results provide proof of concept for SRY inhibition as a therapeutic target for male PD, the rapid onset of motor deficits and neurodegeneration observed in these models do not reflect the progressive nature of clinical PD, which impedes effective screening of neuroprotective targets at clinically relevant time-points. Thus, the translational potential of *Sry* ASO-infusion in male PD was investigated in the chronic rotenone rat model of PD in male rats, which closely mimics the progressive pathological and phenotypic features of PD.

Repeated intraperitoneal administration of rotenone in male rats induces progressive degeneration of motor function and nigral DA neurons

In line with previous studies from the Greenamyre laboratory (Cannon et al., 2009, Van Laar et al., 2016), repeated intraperitoneal rotenone treatment in male rats induced a slow and gradual decline in motor function that was associated with greater than 50% nigral DA cell loss (Fig. 3.11 and 3.12). The work from the current chapter expanded upon the study by Cannon and colleagues (2009) which described a rotenone PD model that was ceased at a predefined clinical endpoint of 2 weeks. In contrast, the chronic rotenone model described in this chapter exhibited neurodegeneration and motor deficits that progressed for more than 2 months (and likely longer) following the initial rotenone administration, as described

by Van Laar et al (2016). Thus, the refined rotenone rat model of PD provides various advantages over current toxin models as it replicates multiple key features of PD over an extended progression of neurodegeneration, which allows for the temporal delineation of individual pathogenic events that may underlie DA cell death.

Nigral Sry ASO-infusion in male rats slows the progression of rotenone-induced motor deficits and nigral degeneration

Repeated rotenone treatment in male rats induced significant elevation of nigral *Sry* mRNA expression at day 10 of treatment, occurring prior to nigral DA cell loss. This is in keeping with the findings from *in vitro* (Czech et al., 2014) and *in vivo* models of PD (Chapter 2), proving further support for a role for SRY in the cellular event(s) underlying PD in males. Moreover, reducing nigral SRY expression, via intranigral *Sry* ASO infusion, significantly diminished the progression of rotenone-induced motor deficits and nigral DA cell loss. The ASO infusion regimen was initiated once the progression of rotenone-induced symptoms was evident (i.e. to closely mimic the time of clinical diagnosis in PD patients), which provided a greater level of clinical relevance for PD patients. Importantly, the neuroprotective effect of ASO infusion was observed at week 11 and 12 - i.e. more than 8 weeks following the last rotenone administration – demonstrating that the neuroprotection occurred via direct targeting of the degenerative disease process rather than an indirect effect of interaction with the toxin. Surprisingly, ASO infusion demonstrated lower efficacy in the shorter 7 or 10-day rotenone treatment regimens. ASO infusion at 4 to 5 weeks following 7 or 10-day rotenone treatment did not have any significant effect on rotenone-induced motor deficits and DA cell loss. However, there was a trend in increased motor

function following ASO infusion towards the latter stages of disease progression of the 7 or 10-day rotenone treatment, highlighting the need to extend the experimental period to fully observe the extent and trajectory of the neuroprotection following ASO infusion.

In contrast to the progressive stage of neurodegeneration, ASO-infusion had lesser efficacy on the onset of rotenone-induced deficits. For example, ASO-infusion prior to rotenone provided initial protection against increases in time stationary, whilst ASO-infusion 1 day following rotenone reduced the onset of deficits in rearing, and reduced increases in mitochondrial degradation. However, ASO had no effect on DA cell loss in either scenario. Indeed, the lower efficacy of ASO during onset of rotenone-induced deficits could be attributed to various reasons. For instance, given the transient effects of ASO infusion on nigrostriatal DA biosynthesis in normal male rats, ASO infusion may have induced compensatory effects on DA synthesis and motor function during the time when rotenone-induced motor deficits were expected. Additionally, compensatory effects are likely to be induced by neuroplasticity events that balance neuronal death during the initial phase of rotenone-induced DA cell loss. For instance, pre-clinical studies show that TH is up-regulated as a response to initial DA cell loss (Zigmond et al., 1984, Mogi et al 1988). Moreover, compensatory effects on motor function following the end of rotenone treatment have been previously described in rotenone rat models of PD that assessed motor deficits during the initial phase of injury (Morais et al., 2012, Bassani et al., 2014) These findings highlight that assessing the effects of therapeutic agents such as *Sry* ASO infusion during the onset stage of the rotenone model of PD may induce confounding compensatory factors that conceal protective effects. Rather, assessing the effects of ASO infusion during progression of chronic rotenone-

induced neurotoxic effects may be the most clinically relevant design to investigate the translational potential of nigral SRY inhibition in the injured male SNc.

Nigral Sry ASO-infusion broadly suppresses rotenone-induced increases in pathogenesis mechanisms during the progression of nigral degeneration

Post mortem studies in this chapter showed that the protective effects of ASO-infusion against progression of rotenone-induced neurodegeneration was associated with reductions in mitochondrial degradation, DNA damage, and neuroinflammation. These findings are consistent with the results from acute rat PD models in chapter 2, which showed similar profile of ASO-mediated neuroprotection against acute-6-OHDA or rotenone induced increases in pathogenic mechanisms. Thus, the broad anti-oxidant, anti-apoptotic, and anti-inflammatory effects observed following ASO-infusion might be mechanistic pathways through which ASO exerts protection against DA cell loss in males. Indeed, these results are in line with the increased oxidative stress and mitochondrial dysfunction (Misiak, 2010, Kenchappa et al., 2004), apoptosis (Rodriguez-Navarro et al., 2008), and neuroinflammation (Joniec et al., 2009, Mitra et al., 2015, Villa et al., 2016) in males compared to females in experimental models of PD and neurodegeneration.

Interestingly, ASO infusion in male rats during the onset phase of rotenone-induced deficits mitigated increases of mitochondrial degradation but had no effect on DNA damage and neuroinflammatory markers. Given that rotenone treatment leads to inhibition of mitochondrial complex I activity (Greenamyre et al., 2010) as well elevation of *Sry* expression during the initial stages of degeneration, interactions between elevated SRY and mitochondrial proteins may underlie the initial trigger of neurodegeneration in male DA neurons. Considering that SRY

164

exerts its actions as a transcription factor, elevation of SRY during pathological conditions may lead to increased binding of mitochondrial proteins involved in folding degradation stability, metabolism, protein synthesis, signalling and transport (Jin et al., 2007, Heinz et al., 2017). In support, chromatin immunoprecipitation and whole-genome promoter tiling microarray analysis on mouse gonadal cells at E11.5 (i.e. when gonadal SRY levels are elevated) revealed that SRY bound to promoters of several mitochondrial genes, including mitochondrial membrane proteins 6, 7, 13 34, and 40 (Li et al., 2014). Moreover, acute 6-OHDA and rotenone intranigral injection in male and female rats induced male-biased elevations in genes closely linked with mitochondrial function such as superoxide dismutase 2 (*Sod2*) and glutathione peroxidase 1 (*Gpx1*) (Supplementary Fig. 2), which was suppressed with ASO infusion. These findings highlight the need for further studies that assess the anatomical and functional relationship between SRY and mitochondrial genes in midbrain DA neurons. In turn, these studies may provide previously unconsidered insights into the role of SRY in mitochondrial function not only in the healthy and Parkinsonian male brain, but potentially other male-biased neurodegenerative disorders such as motor neurone disease or Huntington's disease.

Potential role of Sry inhibition as a disease modifying therapy in male PD

Whilst multiple promising disease-modifying agents that showed promising preclinical results have been studied in clinical trials, all attempts to translate these findings into neuroprotective therapies in human PD patients have failed (Meissner et al., 2011, Lang and Espay, 2018, Kalia et al., 2015). Indeed, the failure of translating pre-clinical findings could be due in part to the complexity of mechanisms underlying PD, as well as lack of a clinically-relevant model of PD that fully and accurately recapitulates the pathobiology of PD. Whilst neurodegeneration in PD is

progressive, the majority of putative agents have been tested using acute neurotoxin-based models, with many regimens beginning prior to an SNc lesion (Bezard et al., 2013). This is in direct contrast to the clinical situation, where PD patients are likely to receive therapy following diagnosis, i.e. when the extent of dopamine neuron degeneration is already approximately 50%. For example, whilst multiple *in vivo* preclinical studies suggested that DA agonist pramipexole protected against DA cell loss in multiple acute toxin-based models of PD (Gu et al., 2004, Iravani et al., 2006, Joyce et al., 2004), clinical trials subsequently showed that pramipexole lacked disease-modifying effects in PD patients (Schapira et al., 2013). Similarly, despite the neuroprotective effects of the peroxisome proliferator-activated receptor-gamma (PPAR- γ) agonist pioglitazone in 6-OHDA and MPTP rodent models of PD (Breidert et al., 2002, Quinn et al., 2008, Sadeghian et al., 2012), a recent phase 2 randomised-controlled trial failed to show any disease-modifying effects in PD patients (Luo, 2015). Moreover, whilst both coenzyme Q10 and creatine, known to maintain normal mitochondrial function, provided therapeutic effects via pre-treatment in 6-OHDA and MPTP rodent models (Matthews et al., 1999, Yang et al., 2009, Beal et al., 1998) phase 3 clinical trials were ended prematurely ended due to lack of therapeutic benefit (Beal et al., 2014, Kieburtz et al., 2015). Thus, the questionable clinical relevance of pre-treatment regimens, and the lack of validation of putative therapeutic strategies in progressive models, may underlie why these and other potentially therapeutic agents fail in the clinic.

In the current chapter, I was able to utilize the slow progressive features of the modified chronic rotenone PD model to delineate the effects of *Sry* ASO-infusion in male rats at both onset and progression phases of rotenone-induced deficits. Here, we confirmed and extended the conceptual findings from chapter 2 and

showed that the ASO-infusion regimen exerted neuroprotection against rotenone-induced deficits even at during the progression phase of injury, a time when therapeutic strategy is most likely to be given in the clinic. Notably, the protective effects of ASO-infusion during the progression phase of rotenone-induced injury also extended to protection against the progression of mitochondrial degradation, DNA damage and neuroinflammation induced by rotenone. Indeed, the broad multi-target effects of ASO-infusion are in line with recent studies that now focus on elucidating whether putative drug targets are equally efficacious against multiple cell death pathways in refined animal models of PD (Bido et al., 2017, Zhang et al., 2017, De Miranda et al., 2018). Given the multi-factorial nature of PD pathogenesis, and their relative contributions of each that inevitably lead to a cascade of events to further promote cell death, it is important to place importance on targeting a broad range of cellular mechanisms, rather than agents that primarily modulate singular pathogenic mechanisms, which may have led to past failures in clinical trials. For instance, whilst creatine and coenzyme q10 are known to be effective promoters of mitochondrial homeostasis, their lack of therapeutic benefit in the clinic (Beal et al., 2014, Kieburtz et al., 2015) could likely have been due to contributions of mitochondrial-independent cell death mechanisms such as DNA damage and apoptosis.

Results in this chapter further support the notion that SRY is detrimental in the injured male SNc, and the translational potential for nigral SRY inhibition as a male-specific neuroprotective therapy in PD. Along these lines, the neuroprotective effects of oestrogen in females are well established in preclinical models of PD (Gillies et al., 2004, Murray et al., 2003, Dluzen, 2005, McArthur et al., 2007). Whilst oestrogen replacement therapy has been shown to improve symptoms in female PD

patients (Saunders-Pullman et al., 1999a, Tsang et al., 2000a, Evatt, 2011), the neuroprotective potential for oestrogen therapy remains unaddressed in large-scale clinical trials. The p38 MAPK antagonist SB202190 has shown to inhibit SRY expression in male mice gonads (Gierl et al., 2012) and human male neuronal cell line (Czech et al., 2014). However, the potential off-target effects induced by broad pharmacological modulators of SRY, such as SB202190, are currently unknown, and thus may limit their use in the clinic. Given the relative role of SRY and oestrogen on male and female DA neurons (Loke et al., 2015, Pinares-Garcia et al., 2018, Gillies et al., 2014a), therapeutic strategies to target SRY in male and oestrogen in female PD patients may lead to optimal neuroprotective therapies for each sex.

Limitations and future studies

In line with the results from acute rat models of PD, the results the current chapter demonstrate that *Sry* mRNA expression is highly up-regulated during rotenone treatment in male rats, occurring prior to DA cell loss, However, the expression profile of *Sry* throughout the progression of rotenone-induced degeneration remains unclear. Better understanding the temporal relationship between *Sry* levels and nigral DA cell loss will assist in identifying the stage of nigral degeneration at which ASO-infusion will be most effective at slowing or stopping PD in males. Moreover, parallel studies in female rats - to measure motor deficits and nigral DA cell loss, as well as oestrogen signalling - following rotenone treatment would provide critical insight into the sex differences in disease progression, as well as the relative contributions from sex-chromosome genes and sex hormones.

Results from my onset studies in this chapter suggest that interactions between elevated SRY and the mitochondrial pathway may underlie the initial trigger of neurodegeneration in male DA neurons. However, our studies have yet to determine the exact downstream mechanisms affected by this interaction in male DA neurons. Thus, to better understand the role of SRY in mitochondrial dysfunction in the injured male SNc, future studies are required to assess both the anatomical and functional relationships of Sry in the mitochondria. Specifically, the ultrastructural co-localisation between SRY and mitochondrial proteins can be investigated via immunogold-electron microscopy, a technique capable of detecting antigens both on and within subcellular structures with greater spatial resolution. Moreover, assessing the effect of SRY knockdown or SRY over-expression on complex I activity, mitochondrial viability and/or mitochondrial membrane potential in healthy and rotenone-treated DA cells will reveal the functional relationship between SRY and mitochondria.

In addition to testing SRY inhibition in toxin-induced rat models of PD, studying the effect of SRY inhibition in genetic PD models may highlight convergent pathogenesis pathways downstream of SRY elevation, given that both forms have largely similar clinical phenotype. Indeed, the α-synuclein-overexpressing rodent models provide good predictive value for screening neuroprotective compounds, considering that α-synuclein is the key component of Lewy bodies, the pathological hallmark of PD (Spillantini et al., 1997). Recently, adeno-associated virus (AAV)-mediated α-synuclein overexpression (Decressac et al., 2012, Ip et al., 2017, Bourdenx et al., 2015) and α-synuclein pre-formed fibril (Volpicelli-Daley et al., 2016, Paumier et al., 2015) approaches in rodents have been used to induce motor deficits, striatal DA terminal loss, Lewy body pathology, and a progressive loss of

nigral DA cells Given that PD-linked genes such as *PINK1, DJ-1* and *parkin* are associated with mitochondrial function, knockout mice of these genes may prove to be useful in assessing the potential interactions between SRY and mitochondria.

The Braak model of PD pathology indicates that neuropathology originates in olfactory and dorsal motor nucleus nuclei before reaching the midbrain (Braak et al., 2003). Thus, it is tempting to speculate that dysregulation of SRY in brain regions affected during early Braak stages may precede up-regulation of SRY in the SNc. Indeed, the chronic rotenone model is an ideal tool to assess these changes, as it reproduces widespread LB pathology in the SNc (Cannon et al., 2009, Di Maio et al., 2016), enteric nervous system (ENS) and olfactory regions (Drolet et al., 2009, Voronkov et al., 2017). Importantly, rotenone also closely mimics Braak staging features in rodents, with LB pathology observed in the gut and brainstem prior to the midbrain (Pan-Montojo et al., 2010). Given that the prevalence and severity of non-motor symptoms of PD such as mood, olfaction and sleep disturbances is higher in men than women (Liu et al., 2015, Martinez-Martin et al., 2012, Solla et al., 2012), and that homeostatic control of these functions involves brainstem nuclei (Grinberg et al., 2010), assessing the regulation and function of SRY in other brain regions and peripheral tissues may reveal extranigral roles for SRY in PD.   In particular, mapping studies that determine SRY expression and co-localisation with LB's in regions affected during presymptomatic Braak stages such as the brainstem, olfactory bulb, and gut during the onset of rotenone-induced injury, may reveal the potential role of SRY up-regulation on the male-bias in non-motor symptoms of PD.

Conclusion

In conclusion, findings from studies in this chapter highlight the translational potential of reducing nigral SRY expression, as *Sry* ASO infusion in male rats exerted neuroprotective effects during the progression phase of injury in the chronic rotenone rat model of PD. Furthermore, the neuroprotective effects of *Sry* ASO infusion during the onset phase of rotenone-induced injury showed initial protection against mitochondrial degradation prior to effects on DNA damage or inflammation, suggesting a role for SRY up-regulation in the mitochondria. Given that normalization of SRY expression protects DA neurons against toxin-induced cell death even at the time of symptom diagnosis, these studies further support the notion that nigral SRY inhibition may be an effective disease-modifying therapy in the treatment of PD in males.

Chapter 4:
Assessment of human SRY expression in the post-mortem male Parkinson's disease and age-matched control substantia nigra

4.1 Introduction

Parkinson's disease (PD) is a debilitating neurodegenerative disorder characterized by motor symptoms that include tremors, rigidity, slowness of movement and postural instability (Jankovic, 2008, Lang and Lozano, 1998). Motor symptoms of PD are caused by the progressive loss of DA neurons in the SNc that project to the striatum (Lang and Lozano, 1998) which are distinguishable by dark neuromelanin (NM)-pigmented granules (Fearnley and Lees, 1991). The motor symptoms of PD first appear when nigral DA cell loss exceeds 50% (Marsden, 1990, Fearnley and Lees, 1991, Lang and Lozano, 1998) and striatal DA terminal loss exceeds 80% (Bernheimer et al., 1973, Kish et al., 1988). This indicates that the nigrostriatal pathway has a remarkable capacity to compensate for the loss of DA neurons (Lee et al., 2008, Stanic et al., 2003, Finkelstein et al., 2000). The compensatory response of the nigrostriatal pathway reflects a presymptomatic phase of PD that is absent of motor symptoms and has a mean duration of 5 to 12 years (Fearnley and Lees, 1991, Hilker et al., 2005, Hawkes, 2008). However, clinical studies have identified non-motor markers during pre-symptomatic PD such as olfactory dysfunction and gastrointestinal disturbances, which can precede PD motor symptoms by up to 10 years (Chaudhuri et al., 2006, Abbott et al., 2001). In support, Braak et al. (2003) have identified a caudal-rostral spread of neuropathology in PD, where neurodegeneration in the enteric nervous system, lower brainstem, and olfactory bulb precedes the onset of neuronal loss in the SNc.

Whilst the underlying cause(s) of DA cell loss in PD remains unclear, the male-sex is a strong risk factor for PD. For instance, epidemiological and clinical data indicates that men have a 1.3 to 3.7-fold higher incidence of PD (Wooten et al., 2004, Baldereschi et al., 2000, Van Den Eeden et al., 2003, Shulman and Bhat,

2006, Tanner and Goldman, 1996) and a 2-fold higher prevalence (Elbaz et al., 2002), which is as high as 3-fold in the 50 to 59 age group (Pringsheim et al., 2014). The onset of PD occurs earlier in men than women, suggesting that the development of symptomatic PD is delayed in women (Haaxma et al., 2007). In support, women are more likely than men to present with a tremor dominant subtype of PD which is associated with slower deterioration of clinical symptoms (Haaxma et al., 2007). Furthermore, PET imaging in disease duration-matched male and female PD patients demonstrated that male PD patients exhibited greater striatal DA denervation than their female counterparts (Kotagal et al., 2013), indicating a faster disease progression in men.

Although the mechanisms underlying the male preponderance to PD are unclear, evidence from brain imaging studies demonstrate sex differences in the healthy and Parkinsonian nigrostriatal DA pathway in humans. For instance, positron emission tomography (PET) imaging studies showed that healthy pre-menopausal women have higher DA synthesis capacity compared to healthy men (Laakso et al., 2002), whilst striatal D_2 receptor affinity was lower in women compared to men (Pohjalainen et al., 1998). Moreover, single photon emission computed tomography (SPECT) imaging revealed that healthy women have higher striatal DA transporter (DAT) binding than men (Lavalaye et al., 2000, Mozley et al., 2001), indicating greater DA uptake in females. Imaging studies in PD patients reveal that female PD patients have higher levels of striatal DAT binding than males (Haaxma et al., 2007, Kaasinen et al., 2015, Lee et al., 2015), reflective of greater DA terminal loss in male PD patients.

In addition to the nigrostriatal DA system, striking sex differences also exists in cellular pathways associated with PD pathogenesis in the healthy and

Parkinsonian human SNc. For instance, microarray analysis of laser-dissected single nigral DA neurons revealed that genes involved in signal transduction and neuronal maturation are up-regulated in the healthy and PD female SNc compared to males, whilst expression of PD-associated genes such as α-synuclein and PINK1 are higher in the healthy and PD male SNc compared to females (Cantuti-Castelvetri et al., 2007). Moreover, genes linked to apoptosis, oxidative phosphorylation, and synaptic transmission were downregulated in the male PD SNc compared to the female PD SNc, whilst the opposite was observed for mitochondrial respiration genes (Simunovic et al., 2009, Simunovic et al., 2010). Thus, better understanding the biology underlying the sex differences in PD may reveal novel cause(s) of PD that are specific to each sex.

Given the well-established neuroprotective actions of oestrogen (Quinn and Marsden, 1986, Benedetti et al., 2001), the male susceptibility to PD was traditionally explained solely by the prevailing levels of oestrogen between sexes (Quinn and Marsden, 1986, Currie et al., 2004, Saunders-Pullman, 2003). Indeed, menopause, ovariectomy and withdrawal of oestrogen-replacement therapy have been associated with increased PD symptom severity in women with PD (Quinn and Marsden, 1986, Shulman, 2007, Benedetti et al., 2001). Conversely, oestrogen replacement therapy in post-menopausal women is associated with reduced PD symptom severity (Tsang et al., 2000b, Saunders-Pullman et al., 1999b), and is also associated with reducing the risk of developing PD in women (Currie et al., 2004). However, emerging evidence indicates that nigral DA neurons in the SNc have intrinsic sex differences that may influence the pattern of gene expression (Simunovic et al., 2009, Simunovic et al., 2010), predisposing males to developing PD. Although the genetic factors driving the male bias in the nigrostriatal DA system

are unknown, sex-chromosome genes are ideal candidates. In particular, the Y-chromosome gene, *SRY*, is an interesting candidate based on its male-specific expression and function in midbrain DA neurons.

Human *SRY* encodes a 36-kDA transcription factor (Sinclair et al., 1990) that up-regulates SOX9 transcription in the bipotential gonad to initiate testis formation during embryonic development (Sekido and Lovell-Badge, 2008). In humans, *SRY* mRNA is expressed in the bipotential gonadal ridge between 41 and 44 *days post coitum* (Hanley et al., 2000), with peak *SRY* mRNA expression occurring at 44 *days post coitum*. *SRY* transcripts are also present in non-reproductive tissues such as the heart, liver, kidneys and brain in males (Milsted et al., 2004, Clepet, 1993, Dewing et al., 2006), suggesting that *SRY* exerts functions outside of male-sex determination. *In situ* hybridisation studies in human brain sections revealed that *SRY* mRNA is expressed in regions such as the pre-frontal cortex (PFC), temporal cortex, and hypothalamus (Mayer et al., 1998). Subsequent immunohistochemistry studies in human male midbrain sections revealed that SRY protein is expressed in DA-abundant regions such as the SNc (Czech et al., 2012), where SRY co-localised with 43% of TH-positive neurons, and not found in non-neuronal cells (Czech et al., 2012). Moreover, an *in vitro* study in a human male DA cell line showed that *SRY* positively regulates DA biosynthesis and metabolic gene expression, and consequently DA levels (Czech et al., 2012), demonstrating a functional role for *SRY* in male DA neurons.

Given the presence and function of SRY in male DA neurons, our laboratory assessed the regulation of *SRY* expression in a human cell culture model of PD (Czech et al., 2014). We showed that *SRY* mRNA expression was highly elevated in response to dopamine toxin, 6-OHDA, treatment in M17 cells (Czech et al., 2014).

This *SRY* up-regulation occurred prior to and during DA cell death, indicating a role for SRY in the pathogenic events underlying DA cell loss. In support, nigral *Sry* mRNA expression was aberrantly elevated in the 6-OHDA induced rat model of PD, which occurred before and during the loss of nigral TH-positive neurons (Ch.2, Fig. 2.5A). Moreover, reducing nigral SRY expression diminished motor deficits and nigral DA cell loss in both acute (Chapter 2) and chronic rotenone rat models of PD (Chapter 3), providing compelling evidence that *Sry* up-regulation in male rats is detrimental in pre-clinical models of PD.

Based on the findings that:

- SRY co-localizes with DA neurons in the human male SNc (Czech et al., 2012)

- *Sry* is up-regulated, prior to and during DA cell death, in *in vitro* and *in vivo* pre-clinical models of PD (Czech et al., 2014, Chapters 2 and 3)

- *Sry* up-regulation is detrimental in acute and chronic rat models of PD (Chapters 2 and 3)

I hypothesise that:

i)   SRY expression is up-regulated in nigral DA neurons during the onset of PD in males (Figure 4.1, Presymptomatic male SNc)

ii)   SRY up-regulation in the remaining DA neurons is detrimental, leading to a preferential loss of SRY-positive DA neurons in male PD (Figure 4.1, PD male SNc)
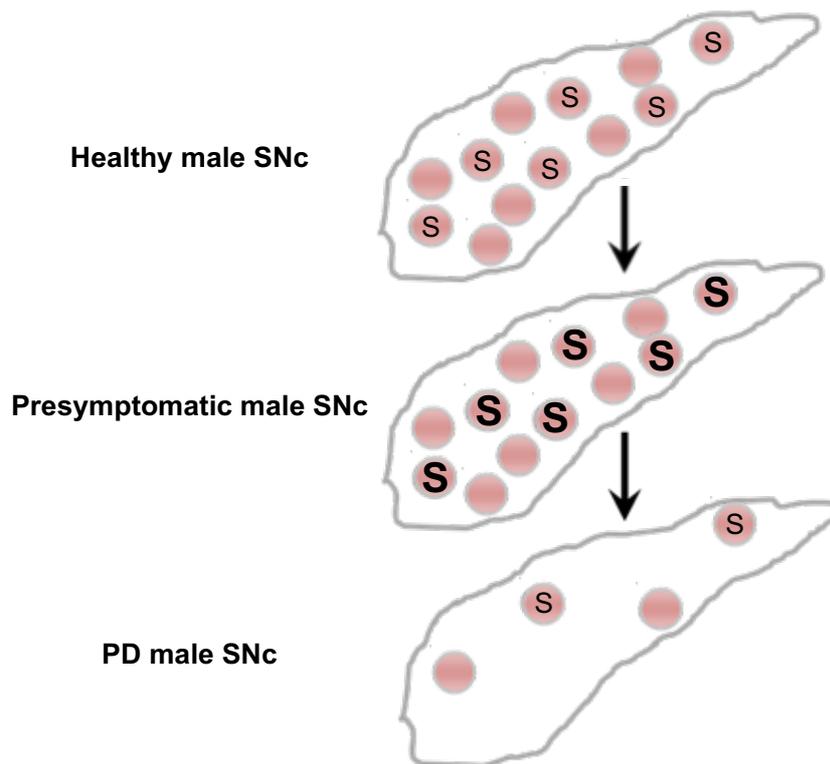
Figure 4.1. Proposed model of SRY expression during the progression of PD in males. In healthy males, SRY protein is expressed in a sub-population of nigral DA neurons in males (Healthy male SNc). We hypothesise that SRY protein expression is up-regulated in nigral DA neurons during the onset of PD (Presymptomatic male SNc). Ultimately, the up-regulation of SRY is detrimental, leading to the preferential loss of SRY-positive DA neurons in male PD (PD male SNc) (S = SRY-positive DA neuron).

To test these hypotheses, the main aims of Chapter 4 were to assess SRY protein expression in human post-mortem SNc from:

- male PD patients
- male LBD patients (as a surrogate for pre-symptomatic PD)
- male age-matched controls

4.2 Materials and Methods

Human ethics

Adult male human post-mortem SNc tissues were obtained with informed consent from the individual from the Victorian Brain Bank Network (VBBN) (table 4.1) (Southern Health Human Research Ethics Committee, project 05073C). A summary of demographics of cases is shown in table 4.1, including: sex, age at death, post-mortem interval (PMI), disease duration and cause of death.

Human post-mortem SNc tissues

Paraffin-embedded unilateral coronal sections of the SNc (10μm) obtained from male controls (n=10), and clinically diagnosed male PD patients (n=9) were used in the study (Table 4.1). Given the difficulty of obtaining presymptomatic or early PD tissues, nigral sections from male incidental Lewy body disease (LBD) patients (n=5) were used as a surrogate for presymptomatic PD (Table 4.1). Whilst LBD patients have similar distribution of Lewy bodies as PD patients, they are asymptomatic and exhibit minimal nigral degeneration suggesting that LBD cases are representative of pre-symptomatic PD (Gibb and Lees, 1988, Dickson et al., 2008, Forno, 1996). Duration of disease in PD patients was calculated from the first mention of Parkinsonian symptoms in individual clinical notes. Age-matched male SNc control tissues were evaluated to confirm that they were not affected by neurodegenerative disease. All post-mortem human SNc sections were cut at the same coronal level, taken just above the cerebellar peduncle, and fixed in paraffin. Each region was anatomically delineated by the VBBN neuropathologist (http://www.mhri.edu.au/vbbn-for-researchers). Haematoxylin and eosin staining (Fig. 4.2) identified the SNc region by neuromelanin (NM)-pigmented neurons which

were densely packed (Fig. 4.2, SNc) and the substantia nigra pars reticulata (SNr) region by neurons which were loosely packed (Fig. 4.2, SNr).
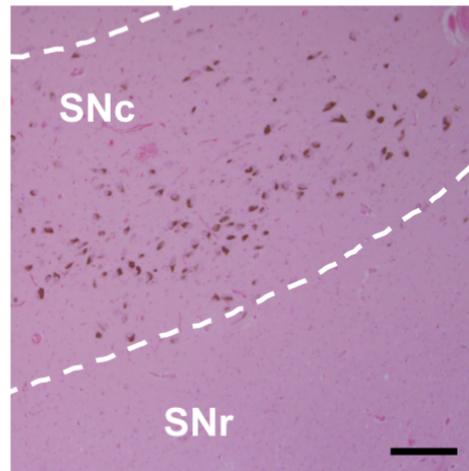
Figure 4.2. Haematoxylin and eosin (H&E) stain of a human male substantia nigra (SN) section (10µm) showing the densely packed neuromelanin-pigmented neurons of the substantia nigra pars compacta (SNc) (outlined by the white dotted line), and loosely packed neurons of the substantia nigra pars reticulata (SNr) (scale bar = 300µm).

Immunohistochemical detection of SRY protein in the human SNc

Paraffin-embedded human SNc sections (10µm) covering the whole SNc were dewaxed in 100% xylene, and rehydrated in a series of 100%, 70% and 50% ethanol washes. For antigen retrieval, tissue sections were placed in citrate buffer (0.1M, pH 6.0) for 3h in a water bath at 95°C. Tissues were then cooled at 21°C for 30 min before washing in phosphate-buffered saline (PBS) and blocked for 60 min at 21°C (CAS-Block, Invitrogen). Sections were incubated with primary antibody against human SRY (1:100, rabbit polyclonal; AVIVA Systems Biology) in CAS-block overnight at 4°C. Autofluorescence blocking kit (Chemicon) was used to reduce auto-fluorescence of endogenous lipofuscins. Secondary antibody (HRP conjugated,1:400, Sigma-Aldrich) was incubated with sections for 60 min at 21°C. Tissue was reacted with cobalt and nickel-intensified diaminobenzidine (DAB, Sigma-Aldrich). DAB-immunostained sections were counterstained with neutral red

for visualisation of nuclei. Sections were examined under bright-field microscopy, using an Olympus microscope equipped with Olympus cellSens (v.1.7.1) image analysis software, and photo-micrographed at 20X, 40X, and 100X magnification. NM pigmentation was used as an endogenous marker for nigral DA neurons in the SNc (Hansen et al., 2016, Gibb and Lees, 1991). Total number of nigral DA neurons per section was estimated by manual whole-section counts of NM-positive neurons stained with neutral red on 1 representative SNc section (Gibb and Lees, 1991, Hirsch et al., 1988). Total number of nigral SRY-positive DA neurons per section was estimated by manual whole-section counts of SRY and NM co-positive neurons stained with neutral red on 1 representative SNc section. Raw histological counts for each sample are provided in supplementary table 2.

Statistical analysis

All values are expressed as the mean ± S.E.M. All data was analysed using tools within Graphpad Prism 7 software (v. 7.0.1). The distribution of the datasets was established by the Shapiro-Wilk normality test. When data was not normally distributed, $\log_{10}$ transformation was carried out, followed by re-testing for normality and parametric analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA), and post-hoc Tukey's multiple comparisons test, to establish significance between diagnostic groups. Pearson product-moment correlation coefficients were used to compute associations of age, PMI, NM, and SRY immunoreactivity between diagnostic groups. The exact *P*-values of the ANOVAs are given in the figure legends. Probability level of 5% ($P < 0.05$) was considered as statistically significant.

Case n#	Sex	Age at death	PMI (hrs)	control / PD / LBD	Disease duration (yrs)	Cause of death
04 / 250	M	79.6	31.5	control	n/a	n/a
07 / 634	M	69.1	31	control	n/a	Myocardial ischaemia
05 / 470	M	48.7	50	control	n/a	Cardiac failure
07 / 764	M	57.6	20.5	control	n/a	Intra-abdominal haematoma
07 / 022	M	81	36.5	control	n/a	n/a
03 / 481	M	73.6	49	control	n/a	n/a
07 / 445	M	61.1	24	control	n/a	Ischaemic heart disease
06 / 972	M	75.6	46	control	n/a	n/a
07 / 635	M	72.6	42.5	control	n/a	n/a
07 / 809	M	80.1	26	PD	9	Hanging
09 / 347	M	79.5	28	PD	31	Myocardial infarction
11 / 042	M	72.1	25	PD	13	Prostate cancer
06 / 437	M	76.8	23	PD	3	Ischaemic heart disease
11 / 073	M	80.1	65	PD	22	Chest infection
08 /319	M	70	32.5	PD	14	n/a
12 / 012	M	79.5	20	PD	35	Respiratory failure
10 / 002	M	79.5	39	PD	26	n/a
10 / 142	M	73	53	PD	2+	Septicaemia
05 / 413	M	72.7	45	PD	n/a	Pulmonary thromboembolism
V11/049	M	71.8	4.5	LBD	n/a	Pneumonia
V05/050	M	82.2	25.5	LBD	n/a	Cardiac failure
V11/029	M	81	15	LBD	n/a	Pneumonia
V07/282	M	77.4	48	LBD	n/a	Pneumonia
07 / 424	M	84.6	21	LBD	n/a	Arteriosclerosis

Table 4.1. Clinical information of 9 male controls, 10 male PD and 5 male Lewy body disease (LBD) patients included in this study.

4.3 Results

Study population

Table 4.2 summarises the clinical variables and immunohistochemical measures from the 10 male controls, 5 male LBD, and 9 male PD patients included in this study. Clinical and histological data including age at death, PMI, total NM neuron counts, and proportion of SRY-positive NM neurons were assessed for normal distribution via the Shapiro-Wilk normality test in all 3 groups.

All clinical and histological data was normally distributed for all 3 groups, except for both age and total NM neuron counts in male PD patients ($P< 0.05$, skewness= -0.95, kurtosis= 0.44; $P< 0.05$, skewness= 1.68, kurtosis= 2.61 respectively). Age in the PD group was negatively skewed with a positive kurtosis, indicating that age at death in PD was significantly clustered and peaked towards the high end of the distribution range. NM counts were positively skewed with positive kurtosis, indicating that values were clustered and peaked towards the low end of distribution range. Datasets for all groups were $\log_{10}$ transformed to account for non-normally distributed data. Datasets were normally distributed following the $\log_{10}$ transformation and were therefore used in the analyses that followed.

One-way ANOVA tests revealed there were no significant differences in either age or PMI between male controls, male LBD and male PD patients (Table 4.2). The average disease duration for the PD group was 19.1 ± 4.0 years (Table 4.2), which is indicative of advanced PD according to the Hoehn and Yahr scale (>15 years, stage 4 or 5) (Hoehn and Yahr, 1967).

	Control	LBD	PD
n	9	5	10
Age at death (yrs)	68.7 ± 3.6	79.4 ± 2.2	76.3 ± 1.2
PMI (hrs)	36.8 ± 3.6	22.8 ± 7.2	35.7 ± 4.6
Disease duration (yrs)	n/a	n/a	19.1 ± 4.0

Table 4.2. Summary of clinical data in male controls, male Lewy body disease (LBD), and male PD patients.

Nigral DA neurons are significantly lost in male PD and LBD

DA neurons in the human male SNc were identified by the endogenous presence of cytoplasmic brown neuromelanin (NM)-pigmented granular bodies, as described previously (Gibb and Lees, 1991). Visualisation at low magnification (20X) revealed that DA neurons were highly abundant in the SNc of male controls, as evident by the abundant distribution of NM pigmentation throughout the SNc (Fig. 4.3A). Quantification of NM-pigmented neurons revealed 268 ± 25 NM neurons / SNc section in the male control group (Fig. 4.4).

In contrast, DA neurons were visibly depleted in the male PD group, as indicated by the sparse distribution of NM pigmentation throughout the SNc (Fig. 4.3B). Quantification revealed a significant reduction in the total number of NM neurons in male PD compared to male controls (77 ± 12 NM neurons / section, one-way ANOVA, $P < 0.0001$ vs control, Fig. 4.4), which was calculated as 29% of the total number of NM neurons in male controls. The loss of over 70% of DA neurons in PD compared to controls is in line with the expected > 50% depletion of DA neurons seen in symptomatic PD (Marsden, 1990, Fearnley and Lees, 1991).

Previous studies have shown that despite the significant deposition of Lewy bodies in the SNc (Gibb and Lees, 1988, Dickson et al., 2008, Forno, 1996), there is a minimal degree of nigral DA cell loss of LBD patients, making it an ideal surrogate for pre-symptomatic PD (Gibb and Lees, 1988, Iacono et al., 2015, Dickson et al., 2008, Forno, 1996, Dijkstra et al., 2014). However, the 5 male LBD patients analysed in the current chapter revealed significant depletion of nigral DA neurons, as indicated by sparse distribution of NM pigmentation in the LBD nigral sections (Fig. 4.3C). Counting of NM neurons revealed a significant reduction in total number of NM neurons in LBD compared to controls (97 ± 29 NM neurons / section,

one-way ANOVA, $P < 0.001$ vs control, Fig. 4.4), which was calculated to be 36% of the total number of NM neurons in male controls. The total number of NM neurons between PD and LBD patients were not statistically different ($P = 0.84$; Fig. 4.4), suggesting that the LBD patients studied had a similar rate of pathogenic progression as PD. This finding was unanticipated, given that LBD patients typically have a slower rate of neuropathological progression compared to PD (Gibb and Lees, 1988, Dickson et al., 2008, Forno, 1996, Dijkstra et al., 2014). Based on this finding, the LBD patients studied in the current chapter were not representative of presymptomatic PD.
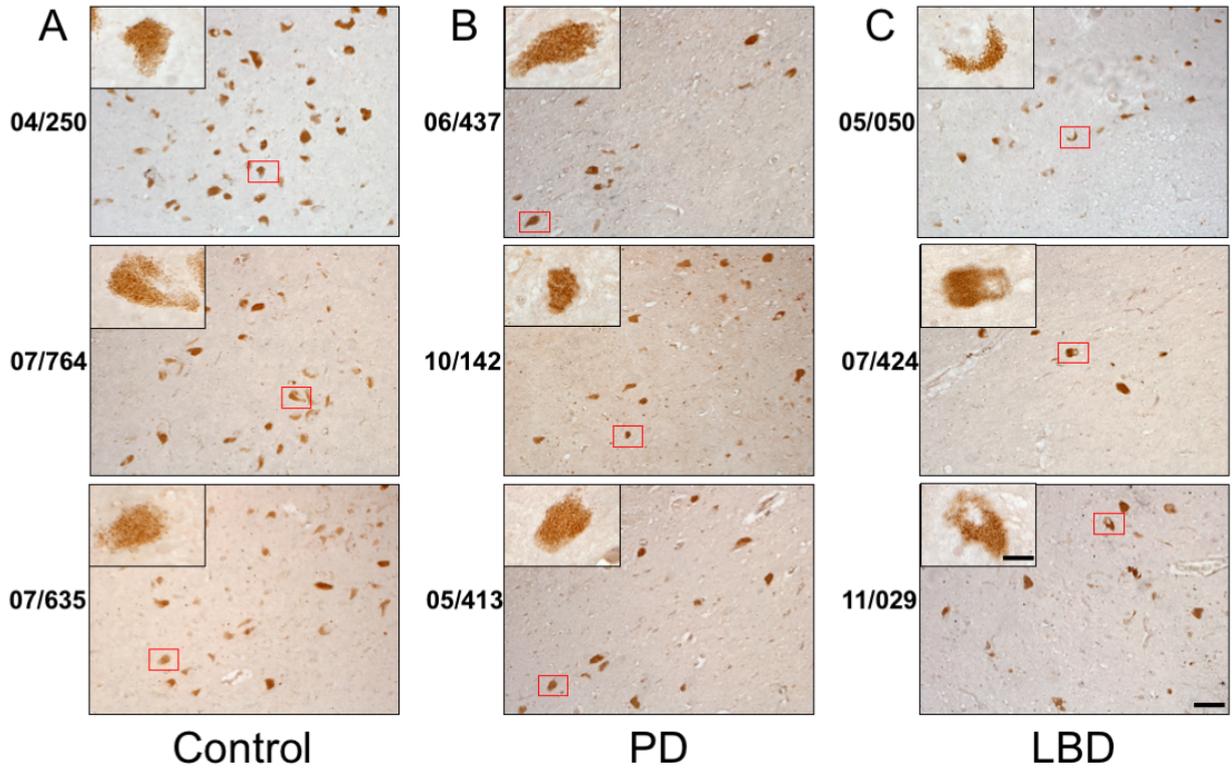
Figure 4.3. Representative photomicrographic images (outset panels, 20X; inset panels, 100X) of post-mortem SNc sections from **A)** male control (x3), **B)** male PD patients (x3), and **C)** male LBD patients (x3) counterstained with neutral red. Nigral DA neurons were identified by the presence of endogenous brown neuromelanin (NM) pigmentation (red boxes indicate magnified area; scale bar = 40µm, inset scale = 10µm; numbers left to the images represent case numbers for each patient).
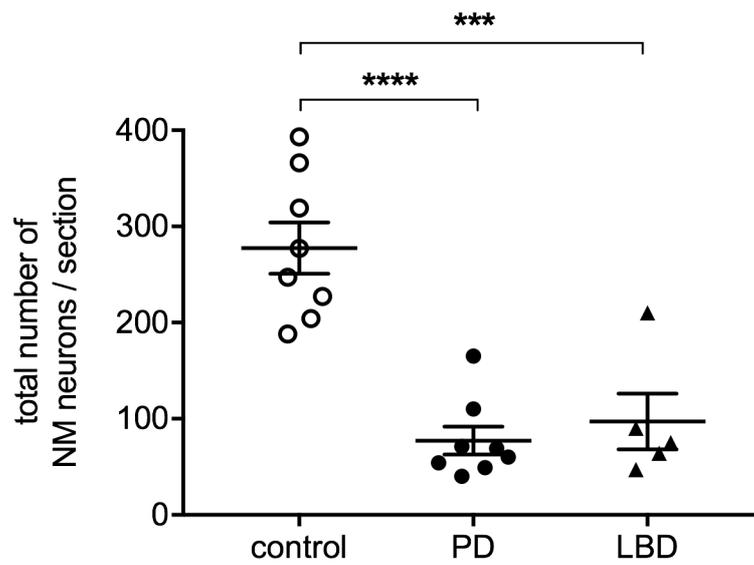
Figure 4.4. Nigral DA neurons are significantly reduced in male PD and LBD. Total number of NM neurons / SNc section from male controls, male PD, and male LBD patients (one-way ANOVA, n≥5/ group, [***] P<0.001; [****]P<0.0001 vs control).

Nigral SRY-positive DA neurons are preferentially lost in male PD and LBD

To determine whether SRY-positive DA neurons are preferentially lost in male PD, SRY immunoreactivity was assessed in male control, male PD and male LBD nigral sections (Fig. 4.5). At 40X magnification (Fig. 4.5A, outsets), SRY immunostaining was found in the majority of NM neurons in the control SNc. Visualisation at 100X magnification (Fig. 4.5A, insets) revealed that granular SRY immunostaining was localised to the cytoplasm of NM neurons, identified by the presence of brown NM pigmentation. SRY protein expression was restricted to the sub-population of NM neurons and was not found in non-NM (i.e. non-DA) cells in the SNc (Fig. 4.5). Quantification of SRY-positive NM neurons revealed 193 ± 14 SRY-positive NM neurons / section in the male control group (Fig. 4.6A). When calculated as a percentage of total number of NM neurons in the PD SNc, results showed that 68 ± 5% of NM neurons co-expressed SRY in the control group (Fig. 4.6B).

In the male PD group, the sub-population of SRY immunoreactive NM neurons was visibly depleted (Fig. 4.5B, 40X outsets). At higher magnification, SRY immunoreactivity was localised to the cytoplasm of surviving NM neurons, and not found in non- NM cells (Fig. 4.5B, 100X insets). Quantification of SRY-positive NM neurons in male PD sections revealed a significant reduction in the total number of SRY-positive NM neurons (34 ± 14 SRY-positive NM neurons/ section, one-way ANOVA, $P < 0.0001$ vs control, Fig. 4.6A). When calculated as a percentage of total number of NM neurons in the SNc, 40 ± 8% of NM neurons co-expressed SRY in the PD group, indicating that the proportion of SRY-positive NM neurons was reduced in PD compared to controls (one-way ANOVA, $P<0.05$ vs control, Fig. 4.6B), Similarly, SRY immunoreactivity was visibly depleted in the male LBD SNc (Fig. 4.5C, 40X outsets). Quantification of SRY immunoreactivity showed a

significant reduction in the total number of SRY-positive NM neurons (11 ± 2 SRY-positive NM neurons/ section, one-way ANOVA, $P < 0.0001$ vs control, Fig. 4.6A). Similar to the male PD group, proportion of SRY-positive NM neurons was reduced in LBD compared to controls (14.4 ± 3% of NM neurons, one-way ANOVA, $P < 0.001$ vs control, Fig. 4.6B). The proportion of SRY-positive NM neurons between male PD and LBD was not significantly different ($P = 0.84$; Fig. 4.6B). In summary, these results indicate that SRY-positive DA neurons are preferentially lost in male PD, and unexpectedly in male LBD, compared to male controls.
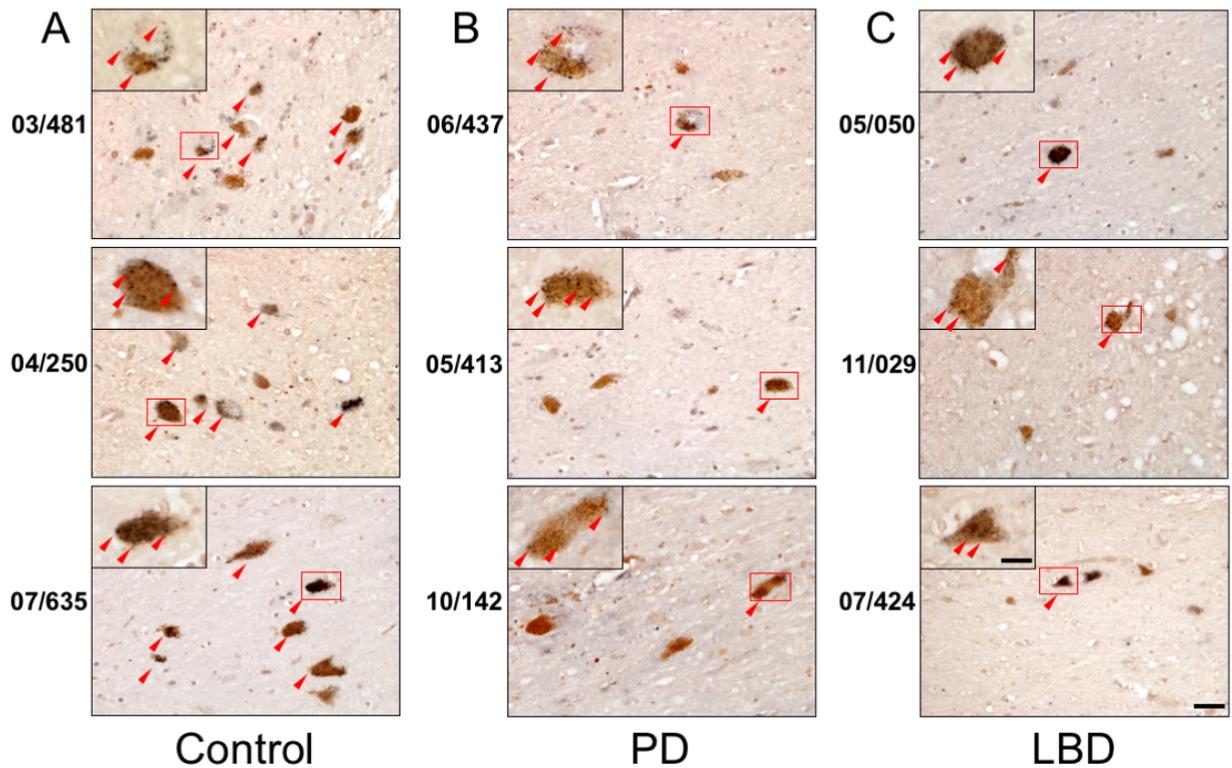
Figure 4.5. Representative photomicrographic images (outset panels, 40X; inset panels, 100X) of post-mortem SNc sections from **A)** male control (x3), **B)** male PD patients (x3), and **C)** male LBD patients (x3). Sections were immunostained for SRY and counterstained with neutral red to visualise nuclei (red boxes indicate magnified area and red arrows indicate SRY-positive NM neurons; outset scale=20μm, inset scale = 10μm; numbers left to the images represent case numbers for each patient).
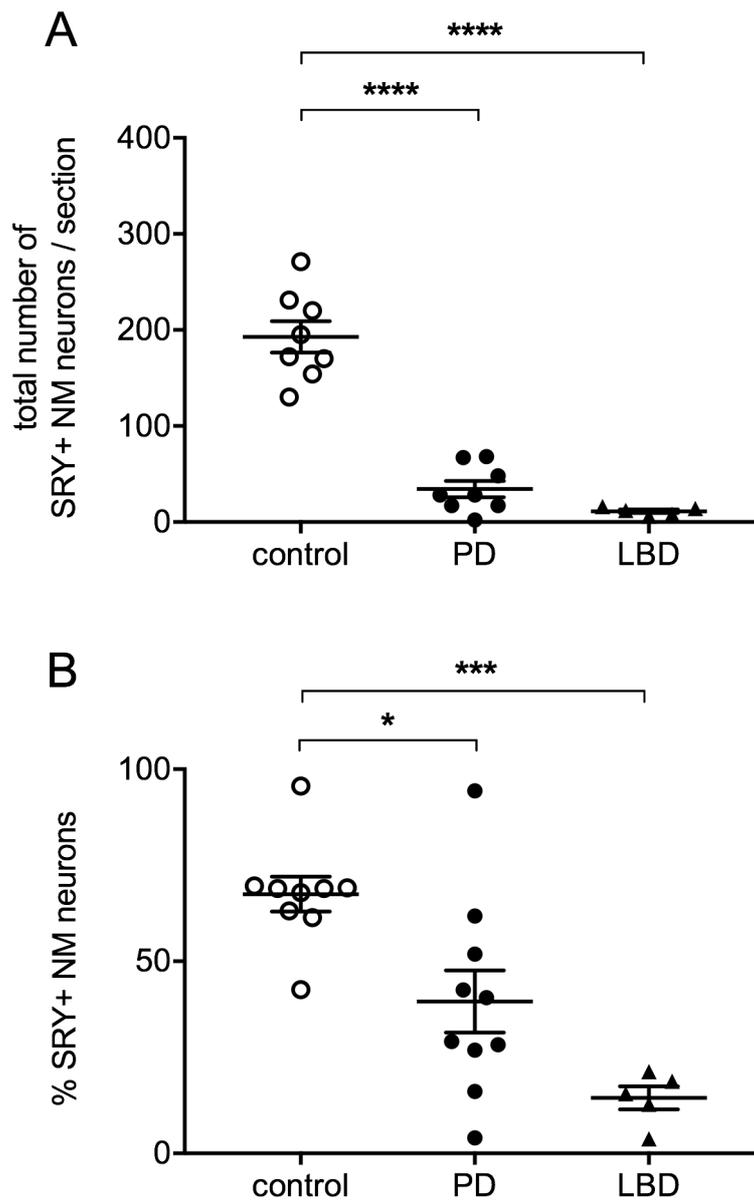
Figure 4.6. Proportion of SRY-positive nigral DA neurons are reduced in male PD and LBD. **A)** Total number of SRY-positive NM neurons / SNc section from male controls, male PD patients, and male LBD patients (one-way ANOVA, n≥5/group, [****] P<0.0001 vs control). **B)** Percentage of SRY-positive NM neurons (as a % of total NM neurons) in post-mortem SNc sections from male controls, male PD, and male LBD patients (one-way ANOVA, n≥5/group, [*] P<0.05; [***]P<0.001 vs control).

Correlations between clinical variables and immunohistochemical measures

To determine the linear relationships between clinical variables (e.g. age at death, disease duration) and immunohistochemical measures (i.e. SRY or NM-positive cells) in the male PD, male LBD and male control groups, correlation analyses were carried out between age, total NM neuron counts, post-mortem interval (PMI), proportion of SRY-positive NM neurons, and disease duration across the three groups. The significant correlation statistics, *r* and *p* values, are highlighted in bold and shown in table 4.3. Correlation analysis between NM and age in PD patients revealed a strong inverse correlation (r=-0.857, *P* < 0.001, Table 4.3), which is in line with the progressive loss of DA neurons seen in PD patients (Marsden, 1990, Fearnley and Lees, 1991). There were no significant correlations between age or PMI and the proportion of SRY-positive NM neurons in either the male control, LBD or PD patient group (Table 4.3), suggesting that neither age nor PMI had an effect on the proportion of SRY-positive NM neurons. Similarly, there was no correlation between illness duration and proportion of SRY-positive NM neurons in male PD patients. Together, these findings suggest that the proportion of SRY-positive NM neurons was related to disease state, and not to age, PMI, or disease duration in the patients studied.

	Age			NM neurons			SRY+ NM neurons			Illness duration
	Control	PD	LBD	Control	PD	LBD	Control	PD	LBD	PD
Age PD (R)	0.350									
(P)	0.356									
Age LBD (R)	-0.123	-0.133								
(P)	0.844	0.831								
NM control (R)	0.157	-0.551	0.355							
(P)	0.474	0.614	0.617							
NM PD (R)	-0.591	**-0.857**	-0.230	-0.330						
(P)	0.123	**0.006**	0.710	0.425						
NM LBD (R)	0.008	0.237	-0.437	0.703	-0.260					
(P)	0.990	0.701	0.461	0.186	0.673					
SRY+ NM Control (R)	0.239	0.482	-0.285	**0.867**	-0.553	0.611				
(P)	0.568	0.226	0.642	**0.005**	0.155	0.273				
SRY+ NM PD (R)	-0.117	-0.483	-0.573	-0.415	0.594	0.060	-0.512			
(P)	0.782	0.225	0.313	0.307	0.121	0.923	0.195			
SRY+ NM LBD (R)	0.865	**0.919**	-0.097	0.632	-0.788	0.013	0.720	-0.542		
(P)	0.058	**0.027**	0.877	0.252	0.113	0.984	0.171	0.345		
Illness duration (yrs) PD (R)	0.103	0.244	0.592	-0.406	-0.353	-0.789	-0.148	-0.456	0.494	
(P)	0.791	0.497	0.293	0.319	0.391	0.113	0.726	0.256	0.397	
PMI Control (R)	0.310	-0.595	0.372	-0.324	0.430	-0.987	-0.597	0.209	-0.009	0.010
(P)	0.417	0.090	0.537	0.432	0.287	0.002	0.118	0.620	0.988	0.980
PMI PD (R)	0.622	-0.110	0.591	-0.089	-0.387	-0.365	-0.290	-0.162	0.298	-0.016
(P)	0.073	0.760	0.294	0.832	0.343	0.545	0.486	0.702	0.626	0.966
PMI LBD (R)	-0.380	-0.174	0.592	0.242	-0.212	0.420	0.169	-0.482	-0.278	-0.174
(P)	0.527	0.780	0.293	0.695	0.732	0.481	0.786	0.411	0.651	0.779

Table 4.3. Correlation between age, total NM neuron counts, proportion of SRY-positive NM neurons, disease duration and PMI in male controls, male PD, and male LBD patients. Pearson correlation coefficient 'R' and *P* values are provided for each association. Statistically significant correlation values between measures are highlighted in bold.

4.4 Discussion

Whilst the underlying cause(s) for PD remains unclear, the male-sex is a significant risk factor for PD. Men have a higher incidence and prevalence, earlier age of onset, rapid symptom progression, and greater nigrostriatal degeneration in PD than women (Van Den Eeden et al., 2003, Elbaz et al., 2002, Haaxma et al., 2007, Kotagal et al., 2013). Work from our group and others suggest that *SRY* may underlie the male-bias in PD. For instance, we showed that *Sry* up-regulation occurs prior to and during DA cell loss in *in vitro* and *in vivo* pre-clinical models of PD (Czech et al., 2014; Chapters 2 and 3) and that up-regulation of *Sry* is detrimental in acute (Chapter 2) and progressive (Chapter 3) toxin-induced rat models of PD. To determine the clinical relevance of our previous findings, SRY protein expression was assessed in post-mortem SNc sections from male PD patients, male LBD patients and age-matched controls.

SRY-positive DA neurons are preferentially lost in male PD patients

In line with previous studies (Marsden, 1990, Fearnley and Lees, 1991), results from the current chapter demonstrated that nigral DA neurons are markedly depleted in male PD patients when compared to male controls. Results in this chapter also revealed, for the first time, a reduction in the total number of SRY-positive neurons in male PD patients when compared to male controls. Moreover, the proportion of SRY-positive DA neurons was also reduced in male PD patients compared to controls, suggesting that SRY-positive DA neurons are preferentially lost in male PD (Fig. 4.7). Whilst the reduced proportion of SRY-positive DA neurons in male PD may also be explained by non-disease factors such as age or post-mortem interval (PMI), there were no significant differences in age or PMI between the male PD and

control group (Table 4.2). Moreover, cause of death as a non-disease factor is unlikely, given that there was no significant bias in the range of causes observed in male PD patients and controls (Table 4.1). These findings indicate that neither age or PMI had an effect on the proportion of SRY-positive DA neurons in male PD patients or controls, which supports an effect of disease state.
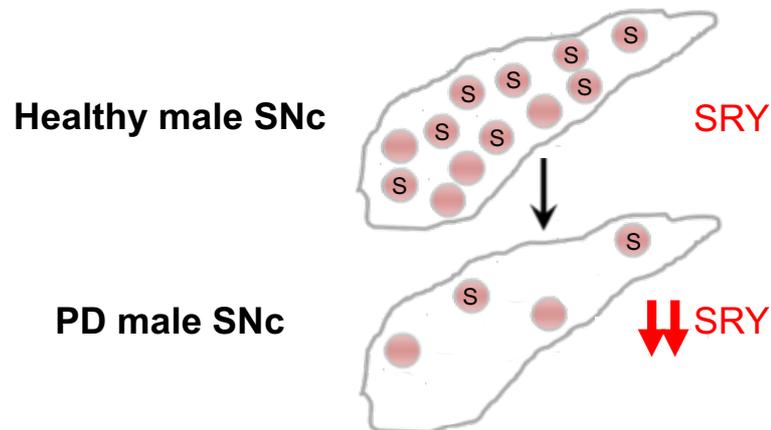
Figure 4.7. In healthy male controls, SRY is present in 68% of total number of nigral DA neurons. The proportion of SRY-positive DA neurons is reduced to 40% in the male PD SNc, suggesting that SRY-positive DA neurons are preferentially lost (S = SRY+ DA neuron)

The reduced proportion of SRY-positive DA neurons in male PD may reflect an intrinsic vulnerability of SRY-positive DA neurons to cell death. Microarray analysis of single nigral DA neurons from human post-mortem brain sections have revealed significant differences in gene expression patterns between males and females in both the healthy and Parkinsonian SNc (Cantuti-Castelvetri et al., 2007, Simunovic et al., 2010). In particular, PARK genes such as α-synuclein and PINK1 were up-regulated in healthy and PD males compared to their female counterparts (Cantuti-Castelvetri et al., 2007), whilst genes involved in apoptosis, neuronal maturation, and oxidative phosphorylation were down-regulated in males compared to females (Cantuti-Castelvetri et al., 2007, Simunovic et al., 2010). Similarly, key markers of

oxidative stress (Misiak, 2010), apoptosis (Rodriguez-Navarro et al., 2008), and neuroinflammation (Joniec et al., 2009, Mitra et al., 2015, Villa et al., 2016) exhibit greater expression in the male SNc compared to females in experimental models of PD and neurodegeneration. Moreover, results from chapter 2 and 3 demonstrate that reducing nigral SRY expression diminished or abolished the expression of markers of mitochondrial degradation, apoptosis, and neuroinflammation in acute and chronic rat models of PD, suggesting that these cellular events may be involved in the vulnerability of SRY-positive DA neurons in male PD. Hence, determining whether the SRY protein preferentially co-localises with markers of DNA-damage, neuroinflammation and/or oxidative stress in male PD and control sections may highlight the mechanism(s) underlying the vulnerability of SRY-positive DA neurons.

SRY-positive DA neurons are preferentially lost in male LBD patients

Previous results from in *vitro* (Czech et al., 2014) and *in vivo* models of PD (chapters 2 and 3) revealed that *Sry* expression is up-regulated prior to DA cell death, suggesting a role for SRY in the presymptomatic phase of male PD. To determine whether a similar up-regulation occurs in the early stages of clinical PD in males, nigral SRY expression was assessed in male LBD patients, previously considered to be a surrogate marker of presymptomatic PD (Gibb and Lees, 1988, Dickson et al., 2008, Forno, 1996). However, quantification of SRY immunoreactivity in male LBD patients revealed a marked reduction in the total number of SRY-positive DA neurons, as well as a reduced proportion of SRY-positive DA neurons compared to male controls (Fig. 4.8). Similar to male PD, there were no significant differences in age or PMI between male LBD patients and controls (Table 4.2) indicating an effect of disease state.
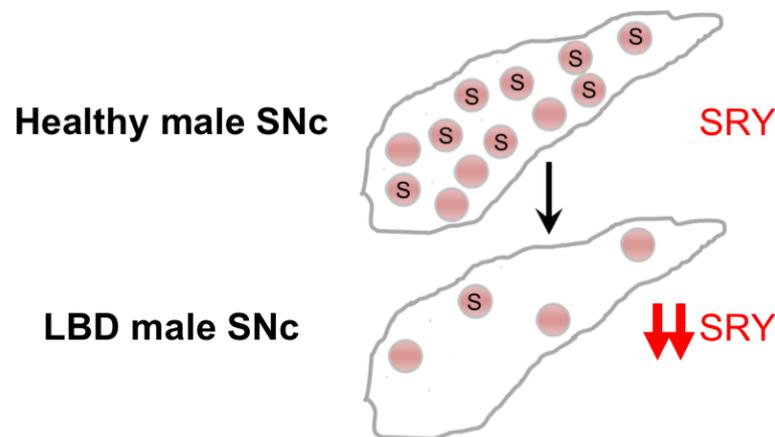
Figure 4.8. The proportion of SRY-positive DA neurons is reduced to 14% of male LBD patient, indicating preferential loss of SRY neurons in male LBD (**S** = SRY+ DA neuron)

Whilst the reduced proportion of SRY-positive DA neurons in male LBD patients was unexpected, it is in line with the extensive loss of DA neurons observed in our male LBD patient sections. In contrast to previous studies (Iacono et al., 2015, Dijkstra et al., 2014), the LBD patient sections studied in this chapter revealed extensive nigral DA cell loss with greater than 70% DA cell loss in 4 of 5 patients. However, recent changes to the neuropathological criteria for LBD suggest that LBD consists of subtypes that differ in their extent of neuropathology, symptoms and severity of disease (McKeith et al., 2017). For instance, LBD patients with neocortical-predominant LB pathology have a shorter disease duration and higher likelihood of parkinsonism compared to limbic-predominant LBD (Graff-Radford et al., 2017). Considering that degeneration in the brainstem and olfactory bulb precedes nigral DA cell loss in PD (Braak et al., 2003), brainstem and olfactory bulb predominant forms of LBD may exhibit minimal DA cell loss, and thus may be an ideal subtype of LBD for assessing pre-symptomatic changes in nigral SRY expression. Interestingly, the incidence of LBD is 2.2-fold higher in males compared to females (Savica et al., 2013), and male LBD patients exhibit greater LB pathology

(Nelson et al., 2010) and a shorter disease duration from the onset of cognitive symptoms to death compared to females (Graff-Radford et al., 2017). Hence, the loss of SRY-positive DA neurons in male LBD patients may underlie the male-bias in incidence and progression of LBD, further supporting the need to examine SRY expression in greater number of brain regions and subtypes of LBD.

Limitations and Future studies

Assessment of SRY protein expression in human post-mortem SNc sections revealed that SRY-positive DA neurons are preferentially lost in male PD, which may contribute to the susceptibility of males to PD. However, further work is needed in determining the expression profile of nigral *SRY* at various stages of PD. Identifying the stage of disease at which SRY is maximally expressed (i.e. optimal time for therapeutic intervention) will be critical in translating the pre-clinical findings to the clinic. Given the difficulty of obtaining presymptomatic or early PD brain samples, greater number of age groups and subtypes of PD and LBD brain sections (n ≥20 per group) will need to be sourced from a variety of brain bank sources outside of Victoria and Australia. In particular, brain samples acquired from patients over the 70 years may exhibit signs of early PD or presymptomatic PD, given that nigral DA cell loss occurs with normal ageing (Ma et al., 1999, Gibb and Lees, 1991).

The results from current chapter also demonstrate that SRY-positive DA neurons are preferentially lost in not only male PD, but also in male LBD patients, when compared to age-matched male controls. Given that presence of Lewy bodies is a common pathological feature of both LBD and PD, better understanding the relationship between SRY and Lewy body pathology in males may reveal novel pathogenic mechanism(s) that underlie the male-bias in PD and LBD. Further work

is needed to determine the extent of co-expression of SRY and LBs in male PD and LBD nigral sections, and to assess the potential role of SRY up-regulation in the misfolding of α-synuclein that exerts toxic effects on nigral DA neurons in PD, and to lesser extent in LBD.

Whilst the results from this chapter suggest that reduced expression of nigral SRY protein is associated with the loss of DA neurons in male PD, the downstream pathways(s) activated by SRY during DA cell loss in males remain unclear. Specifically, studies utilising laser-capture microdissection to isolate SRY-positive DA neurons from male PD and control human SNc sections for single cell RNA and/or chromatin immunoprecipitation (ChiP) sequencing will be address these issues. Results from these studies will provide valuable insight into the transcriptomic profile of SRY-positive neurons in normal and Parkinsonian male brain and have the potential to reveal the distinct mechanism(s) underlying the divergent role of SRY in the healthy and injured male SNc.

Conclusion

The results from this chapter demonstrate that SRY-positive nigral DA neurons are preferentially lost in male PD patients, and unexpectedly in male LBD patients, when compared to age-matched male controls. These results confirm and extend our previous results in male post-mortem SNc sections (Czech et al., 2012), by demonstrating the clinical relevance of *SRY* in neurodegenerative disorders such as PD and LBD. Moreover, these results support the findings from chapters 2 and 3, which suggest a detrimental role for *Sry* up-regulation in acute and chronic rat models of PD, providing support to the notion that *SRY* contributes to the vulnerability of male DA neurons to injury and thereby PD. These exciting preliminary findings pave the way for future studies to reveal novel target gene(s) of SRY and/or pathways activated by SRY that could drive the preferential loss of SRY-expressing DA neurons in male PD. Furthermore, studies to identify the temporal and regional profile of *SRY* expression in the normal and PD human male brain will undoubtedly reveal important insights into when and where *SRY* expression is dysregulated in males. This information will be critical for establishing the optimal time and site for translating *SRY* as a therapeutic target for male PD, and potentially other male-biased neurodegenerative disorders.

Chapter 5:
General Discussion and Conclusion

PD is a debilitating neurodegenerative disorder that affects more than 10 million people worldwide (de Lau and Breteler, 2006). The prevalence of PD rises from 1% for people over the age of 60 to 4% over the age of 80, demonstrating that ageing is a major risk factor (Pringsheim et al., 2014, Ascherio and Schwarzschild, 2016). The population living with PD worldwide is expected to double between 2005 and 2030 (Dorsey et al., 2007, Kowal et al., 2013, Deloitte, 2015), highlighting that the significant personal, societal and economic burden already imposed by PD will continue to escalate as the world's population ages. The motor symptoms of PD result from the progressive loss of DA neurons in the SNc, and the subsequent loss of striatal DA content (Lang and Lozano, 1998). Whilst current treatments such as levodopa and DA agonists effectively treat the symptoms of PD, the progressive nature of PD limits the success these symptomatic treatments provide (Connolly and Lang, 2014). To date, all attempts to develop therapies that can effectively change the course of the disease by slowing or halting its progression have failed (Lang and Espay, 2018, Kalia et al., 2015). One of the critical factors hindering the development of neuroprotective therapies is the relatively poor understanding of the molecular mechanisms that underlie the selective vulnerability of DA neurons in PD. Several lines of evidence support a role for mitochondrial impairment, oxidative stress, protein misfolding, neuroinflammation, and unique phenotypic traits of DA neurons in the pathogenesis of PD (Dauer and Przedborski, 2003, Schapira, 2008, Surmeier et al., 2017, Sulzer and Surmeier, 2013). Moreover, the identification of PD associated genetic mutations (Klein and Westenberger, 2012, Trinh and Farrer, 2013) and predisposing factors such as ageing (Nussbaum and Ellis 2003, de Lau and Breteler, 2006) support the notion that PD is a complex disease that likely occurs from a convergence of environmental and genetic factors.

Whilst the underlying cause(s) of DA cell loss remain unresolved, epidemiological studies have demonstrated that the male-sex is a prominent risk factor in PD. PD occurs more often in men than in women, with multiple studies reporting that the incidence rate is at least twice as high in males compared to females (Wooten et al., 2004, Baldereschi et al., 2000, Van Den Eeden et al., 2003, Shulman and Bhat, 2006, Tanner and Goldman, 1996). The male bias is exhibited in the clinical profile of PD, as men have earlier age of onset than women and are less likely to initially present with tremors, a symptom that correlates with slower rate of decline in motor impairment (Haaxma et al., 2007). The male bias also extends to the nigrostriatal DA system, as men undergo a faster rate of nigrostriatal degeneration in PD compared to women (Kotagal et al., 2013, Haaxma et al., 2007). The classical view is that sex differences in PD arise solely from the neuroprotective actions of oestrogens in females (Quinn and Marsden, 1986, Sandyk, 1989, Murray et al., 2003, Benedetti et al., 2001). However, emerging evidence has revealed that genetic factors, such as sex chromosome genes, can also contribute to the inherent sex differences in the DA pathway (Carruth et al., 2002, Dewing et al., 2003, Beyer, 1992). Together, these studies suggest there are intrinsic sex differences in DA cells which could predispose the male-sex to PD.

Although the genetic factors driving the sex dimorphism in the nigrostriatal DA pathway are unknown, sex-chromosome genes, such as the Y-chromosome gene, SRY, are ideal candidates. Classically, the role of SRY was thought be restricted to sex determination during development, due to its function in the sex determination pathway and gonadal development (Ono and Harley, 2013). However, SRY mRNA and protein is also expressed in a number of non-reproductive tissues in males, such as the adrenal glands, kidneys, lung, heart and

the brain (Milsted et al., 2004, Clepet, 1993, Dewing et al., 2006), suggesting a role for SRY outside of male sex determination. In the human and rodent male brain, SRY is expressed in DA-rich regions such as the SNc and VTA (Czech et al., 2012, Dewing et al., 2006). SRY positively regulates transcription of DA machinery genes such as TH and MAO-A (Dewing et al., 2006, Czech et al., 2012, Wu et al., 2009, Milsted et al., 2004), and consequently regulates voluntary movement in males (Dewing et al., 2006). Furthermore, SRY is aberrantly up-regulated in a human cell culture model of PD (Czech et al., 2014), suggesting a role for SRY in the pathogenic events underlying DA cell loss. In order to determine whether SRY contributes to the male susceptibility to PD, my PhD thesis investigated the regulation and function of SRY in experimental PD models and clinical PD in males.

SRY positively regulates nigrostriatal DA and motor function in healthy male rats

To better understand the physiological role of SRY in the male SNc, the effect of reducing nigral SRY expression, via intranigral *Sry* ASO-infusion, was assessed in male rats. Results in chapter 2 confirmed and extended previous studies from our group (Dewing et al., 2006) to show that reducing nigral SRY expression reduces nigrostriatal DA biosynthesis and consequently motor function in healthy male rats. The ASO-induced reduction of motor function in male rats was transient and reversible, indicating that the ASO-infusion did not induce loss of nigral DA neurons. Moreover, ASO-infusion did not affect motor function in female rats, indicating that the ASO-mediated effect was male specific. Given the intimate relationship between Sry and the catecholamine system in the brain and the periphery (Lee and Harley, 2012), better understanding of the role of Sry in other catecholaminergic brain regions such as the VTA and LC could lead to novel insights into the complex

inherent sex differences found in physiological processes such as reward, attention and memory, and stress and pathophysiological processes such as drug addiction and hypertension (Gillies et al., 2014, McCarthy et al., 2012, Loke et al., 2015, Becker, 2008, Balint et al., 2009). Indeed, human post-mortem studies demonstrate that the catecholaminergic neurons in the VTA and LC degenerate in PD, suggesting a potential role in PD (Alberico et al., 2015, Gesi et al., 2000). Given the physiological role of VTA in reward and addiction and LC in autonomic processes such as blood pressure regulation, this may reflect the impulsive traits and postural hypotension observed in PD patients (Senard et al., 1997, Evans et al., 2009).

Reducing nigral SRY expression is neuroprotective in multiple acute and chronic toxin models of PD in male rats

To determine whether *Sry* is involved in the cellular events underlying DA cell loss, the regulation of nigral SRY expression was assessed in acute toxin-induced rat models of PD in males. Results in chapter 2 showed that *Sry* mRNA was highly and persistently up-regulated in response to acute 6-OHDA or rotenone-induced DA cell injury in male rats. The up-regulation of nigral *Sry* occurred prior to and during DA cell loss, suggesting a compensatory increase in response to injury. This is in line with previous findings which showed that SRY is up-regulated in *in vitro* PD models (Czech et al., 2014). To better understand the role of *Sry* up-regulation in the injured male SNc, studies in chapter 2 assessed the effect of reducing SRY expression on toxin-induced motor deficits and DA cell loss in the acute 6-OHDA and rotenone rat models of PD. Our results showed that ASO-infusion, prior to or following acute 6-OHDA injection, attenuated 6-OHDA induced motor deficits in the limb-use and rotation tests (unilateral assessment of motor asymmetry) and nigrostriatal

degeneration in male rats. In contrast, motor function was unaffected by ASO-infusion in 6-OHDA-injected female rats, indicating that the ASO-mediated effect was male specific. ASO-infusion also protected against acute rotenone-induced motor deficits and DA cell loss in male rats, indicating that ASO-mediated neuroprotection may involve suppressing cell death mechanism(s) common to both toxins. Thus, results in chapter 2 provided conceptual data to show that toxin-induced up-regulation of nigral *Sry* is detrimental in males, and suggested a therapeutic potential for inhibition of nigral SRY in male PD. However, given that PD diagnosis in patients does not occur until DA cell loss exceeds 50%, the neuroprotective effects of *Sry* ASO-infusion needed to be validated in a progressive, clinically relevant rat model of PD.

In view of this, the main aims of Chapter 3 was to assess the effects of ASO-infusion on the onset and progression phases of the chronic rotenone rat model of PD, a model that closely reproduces the progressive nature of nigral degeneration as well as key pathophysiological features of clinical PD. Firstly, repeated intraperitoneal rotenone administration in male rats induced robust elevation of nigral *Sry* mRNA, prior to and during DA cell loss, which is in line with previous results from *in vitro* cell culture and acute *in vivo* rat models of PD (Czech et al., 2014, Chapter 2). Whilst numerous pre-clinical PD models have shown therapeutic effects of novel agents on PD-relevant cellular pathways, few address the importance of targeting therapy after inducing DA cell injury, or the most effective clinically relevant period of therapy delivery. However, ASO-infusion provided robust neuroprotection even after the progression of rotenone-induced injury i.e. diagnosis of symptoms has begun, which highlights the strength of ASO-infusion compared to other promising therapies currently in pre-clinical testing. Importantly, ASO-infusion

significantly attenuated the progression of 14-day rotenone-induced motor deficits in the rearing and open-field test (bilateral locomotor assessment) and DA cell loss at week 11 and 12 - i.e. more than 8 weeks following the last rotenone injection. Together, results from chapters 2 and 3 provide compelling evidence that i) up-regulation of *Sry* in response to DA cell injury has a detrimental effect in the male SNc and ii) correcting the aberrant elevation of nigral SRY expression exerts male-specific neuroprotective effects in acute and chronic toxin-induced rat models of PD. Whilst these chapters highlight the therapeutic potential of nigra-specific ASO-infusion in males, development of effective and safe ASO delivery methods into the human SNc will be a fundamental challenge in translating these results to the clinic. To date, the most promising clinical approach to deliver ASOs for treatment of neurological disorders is through direct administration via intrathecal injection. For instance, a recent clinical study reported that sustained ASO delivery (via intrathecal injection) to target huntingtin (HTT) mRNA in Huntington's disease (HD) patients had no adverse effects either during the 4 months treatment period or the 4-months follow up period (Tabrizi et al., 2018). However, ASO Delivery into the SNc requires enhanced delivery methods that are potent and selective in order to reach its target at sufficient concentrations, which may require development of molecular scale targeted ligand-oligonucleotide conjugates, lipid- and polymer-based nanocarriers, or antibody conjugates to transport ASO directly to the SNc (Evers et al., 2015, Juliano, 2016). These findings suggest ASO technology is a safe, promising avenue to provide disease-modifying therapy in neurodegenerative disorders such as PD. Identifying non-invasive routes of delivery is a crucial step to enhance target potency and improve delivery of ASO.

Protective effect of reducing SRY expression is mediated by a broad suppression of pathogenic mechanisms underlying DA cell loss

To identify the mechanism(s) underlying the neuroprotective effect of ASO-infusion, expression of key sensors and mediators of DA cell loss was assessed from nigral samples obtained from acute and chronic rat models of PD. Post-mortem results from chapter 2 revealed that the therapeutic effects of ASO-infusion in acute 6-OHDA and rotenone-induced rat models of PD in male rats were associated with attenuation of toxin-induced increases in markers of mitochondrial degradation, DNA damage, and neuroinflammation. Moreover, 6-OHDA induced increase in DNA damage and inflammation was higher in male rats compared to females, which was normalized to female levels by ASO-infusion in male rats. Parallel studies in our group assessed the effect of reducing SRY expression on nigral gene expression profiles of key PD pathogenesis pathways in acute models of PD (Supplementary Fig. 2). Acute 6-OHDA or rotenone injection induced robust elevation of genes involved in DNA damage (*Gadd45y, PUMA*) and oxidative stress (*Gpx1, Sod2*) and pro-inflammation (*iNos and Il1β*), which were also higher in males compared to females (Supplementary Fig. 2). These findings are in line with previous findings in experimental models of PD and neurodegeneration, which have shown increased expression of markers of oxidative stress (Misiak, 2010, Demarest and McCarthy, 2015, Guevara et al., 2011), apoptosis (Rodriguez-Navarro et al., 2008), and neuroinflammation (Joniec et al., 2009, Mitra et al., 2015, Villa et al., 2016, Bian et al., 2009) in males compared to females, suggesting these pathogenesis mechanisms are inherently male-biased. Whilst the sex differences observed in oxidative stress, mitochondrial dysfunction and inflammation have previously been attributed to differences in sex hormones (Arnold and Beyer, 2009, Villa et al., 2016),

our studies demonstrate that sex-specific genetic factors may also contribute to the regulation of these mechanisms.

In line with the findings in chapter 2, post-mortem results from chapter 3 also revealed that ASO-mediated protection in male rats was associated with broad suppression of rotenone-induced increases in mitochondrial degradation, DNA damage and neuroinflammation during the progression of the rotenone rat model of PD. In contrast to existing acute PD models, the progressive nature of chronic rotenone-induced deficits provides a realistic time course to establish a timeline of pathogenic events, and opportunities to intervene therapeutically at clinically relevant points during the progression of the disease course. Indeed, preliminary studies from our group indicate that mitochondrial dysfunction and DNA damage are up-regulated early in the disease course and precede increases in neuroinflammation, in the chronic rotenone rat model of PD (Pinares-Garcia et al., unpublished). This is in line with pre-clinical studies that have shown that mitochondrial dysfunction and oxidative stress may precede or even act as primary mediators of downstream mechanisms such as neuroinflammation and DNA damage (Fiskum et al., 2003, Hirsch et al., 2013, Witte et al., 2010). Moreover, the modest protective effect of ASO-infusion during the onset phase of the rotenone-induced deficits was associated with attenuation of mitochondrial degradation but without any effect on DNA damage or neuroinflammation. Taken together, the protective effect of ASO-infusion may be mediated by attenuating a possible deleterious interaction between elevated *Sry* and mitochondrial proteins during the initial stages of rotenone-induced nigral degeneration. However, further studies are required to determine the anatomical and functional relationship between SRY and mitochondrial proteins in the healthy and injured male SNc to confirm this tantalising

possibility. Moreover, establishing the temporal relationship between nigral *Sry* levels, nigral DA cell loss and pathogenic events during the disease progression in the chronic rotenone model of PD may identify the time point at which ASO-infusion will be most effective.

SRY-positive DA neurons are preferentially lost in male PD patients

To demonstrate that findings from pre-clinical models of PD were clinically relevant, Chapter 4 assessed SRY protein expression in post-mortem SNc sections obtained from male PD patients, male LBD patients (as a surrogate marker of presymptomatic PD) and age-matched controls. Based on earlier preclinical findings, I hypothesised that i) nigral SRY expression is up-regulated in DA neurons during initial stages of nigral injury in males, and ii) that the detrimental effect of SRY up-regulation would lead to a preferential loss of SRY-positive DA neurons in male PD. The main finding of chapter 4 was that SRY-positive DA neurons were preferentially lost in the male PD patients, which supports the hypothesis and the pre-clinical studies from chapters 2 and 3. Unexpectedly, SRY-positive DA neurons were also preferentially lost in male LBD patients. This surprising finding may indeed highlight a role for SRY as a contributing factor that underlies sex differences in LBD, a neurodegenerative disease where subtype-specific male-biases remain poorly understood. Recent diagnostic criteria for LBD has been established to classify different sub-types of LBD cases depending on the severity of symptoms, LB pathology and SPECT/ PET scanning to measure DA content (McKeith et al., 2017). Given that brainstem and olfactory bulb predominant forms of LBD exhibit minimal DA cell loss (McKeith et al., 2017), these sub-types may be ideal representative markers to assess pre-symptomatic changes in nigral SRY

expression. Whilst these results provide clinical evidence that supports a detrimental role for SRY in male PD, whether SRY is up-regulated in presymptomatic stages of clinical PD remains unresolved. Thus, further work is still needed in determining the expression profile of nigral *SRY* in a greater number of age groups and subtypes of PD, LBD and age-matched controls. Identifying the stage of disease at which *SRY* is maximally expressed (i.e. optimal time for therapeutic intervention) will be critical in translating the pre-clinical findings to the clinic.

Role of oestrogen and SRY in the healthy and injured female and male SNc
The prevailing view of sex differences in PD is that they result solely from the well-documented neuroprotective actions of oestrogen in females (Quinn and Marsden, 1986, Shulman and Bhat, 2006, Gillies et al., 2004, Murray et al., 2003, Dluzen, 2005, McArthur et al., 2007). Moreover, oestrogen positively regulates the healthy female DA pathway, where it regulates DA synthesis, release, metabolism and receptor binding (Demotes-Mainard et al., 1990, Di Paolo et al., 1985, McDermott et al., 1994, Pasqualini et al., 1995) suggesting that oestrogen has positive regulatory functions in the healthy and injured nigrostriatal DA system in females. However, work from our group and others provide compelling evidence that sex-specific genetic factors also contribute to the inherent sex differences in the healthy and diseased DA system (Carruth et al., 2002, Davies, 2014, McCarthy and Arnold, 2011, Cantuti-Castelvetri et al., 2007, Simunovic et al., 2010). In particular, results from my thesis significantly expand our understanding of *Sry* in the heathy and diseased male brain. In healthy male rats, reducing nigral SRY expression, via repeated ASO-infusion, transiently reduced nigrostriatal DA biosynthesis and consequently motor function, which is in line with previous studies from our group

(Czech et al., 2012, Dewing et al., 2006). However, 6-OHDA or rotenone treatment increases nigral SRY expression, which is accompanied by increased oxidative stress, mitochondrial dysfunction and neuroinflammation. Moreover, reducing nigral SRY expression in acute or chronic rat PD models diminished motor deficits and nigrostriatal degeneration, indicating that SRY elevation has a detrimental role in injured male DA neurons. Thus, the contrasting effect of *Sry* ASO-infusion in healthy and toxin-induced rat models of PD indicates a double-edged role for SRY in the healthy and injured male SNc. Taken together, Figure 5.1 depicts a working model for the divergent role of SRY in the healthy and injured male SNc, which can be compared to the role of oestrogen in female SNc. Based on the pre-clinical and clinical findings from my thesis, nigral SRY inhibition is a novel male-specific, disease-modifying therapy for PD (Fig. 5.1, right). Considering the well-established neuroprotective effect of oestrogen in female PD (Quinn and Marsden, 1986, Shulman and Bhat, 2006, Gillies et al., 2004, Murray et al., 2003, Dluzen, 2005, McArthur et al., 2007), our findings highlight the crucial need for understanding the sex differences in PD, and the growing need in designing sex-specific therapies.
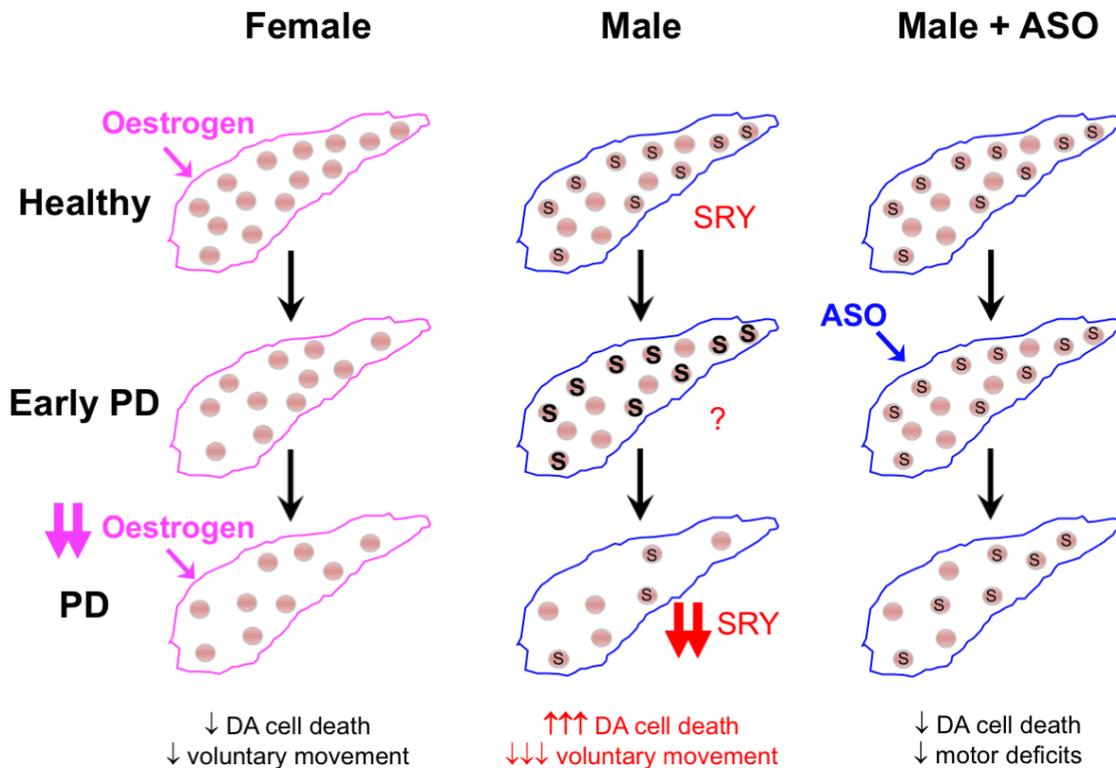
Figure 5.1. The roles of oestrogen and SRY in the healthy and injured female and male SNc. In females (**left**), oestrogen positively regulates the healthy nigrostriatal DA system, and is neuroprotective, reducing the vulnerability of female DA neurons to injury. However, the protective effects of oestrogen is compromised under conditions of reduced oestrogen (i.e. menopause), leading to increased vulnerability of female DA neurons to injury. In males (**middle**), SRY, which is expressed in ~2/3rd of nigral DA neurons, directly regulates nigrostriatal DA system. We postulate that SRY expression is up-regulated in DA neurons during pre-symptomatic or early PD, as a compensatory response to boost DA production. Ultimately, the up-regulation of SRY is detrimental, leading to the preferential loss of SRY-positive DA neurons in male PD. Work from my thesis has shown that nigral *Sry* ASO-infusion is neuroprotective in acute and progressive models of PD **(right)**. Thus. inhibition of SRY expression may be a novel therapeutic strategy to slow the progression of PD in males (S = SRY-positive DA neuron).

Future studies

The results from my PhD thesis have provided valuable insights into the roles of nigral SRY in the healthy and Parkinsonian male SNc. However, these novel findings have generated many critical questions concerning the role of SRY in the male SNc that still remain unaddressed.

Studies in chapters 2 and 3 and parallel studies from our group that linked the dysregulation of nigral SRY with PD-relevant pathways and mitochondrial and inflammation-associated genes were performed using a biased approach, where we focussed on a selection of known male-biased pathogenesis pathways. Thus, it is unclear whether the up-regulation of SRY may also act via additional, as yet unexplored cellular pathway(s). Identifying target genes and DNA binding partners of nigral SRY in the healthy and injured male SNc via unbiased genome-wide approaches such as RNA sequencing and ChIP sequencing is essential to further our understanding of the roles of SRY in the healthy and PD male brain. These approaches have the potential to reveal the distinct mechanism(s) underlying the aberrant up-regulation of SRY expression in male PD, and potentially identify pathways that can be modulated as neuroprotective strategy for PD.

Despite the compelling evidence demonstrating a detrimental role for SRY dysregulation in males in my thesis, and the neuroprotective effects of oestrogen in females, the extent of contributions that sex hormone and genetic factors have on the healthy and diseased DA system in males and females remain unclear. Studies that manipulate hormone levels via gonadectomy or oestrogen treatment in *Sry* ASO-infused male rats, or alternatively in SRY over-expressing female rats, could elucidate the nature of interactions between SRY and oestrogen that contribute to the inherent male bias in PD.

Although work in this thesis provided evidence for the neuroprotective potential of SRY inhibition in pre-clinical PD, translation of this research to clinical trials require validation in humanized models of PD. The development of human induced pluripotent stem cells (hiPSCs) that are directly reprogrammed from patient-derived cells have become a powerful tool to model not only PD, but other neurological disorders (Park et al., 2008, Soldner et al., 2009). HiPSCs derived from PD patients and controls and reprogrammed into DA neurons has provided avenues to explore dysregulated cellular and molecular mechanisms, as well as an innovative platform for screening of novel therapies (LaMarca et al., 2018, Marchetto et al., 2010). Thus, hiSPCs derived from male idiopathic or genetic PD patients may be a clinically-relevant screening tool to address the translational potential of novel human SRY inhibiting molecules (e.g. antisense oligonucleotides, siRNA).

Considering the broad expression of SRY in the male brain (Mayer et al., 1998, Lahr et al., 1995, Dewing et al., 2006), dysregulation of SRY expression may underlie other male-biased neurological disorders. Autism spectrum disorder and attention-deficit hyperactive disorders are both neurodevelopmental disorders characterized by childhood difficulties in learning or impulsivity and/or hyperactivity (Geschwind, 2008). Whilst the underlying cause(s) for either autism or ADHD is unknown, both disorders are significantly more prevalent in males. Indeed, autism is 4 to 11 times more common amongst men than women (Halladay et al., 2015), whilst ADHD occurs 3 times more commonly in boys than girls (Gaub and Carlson, 1997). Whilst the male bias in autism and ADHD has been attributed to abnormal levels of foetal testosterone in males (Baron-Cohen, 2002), sex-chromosome genes are also associated with the increased risk of males to neuropsychiatric disorders (Laumonnier et al., 2004, Schaafsma and Pfaff, 2014, Cohen et al., 2003, Cohen et

al., 2011). Work from our group (Loke et al., in prep) and others (Mayer et al., 1998, Lahr et al., 1995) have shown that SRY is expressed in brain regions closely associated with pathophysiology of neuropsychiatric disorders including the pre-frontal cortex, striatum, hippocampus, and amygdala. Thus, it is plausible that dysregulation of SRY expression may contribute to the abnormal functioning of these brain regions in males and consequently susceptibility of males to these disorders. The (patho)physiological role of Sry in specific brain regions or cell types in the male brain could be investigated via generation of spatiotemporally-restricted SRY conditional knockout and/ or SRY over-expressing mice. Furthermore, the precise role of SRY in the male PD brain could be elucidated via generation of SRY conditional knockout / A53T α-synuclein over-expressing double transgenic mice, as a non-invasive system to spatiotemporally define the therapeutic potential of sustained SRY knockdown in a progressive model of PD.

Motor Neuron Disease (MND) is a neurodegenerative disorder characterized by the rapid atrophy of motor neurons in the brain, where degeneration is localised to regions including the frontal cortex, thalamus, brainstem and spinal cord regions (Leigh and Ray-Chaudhuri, 1994, Brownell et al., 1970). Epidemiological studies indicate that men have a 1.4 times greater prevalence, a 1.6 times higher risk of developing MND, and an earlier onset of MND symptom development compared to women (McCombe and Henderson, 2010, Abhinav et al., 2007, Manjaly et al., 2010, Haverkamp et al., 1995). Whilst the pathogenesis mechanisms that underlie MND remain unclear, increasing evidence has revealed interactions between motor neurons and activated microglia and astrocytes, which is indicative of a neuroinflammatory component to MND (Evans et al., 2013, Shaw, 2006, Komine and Yamanaka, 2015). Given that preliminary studies have revealed the presence

of SRY protein expression in the microglia in the human male frontal cortex and thalamus (Loke et al., in prep), this suggests a potential role for SRY in neuroinflammation that could underlie the male bias in MND.

Conclusion

The work described in my PhD thesis confirm and expand upon our understanding of the role of SRY in the healthy male brain, and for the first time, a role for SRY in the diseased or Parkinsonian male brain. These findings challenge the current dogma that sex hormones are the sole cause for the male-sex bias in PD, and indicate that sex chromosome genes, particularly the Y-chromosome gene, *SRY*, also contribute to the male susceptibility to PD. Asides from a role in sex development, work from my thesis and others demonstrate that SRY play a key role in the regulation of the nigrostriatal DA system in male, potentially as an alternate mechanism to oestrogen in males. Work from my thesis have shown that *Sry* is up-regulated in response to DA cell injury. The protective effect of nigral SRY inhibition in experimental PD models suggests that SRY dysregulation in response to injury is detrimental, and contributes to the male susceptibility to PD. Future research aimed at elucidating the role of SRY dysregulation in the PD will be pivotal in developing SRY inhibition as a novel disease-modifying target for PD in males.

Appendix 1: Supplementary tables

Species	Gene identifier	Gene Name	Forward sequence (5' – 3')	Reverse sequence (5' – 3')
Rat	*Sry*	Sex determining region Y	ttccaggaggcgcagagactga	tgttgaggcaacttcacgctgca
Rat	*Tbp1*	TATA-box-binding protein 1	gggagctgtgatgtgaagt	gtggtcttcctgaatcccttta

Supplementary table 1. Primer sequences used for qRT-PCR studies in chapter 3

Case n#	control / PD / LBD	total number NM neurons	total number SRY+ NM neurons	% SRY+ NM neurons
04 / 250	control	319	220	69.0
07 / 634	control	393	271	68.9
05 / 470	control	188	130	69.2
07 / 764	control	366	231	63.1
07 / 022	control	247	172	69.6
03 / 481	control	277	170	61.4
07 / 445	control	204	195	95.6
06 / 972	control	190	81	42.6
07 / 635	control	227	154	67.8
07 / 809	PD	71	67	94.4
09 / 347	PD	49	2	4.1
11 / 042	PD	165	48	29.1
06 / 437	PD	69	28	40.6
11 / 073	PD	40	17	42.5
08 /319	PD	110	68	61.8
12 / 012	PD	54	28	51.9
10 / 002	PD	60	17	28.3
10 / 142	PD	67	18	26.9
05 / 413	PD	87	14	16.1
V11/049	LBD	90	14	15.6
V05/050	LBD	75	16	21.3
V11/029	LBD	47	6	12.77
V07/282	LBD	210	8	3.81
07 / 424	LBD	64	12	18.75

Supplementary table 2. Raw Immunohistochemical data of total number of NM neurons / section, total number of SRY-positive NM neurons / section, and % of SRY-positive NM neurons (as a % of total NM neurons) from 9 male controls, 10 male PD and 5 male LBD patients included in this study.

Appendix 2: Supplementary figure 1
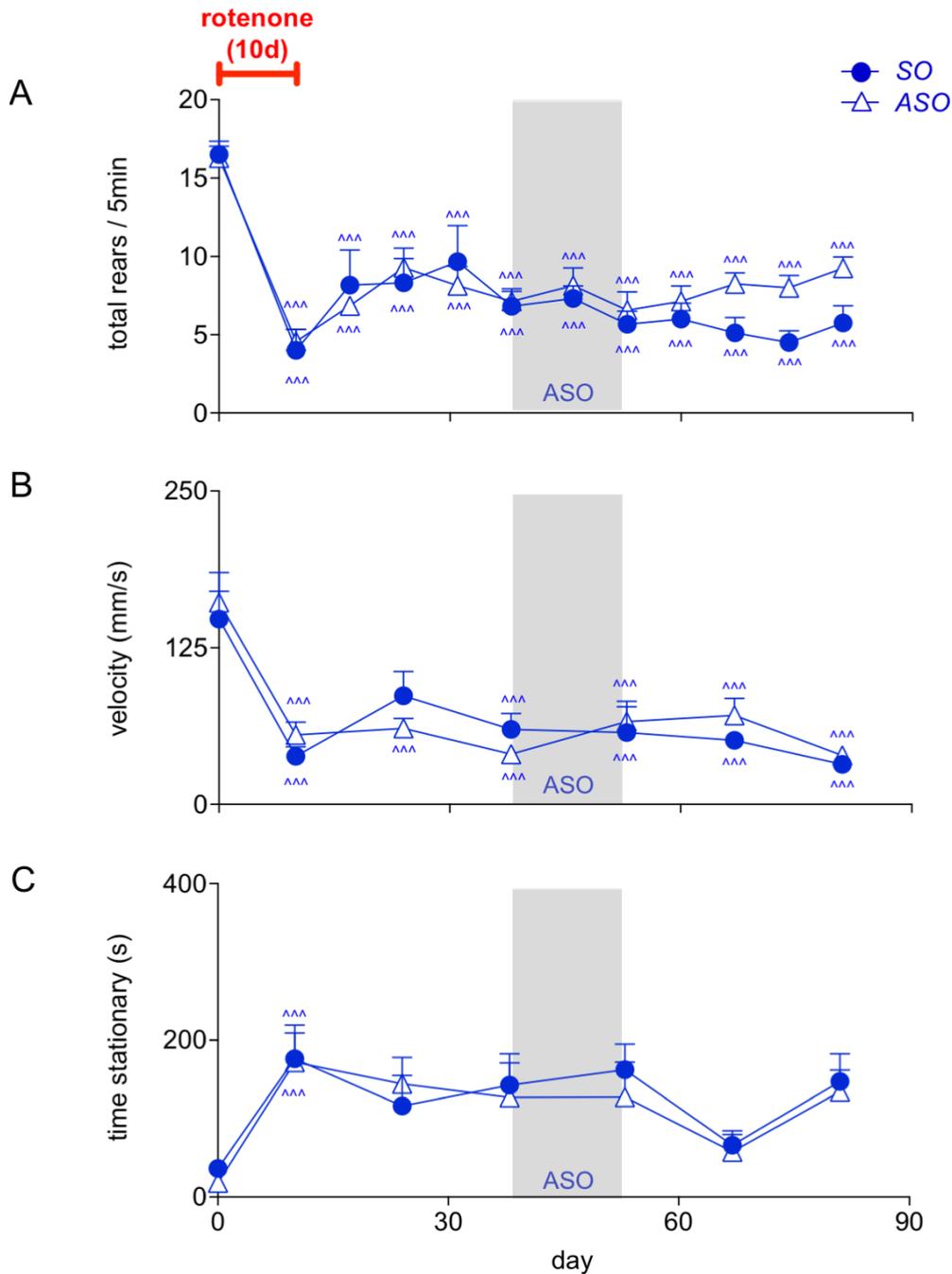
Supplementary figure 1. Nigral Sry ASO-infusion has no effect on the progression of motor deficits or nigral degeneration following 10 days rotenone treatment in male rats. ASO (or SO) was infused bilaterally into the SNc for 14 days, 4 weeks following repeated daily rotenone administration for 10 days in male rats. Motor function was assessed by the **A)** rearing test or the **(B-C)** open-field test to measure **B)** velocity and **C)** time stationary. (n=10/group; two-way ANOVA, ^^^ $P < 0.001$ vs day 0).
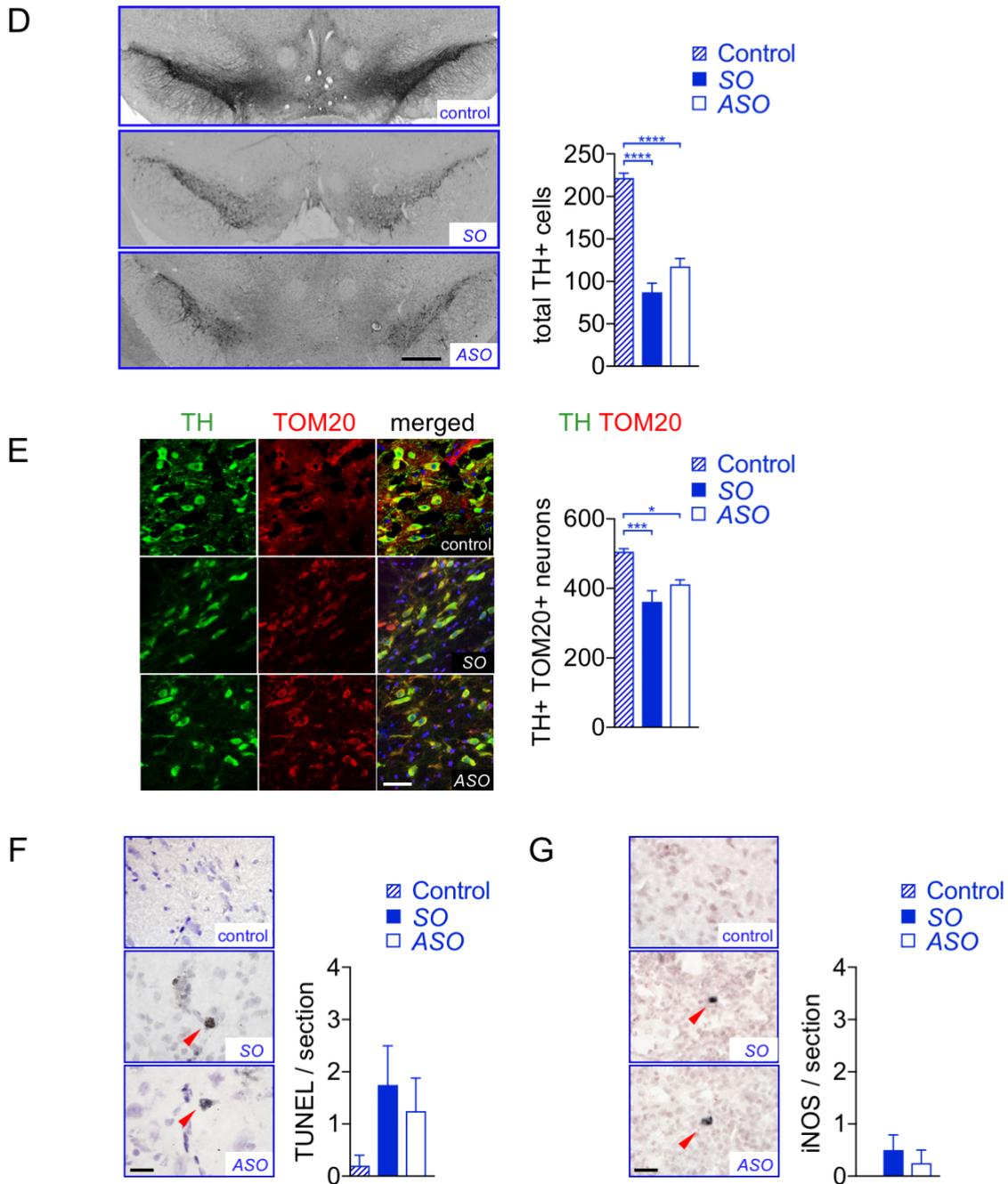
Supplementary figure 1 (continued). Nigral degeneration was assessed by **D)** nigral TH-positive cell counts (n=9/group; one-way ANOVA, **** $P < 0.0001$; scale=400μm). Effect of ASO (or SO) infusion on **E)** nigral TH- and TOM-20-positive (merged) neurons, **F)** TUNEL-positive neurons, and **G)** iNOS-positive neurons (n=5/group; one-way ANOVA, *** $P < 0.001$, * $P < 0.05$ vs control; scale=20μm).

Appendix 3: Supplementary figure 2
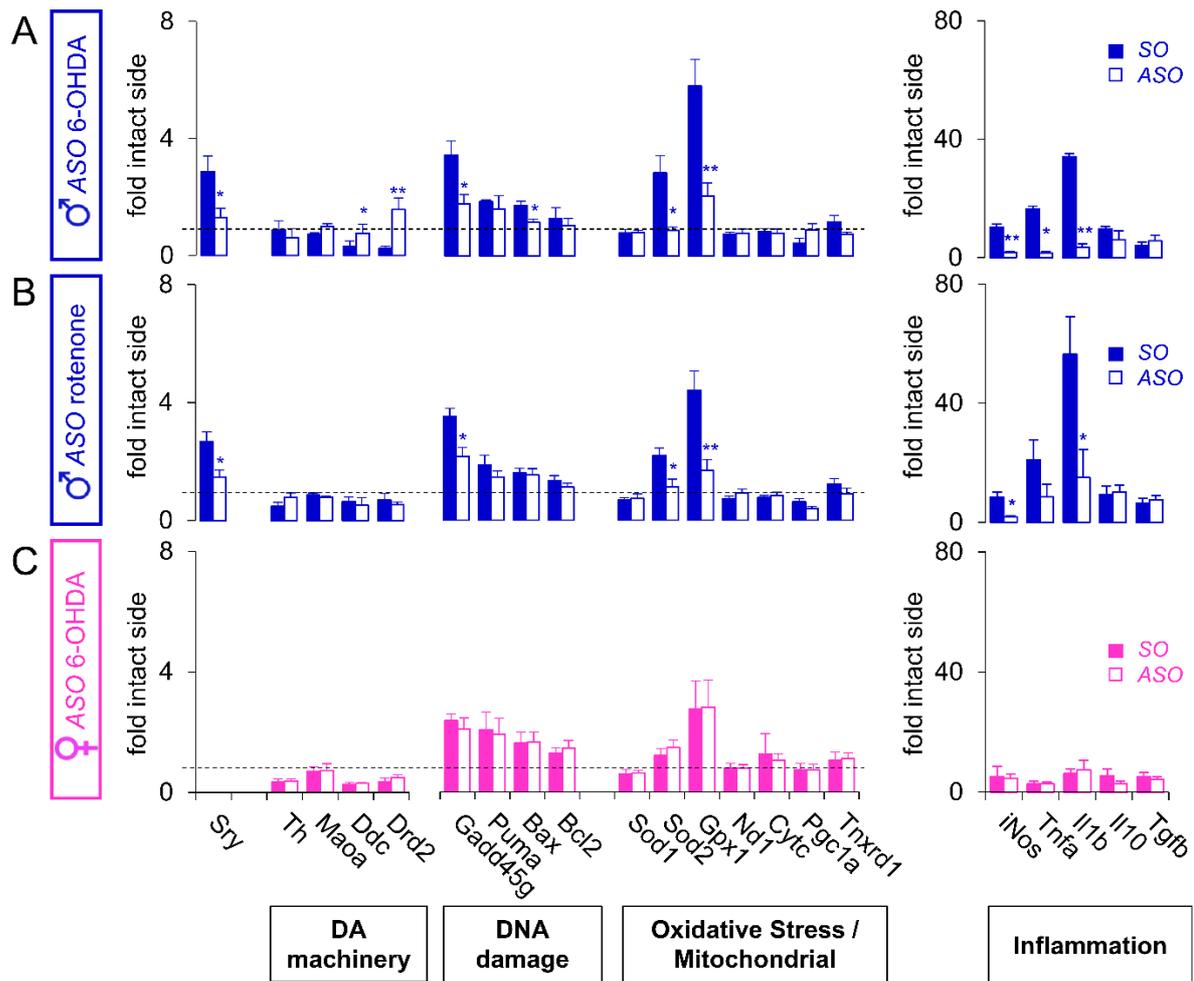
Supplementary figure 2. **Reducing nigral SRY expression normalizes toxin-induced dysregulation of PD pathogenesis genes in male rats.** Effect of ASO (or SO) infusion on expression of nigral genes (fold intact side) involved in DA machinery, oxidative stress and mitochondrial function, and inflammation at two days post **A)** 6-OHDA or **B)** rotenone injection in male rats or **C)** 6-OHDA injection in female rats (n≥5/group; unpaired t-test, * P < 0.05, ** P < 0.01 vs. SO; dashed line = baseline levels).

Appendix 4: Publications during enrolment

Sex: A Significant Risk Factor for Neurodevelopmental and Neurodegenerative Disorders

Paulo Pinares-Garcia [1,2], **Marielle Stratikopoulos** [1,2], **Alice Zagato** [1,3], **Hannah Loke** [1] **and Joohyung Lee** [1,2,*] [iD]

[1] Brain and Gender laboratory, Centre for Endocrinology and Metabolism, Hudson Institute of Medical Research, Clayton, Victoria 3168, Australia; paulo.pinares-garcia@hudson.org.au (P.P.-G.); mstr0005@student.monash.edu (M.S.); alice.zagato@hudson.org.au (A.Z.); hannah.loke@hudson.org.au (H.L.)
[2] Department of Anatomy and Developmental Biology, Monash University, Clayton, Victoria 3168, Australia
[3] School of Life and Environmental Sciences, Deakin University, Burwood, Victoria 3125, Australia
[*] Correspondence: joohyung.lee@hudson.org.au; Tel.: +613-8572-2507

Abstract: Males and females sometimes significantly differ in their propensity to develop neurological disorders. Females suffer more from mood disorders such as depression and anxiety, whereas males are more susceptible to deficits in the dopamine system including Parkinson's disease (PD), attention-deficit hyperactivity disorder (ADHD) and autism. Despite this, biological sex is rarely considered when making treatment decisions in neurological disorders. A better understanding of the molecular mechanism(s) underlying sex differences in the healthy and diseased brain will help to devise diagnostic and therapeutic strategies optimal for each sex. Thus, the aim of this review is to discuss the available evidence on sex differences in neuropsychiatric and neurodegenerative disorders regarding prevalence, progression, symptoms and response to therapy. We also discuss the sex-related factors such as gonadal sex hormones and sex chromosome genes and how these might help to explain some of the clinically observed sex differences in these disorders. In particular, we highlight the emerging role of the Y-chromosome gene, *SRY*, in the male brain and its potential role as a male-specific risk factor for disorders such as PD, autism, and ADHD in many individuals.

Keywords: brain sex differences; estrogen; testosterone; *SRY*; gender-specific medicine; ADHD; Parkinson's disease; Alzheimer's disease; autism; schizophrenia; depression

1. Introduction

Sex is sometimes a significant variable in the prevalence and incidence of neurological disorders [1–7]. In addition, sexual differences also exist in the age-of-onset, progression, disease severity, underlying neuropathology and treatment response of neurological diseases [1,3–5,7–9]. Whilst this attention to sexual dimorphism has traditionally been stronger in fields like cancer, cardiovascular and endocrine disorders [10,11], accumulating evidence demonstrates significant sex differences in brain physiology and behavior throughout development and adulthood [1,4,12]. Indeed, girls are more likely to suffer from depression than boys, and following puberty this female susceptibility for anxiety increases to around twice that for males [13]. Males with ADHD tend to exhibit the hyperactive-impulsive subtype whereas females tend to exhibit the inattentive subtype [14]. Sex differences are also seen in neurodegenerative disorders as the male sex is a significant risk factor for Parkinson's disease [15–17] and motor neuron disease [18,19], whilst females are more susceptible to Alzheimer's disease [20,21] and multiple sclerosis [22,23] (Summarised in Figure 1).

Despite the well-established understanding that males and females differ in their predisposition to neurological diseases, gender is rarely considered when making diagnostic or treatment decisions. Hence, a better understanding of the molecular underpinnings behind these sex differences could help develop more targeted therapies with higher success rates, especially in diseases where sex differences are most prominent.
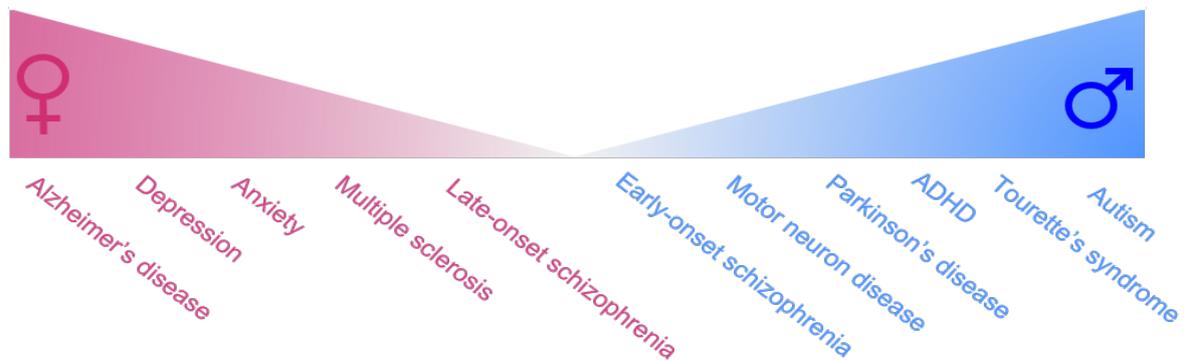
Figure 1. Sex differences in the prevalence of neurodegenerative and neuropsychiatric disorders. Abbreviations: ADHD, Attention-deficit hyperactivity disorder.

Significant increase in research efforts is now starting to unravel the biological mechanisms responsible for promoting sex-specific characteristics in the healthy and diseased brain [7,24–27]. It is now well established that organizational and activational effects of sex steroid hormones play an important role in brain sex differentiation [7,28–31]. Indeed, neuroprotective actions of estrogen in females underlie sex differences in susceptibility to disorders such as PD and schizophrenia [7,32–34], whilst aberrant levels of fetal testosterone have been associated with male preponderance to neurodevelopmental disorders such as autism and ADHD [35,36]. In addition to the influence of gonadal hormones, accumulating evidence demonstrate that sex chromosome genes (i.e., X and Y-linked) can directly influence brain function in normal and pathological conditions [26,37,38]. The Y-chromosome gene, *SRY*, which directly exerts male-specific actions in adult dopamine neurons, may also underlie male preponderance to disorders such as PD and autism. Thus, it is likely that these sex differences in the healthy and diseased brain result from complex interactions between sex hormones, sex chromosomes and epigenetic factors.

In view of these findings, increasing our understanding regarding the molecular basis of sex differences in neurological disorders will be pivotal in identifying sex-specific risk or protective factors and in being able to develop more effective therapies for each sex. The current review will summarise the available evidence on sex differences in neuropsychiatric and neurodegenerative disorders regarding prevalence, progression, symptoms and response to therapy. We will then discuss the potential role of sex hormones and sex chromosome genes in contributing to these sex differences.

2. Female-Biased Brain Disorders

2.1. Female-Biased Neuropsychiatric Disorders

2.1.1. Anxiety Disorders

Anxiety disorders are a group of psychiatric disorders characterized by exaggerated feelings of anxiety and fear responses. These feelings can manifest as physical symptoms, such as increased heart rate, difficulty concentrating or mind going blank, irritability, muscle tension, lethargy, and sleep disturbance [39,40]. Most common types of anxiety disorders include generalized anxiety disorder, social anxiety disorder, post-traumatic stress disorder, obsessive compulsive disorder and panic disorder. People often have more than one type of anxiety disorder. Like depression,

serotonin appears to be involved in the pathogenesis of anxiety disorders and thus therapeutics directed at enhancing synaptic levels of serotonin, such as serotonin-reuptake inhibitors (SSRIs) and serotonin/norepinephrine-reuptake inhibitors (SNRIs), ease symptoms associated with anxiety [41].

Almost all subtypes of anxiety are more likely to be diagnosed in women than in men [42,43]. Lifetime prevalence rates of the major anxiety disorders range between approximately 3 to 12% and are approximately two times greater among women than men [42]. Women seem to be more negatively affected by symptoms of anxiety disorders, often experiencing symptoms to a greater degree [42]. Data on the prevalence of affective disorders such as anxiety and depression mainly come from self-reports and it has been hypothesised that part of the high female prevalence may be due to the unwillingness for males to report their anxiety symptoms [43,44]. Another reason for the higher female prevalence of anxiety disorders has been thought to come from the difficulty men face in expressing their feelings due to masculine sex-role stereotypes [45]. Despite these sociological factors, overwhelming evidence demonstrate that actions of gonadal hormones on brain functions, such as hippocampal neurogenesis [46] and fear conditioning [47], play a significant role in the vulnerability of females to anxiety disorders.

2.1.2. Depression

Depression is one of the most common and debilitating psychiatric disorders, with more than 300 million people affected worldwide [48]. Depression is a leading cause of suicide [49] characterized by symptoms of depressed mood, lack of drive, anhedonia, changes in appetite, sleep disturbances, feelings of guilt and concentration problems [50–52]. These symptoms are thought to largely arise from underactivation of the brain serotonin and norepinephrine transmitter systems [53], although deficiencies in neurotrophic and angiogenic factors [54] and glutamate metabolism [55] are also thought to contribute. Hence, anti-depressant medications such as SSRIs, SNRIs, and monoamine oxidase inhibitors act via enhancing serotonin and/or norepinephrine levels in the brain [56].

Depression is much more common among women than men, with female/male risk ratios roughly 2:1 [57]. This female-bias begins in adolescence and continues to midlife, approximating the span of the childbearing years in women [58]. Like anxiety disorders, sex differences in reporting of symptoms and differential persistence (e.g., sex-roles that create higher stress levels for women leading to higher rates of depression) may contribute to the higher female prevalence of depression [59]. However, increased incidence of depression in women during perimenopause and menopause, as well as following child-birth (i.e., post-partum depression) [43,44,60] suggests that fluctuating levels of sex hormones plays a significant role in female susceptibility to depression.

2.1.3. Late-Onset Schizophrenia

Schizophrenia is a complex, chronic neuropsychiatric disorder that has a population frequency of approximately 1% [61–63]. Schizophrenia is characterized by distortions in thinking, perception, emotions, language, sense of self and behavior, manifested by a mixture of debilitating positive (hallucinations and delusions) and negative symptoms (depression, cognitive impairment, social withdrawal) [64]. Whilst the neuropathology underlying schizophrenia is unclear, most theories center on either an excess or a deficiency of neurotransmitters, including dopamine, serotonin, and glutamate [65–67]. Positive symptoms such as hallucinations and delusions, which are thought to result from excessive dopamine release in the prefrontal cortex [68,69] have been treated by dopamine antagonists such as haloperidol [70,71]. Other theories implicate aspartate, glycine, and gamma-aminobutyric acid (GABA) as part of the neurochemical imbalance of schizophrenia [72].

Late-onset schizophrenia, which classifies individuals who are diagnosed at the age of 45 or older, are more common in women than men [73]. In contrast, early-onset schizophrenia, which refers to those diagnosed before the age of 18, are more common in males than females (discussed later in this review). Women have been shown to be more vulnerable to psychotic breakdown at times of estrogen withdrawal, i.e., menopause or just after giving birth [74]. Females more frequently exhibit depressive

symptoms [73], whilst males tend to have a greater vulnerability to negative symptoms and traits of disorganization. However, studies have found premorbid functioning to be worse in men than in women, with late-onset schizophrenia generally found to have good premorbid competence [75–77]. Overall, the prognosis of the illness, the social functioning, and the response to treatment is generally better in female schizophrenic patients compared to males [73].

2.2. Female-Biased Neurodegenerative Disorders

2.2.1. Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder, with an estimated worldwide prevalence of 24 million people [78]. AD sufferers exhibit gradual cognitive decline, often beginning with memory loss and extending to behavioural disturbances such as apathy and depression [79]. AD is characterized by the accumulation of intracellular hyperphosphorylated tau inclusions termed neurofibrillary tangles, extracellular plaques consisting of beta-amyloid aggregates, and brain atrophy caused by the progressive loss of cholinergic neurons [80–84]. The propagation of neuropathological changes in AD is highly characterized, beginning in the entorhinal cortex, before spreading to the hippocampus, basal forebrain, temporal and parietal lobes, and eventually the neocortex [85,86].

The female sex is a significant risk factor for AD, as two-third of AD patients are women [21,87,88]. Moreover, women have a two-fold higher incidence in AD [89], and two-fold higher lifetime risk of AD compared to men [90,91]. This female-bias is not seen in any other types of dementias [92], although some have reasoned that it is a result of women living longer than men [93]. Women AD patients exhibit faster rate of hippocampal atrophy and greater neurofibrillary tangles than men with AD [94,95]. Women also show more rapid loss of autonomy, greater disability and more rapid cognitive decline whilst men have a higher mortality and comorbidity and later onset [79,96,97]. In particular, female AD patients exhibit a greater cognitive decline in areas of visuospatial abilities, verbal processing, and semantic and episodic memory than male AD patients [98–101]. Clinical and pre-clinical studies demonstrate conflicting evidence on sex differences in response to anticholinesterase medications, a widely used therapy for AD. Some studies have reported that females responded better than males in clinical [102] and in AD models [103], whilst recent meta-analysis study have no significant sex differences [104]. Overall, women show a greater rate of prevalence and incidence, increased cognitive decline and greater rate of neuropathological decline in AD compared to men. Given the differences in sex hormone levels in AD patients compared to controls, this suggests that biological factors could contribute to sex differences seen in AD. Further investigation into the underlying causes of sex differences in AD is a crucial step towards providing specific gender-based therapies and diagnosis.

2.2.2. Multiple Sclerosis

Multiple Sclerosis (MS) is a neurodegenerative disease characterized by autoimmune demyelination of axons and plaque formation, eventually leading to CNS degeneration [105]. Disease course can be classified into 4 subtypes; relapsing-remitting (RR), primary progressive (PP), secondary progressive (SP), and primary relapsing (PR). RR-MS as the name suggests, is characterized by periods of acute disease followed by recovery [106]. RR-MS is the most common type accounting for 85–90% of cases, whilst 10–15% of cases are categorized as PP, where symptoms continually worsen with time [106]. The disease mainly affects young adults with peak symptom onset at 30 years of age [107]. MS symptoms have a wide range and severity, resulting from disparity in lesion development and progression. Most commonly reported are fatigue, visual or sensory impairment and cognitive deficits. Magnetic resonance imaging (MRI) is an essential technique for diagnosis and can be used to determine the presence of lesions, anatomically specific to MS whilst brain atrophy is associated with disease progression and cognitive impairment [107,108]. Unlike most neurodegenerative diseases, disease-modifying treatments are available for MS, although there is still no cure. Disease-modifying

treatments, which are usually drugs that suppress or modulate the immune systems, are efficacious at slowing disease progression or reducing severity or frequency of attacks when implemented early [109].

There is a higher incidence of MS in females compared to males [22], with 3.6 females to 2.0 male cases per 100,000 [23]. RR-MS has approximately two-fold higher incidence in women over men [105]. Unaffected females that carry susceptibility genes are also more likely to transmit to children than males [110]. RR-MS, female sex and early age of onset are all associated with more benign disease course [111] whilst male sex, later age of onset and a high number of early attacks all associated with poorer prognosis [112,113]. Males with MS are prone to develop less inflammatory, but more destructive lesions than women [114]. Women have greater T-cell immunoreactivity to myelin protein than males in both MS cases and healthy controls [115]. Comparison of gene expression in inflammatory lesions suggests MS pathogenesis in males induces estrogen signalling pathways, whilst females exhibit an upregulation of progesterone pathways [116]. Interestingly, female disease incidence is rising, which may be due to an unknown environmental influence or gene-environment interaction [117–123].

3. Male-Biased Disorders

3.1. Male-Biased Neuropsychiatric Disorders

3.1.1. Autism

Autism, or autism spectrum disorders, is a set of heterogeneous neurodevelopmental conditions, characterized by early-onset difficulties in social communication and unusually restricted, repetitive behaviour and interests [124,125]. Symptoms manifest prior to the age of 3, although diagnosis doesn't usually occur until 3–4 years of age [126] which is based primarily on the observation of behavioral problems and atypical language development [127]. Globally, autism is thought to affect around 25 million people as of 2015 [128]. The most recent report by the Centers for Disease Control and Prevention revealed a 15% increase in prevalence of autism in the United States from the previous two years [129]. The recent increase in the diagnosis of autism may be partly due to changes in diagnostic practice, although the question of whether actual rates have increased remains unclear [130]. Autism is thought to affect information processing in the brain by altering neuronal connectivity and organization during development [124,125], although how this occurs is not well understood. Evidence from clinical and animal research also suggests an imbalance in serotonin and dopamine [124,125]. Boys with autism have reduced serotonin synthesis and levels in the frontal cortex and thalamus [131,132], which may underlie impaired language production and sensory integration symptoms [131]. Studies have also shown that autism is a hyperdopaminergic condition, likely due to the atypical neural network between the amygdala and prefrontal cortex, which could underlie the social deficits in autism [133,134].

Whilst the cause of autism remains unknown, it is likely to result from a combination of genetic and environmental factors such as prenatal infections, valproic acid or alcohol use during pregnancy [124,125]. Interestingly, one of the most consistent findings in autism research is the higher rate of diagnosis in males than females [124,125,135,136]. Autism is approximately four times more common among males than females [137], although this ratio is further increased to eleven males to one female in severe autism [138]. Aside from prevalence rates, males and females differ in the presentation of clinical symptoms [139]. Females with autism show less restricted and repetitive behaviours and interests compared to males and tend to have internalising symptoms such as depression and anxiety, whereas males tend to have more externalising symptoms such as aggression, and hyperactivity [140,141]. Several theories exist on the male preponderance of autism—such as a genetic protective effect in females [142,143] or the "extreme male brain" theory [144]—which will be discussed later in this review.

3.1.2. Attention-Deficit Hyperactivity Disorder

Attention-deficit hyperactivity disorder (ADHD) is the most commonly diagnosed psychiatric disorder in children, affecting approximately 5% of children worldwide, particularly boys [145]. ADHD is characterized by symptoms of inattention and/or hyperactivity-impulsivity [146]. Symptoms typically appear the age of twelve years old, persist for more than six months, and cause disruptions in at least two settings (such as school, home, or recreational activities). Symptoms of ADHD have been primarily associated with the hypofunction of catecholamines dopamine and norepinephrine in the frontal-subcortical circuit (i.e., prefrontal cortex and striatum), which are involved in attention, reward and motor activity [146–149]. Thus, the most effective drugs used to treat ADHD, such as methylphenidate and d-amphetamine, are all stimulants, which increase levels of dopamine and norepinephrine to enhance catecholamine signalling in the brain [150,151].

Like autism, ADHD is a neurodevelopmental disorder with a strong male-bias, with a sex ratio of three males to every female [152–156]. Males are likely to exhibit all subtypes of ADHD [157], have higher ADHD symptom scores, and may present with externalising behaviours such as physical abuse, aggression and criminality [158]. On the other hand, females tend to exhibit the inattentive subtype and be at increased risk of developing co-morbid eating and anxiety disorders [14,159–161]. There is also some evidence for sex differences in brain activity in ADHD as electroencephalography (EEG) recordings revealed that girls exhibit abnormally elevated coherence in frontal and temporal regions and localised frontal theta enhancement, whereas boys show little evidence of systematic coherence development, and more widespread theta-wave enhancement [162–164]. These findings are in line with the notion of a more extensive and severe neurodevelopmental phenotype in males with ADHD. A recent genome-wide association study of ADHD patient samples from Psychiatric Genomics Consortium (PGC) and the Lundbeck Foundation Initiative for Integrative Psychiatric Research (iPSYCH) revealed a greater familial burden of risk in female individuals with ADHD, although autosomal common variants did not explain the sex bias in ADHD prevalence [165]. Overall, sex differences observed in ADHD and autism may be partially accounted for by diagnostic and ascertainment biases, but are likely, in large part, to be due to biological differences between males and females.

3.1.3. Tourette's Syndrome

Tourette's syndrome is a neuropsychiatric disorder characterised by recurring motor and phonic tics during childhood and adolescence [166]. Typical onset of Tourette's syndrome occurs around 6 to 7 years old and one-third of Tourette's affected children retain their symptoms into adulthood [167,168]. 90% of Tourette's patients suffer from comorbid psychiatric conditions including ADHD, obsessive compulsive disorder (OCD), aggression and other impulse control disorders [169,170]. Neuroimaging and post-mortem studies have shown excessive activity and/or innervation of the cerebral cortex and basal ganglia of Tourette's patients [171], which may reflect dysregulations in the dopaminergic system [172–175]. Treatment options for Tourette's syndrome include drug treatments such as dopamine antagonists [176,177] or behavioural approaches such as habit reversal therapy [178,179].

Tourette's syndrome is more common in boys with sex ratio of four boys to one girl [180], and is diagnosed earlier in males than females [181]. In males, onset of Tourette's syndrome is characterised by anger-related manifestations and simple tics; conversely, females exhibit complex tics more often than males [181]. Furthermore, male TS patients exhibit significant deficits in cortical and callosal thickness, which are not observed in females [182–184]. Whilst males have increased vulnerability for tics in childhood, females have greater tic severity during adulthood [185].

3.1.4. Early-Onset Schizophrenia

Although schizophrenia has a weak male bias on average (7 males: 5 females), this ratio is increased in younger males (<20 years old) where two males to every female are affected [186–189]. Males also

have an earlier age of onset of schizophrenia, between 18–25 years of age, compared with the female age of onset which is 25–35 years [73]. Males tend to have a greater vulnerability to negative symptoms and traits of disorganization, while females more frequently exhibit depressive symptoms [73].

3.2. Male-Biased Neurodegenerative Disorders

3.2.1. Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after AD, affecting nearly 10 million people worldwide. PD affects 2% of population over the age of 65, increasing to 5% over the age of 85 [190]. PD is characterized by the inability to initiate and maintain voluntary movement [190]. Motor symptoms of PD are associated with the loss of midbrain dopamine neurons [191]. Whilst current therapies based on DA replacement strategies effectively treat motor symptoms, they do not slow down or halt the progression of PD. Furthermore, their therapeutic benefit is eventually marred by the development of debilitating side effects known as dyskinesias [192]. Whilst the cause(s) of PD is unknown, PD is likely to arise from a complex interplay between genetic and environmental factors [193–196].

Aside from aging, the male-sex is the most significant risk factor for PD. Men are twice more likely to develop PD and also have an earlier age of onset and a faster rate of disease progression in PD than women [16,17,197,198]. Evidence from animal models of PD reproduce the sex differences observed in humans, as administration of equal doses of dopamine toxins produce greater motor deficits and nigrostriatal dopamine loss in male rodents and primates than their female counterparts [199,200]. Microarray analysis of single SNc dopamine neurons from healthy post-mortem SNc revealed that expression of genes implicated in PD pathogenesis (e.g., *α-synuclein*, *PINK-1*) was higher in men than in women [201], suggesting that nigral dopamine cells have intrinsic sex differences that may influence the pattern of gene expression, predisposing the male-sex to developing PD. There are also clear differences in expression and function of dopamine machinery genes between males and females. For instance, striatal D2R density and binding potential decline twice as fast with age in males compared to females [202], which is likely to reflect sex differences in symptom severity and response to medication in PD. Together, evidence from animal models and clinical studies suggests that sex differences in PD pathogenesis mechanisms and dopamine machinery genes are likely to contribute to sex differences in prevalence, symptoms severity and medication response in PD.

3.2.2. Motor Neuron Disease

Motor neuron disease (MND), also known as amyotrophic lateral sclerosis (ALS) or Lou Gehrig's disease, is a rapidly degenerative muscular disorder affecting over 2.7 per 100,000 people [203]. Although MND has >99% fatality within 2–5 years [204], one of the most famous sufferer Stephen Hawking survived well over 50 years from his initial diagnosis. The pathological hallmark of MND is the presence of hyper phosphorylated and ubiquitinated aggregates of TAR DNA-binding protein 43 (TDP-43), a ubiquitously expressed nuclear protein involved in transcriptional repression and RNA splicing [205–208]. Whilst the pathogenic role of TDP-43 aggregates remains unclear, it is likely due to either a loss of function, toxic gain of function, or a combination of both [209]. Clinically there is a fast-focal onset but movement impairment eventually spreads all over the body [210], with phenotype heterogeneity attributed to variation in disease progression anatomically.

Around 95% of MND cases are idiopathic in nature, with the remaining 5–10% of cases attributed to familial genetic origins [211]. Males are at greater risk for MND with a sex ratio of 1.6 male: 1 female for prevalence and 1.4 male: 1 female for incidence [18,19,212,213]. The incidence ratio has been reported to be as high as four males: one female in the 20–29-year age of onset group [214]. Men also have an earlier age of onset for MND than women [215–217]. This male-bias is reflected in the superoxide dismutase 1 (SOD1) mouse model of MND as male SOD1 mice had an earlier disease onset than female counterparts [218]. There are sex differences in the clinical features of MND, with

men more likely to have spinal onset MND (associated with limb muscle wasting), whereas bulbar onset MND (associated with dysarthria and dysphagia for solids or liquids) are more common in women [18].

4. Role of Sex Hormones and Sex Chromosome Genes in Susceptibility to Neurological Disorders

Sex differences in brain and behaviour have been largely attributed to the effects of sex hormones, including permanent or "organizational" effects during development and reversible or "activational" effects during adulthood [219]. However, it is becoming increasingly clear that brain sex differences are also mediated by the complement of genes encoded on the sex chromosomes, which are expressed in a sex-specific manner that is independent of the effects of sex hormones [3,26,37,38]. Indeed, sex chromosome gene expression is sexually dimorphic in the brain in a region-specific, cell type-specific, and time-specific manner [220–228]. Here, we will discuss the relative contribution of sex-hormones and sex-chromosome complement genes in the susceptibility to neurodegenerative and neuropsychiatric disorders.

4.1. Influence of Sex Hormones

4.1.1. Estrogen and Estrogen Signalling

Compelling evidence demonstrate that estrogen exerts neuroprotective actions in females [7]. For instance, post-menopausal women also have an increased disease risk of PD and AD as they have the lowest concentrations of circulating estrogen, even lower than males [92]. Similarly, symptoms worsen in female PD or MS patients with the onset of menopause or before the onset of menses during the menstrual cycle [229,230]. Furthermore, early initiation of estrogen replacement therapy at menopause appears to lower the risk of developing AD [79,231], whilst post-menopausal estrogen treatment has been associated with reduced risk of developing PD [232] and reduced symptom severity in female PD patients [233,234]. Likewise, increased levels of estrogen produced during pregnancy are associated with reduced severity of MS [230,235]. Studies in animal models recapitulate these clinical findings as treatment with estrogen or estradiol attenuates neuropathology and symptoms in animal models of PD [199,200,236], MS [237], and AD [238,239]. Estrogen exerts its protective effects by mediating mitochondrial function [240], anti-apoptotic mechanisms [240] and immune responses [230], as well as reducing beta amyloid neurotoxicity [241].

Estrogen also plays a significant role in sex differences in susceptibility, symptom severity and treatment response to affective disorders and neuropsychiatric disorders. Women may have a higher risk for developing anxiety disorders, or exacerbation of their present symptoms, during different phases of their reproductive lives, such as puberty, menses, pregnancy, postpartum, and menopause [242–246], whilst depressed women have significantly lower circulating levels of estrogen [247]. Additionally, depressed women had more favorable response to sertraline than to imipramine and the reverse was found with men [52]. Given that the female response was primarily in premenopausal women, this suggested that female sex hormones may enhance response to SSRIs or inhibit response to tricyclics [248]. Similar to PD, the incidence of schizophrenia in post-menopausal females is higher compared to pre-menopausal females [249]. Women have been shown to be more vulnerable to psychotic breakdown at times of estrogen withdrawal, for example just after giving birth and at menopause [74]. Conversely, increased levels of estrogen during the menstrual cycle is associated with an improvement of schizophrenic symptoms and therapeutic response to treatments [250,251]. In support, a clinical study of female schizophrenic patients demonstrated that estrogen or estradiol treatment with antipsychotic drugs led to significant improvements in acute and severe psychotic symptoms when compared to antipsychotic drugs given alone [252,253].

Whilst the effects of estrogen on neurodegeneration and cognition are well established, the roles of the estrogen receptors alpha (ERα) and beta (ERβ) are less clear. In the brain, ERα mRNA is abundantly

expressed in the hypothalamus and amygdala [254–256], whilst ERβ mRNA is highly expressed in the hippocampus and entorhinal cortex [254–256]. In line with the brain distribution pattern, female ERβ knockout (KO) mice exhibited spatial learning and memory deficits compared to wild-type controls [257], which was not observed in the ERα KO mice [258]. Moreover, treatment with the ERβ (but not ERα) agonist in ovariectomized mice improved performance in spatial memory tasks [259], suggesting a role ERβ in memory and cognitive processing. In contrast, ERα, but not ERβ, agonist exerted neuroprotective effects in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mice model of PD [260]. Furthermore, ERα KO mice exhibited greater vulnerability to MPTP-induced DA depletion compared to WT mice, which was not observed in the ERβ KO mice [261]—suggesting a role for ERα in estrogen-mediated neuroprotection. Taken together, greater understanding of the nature of ER selective ligands and the function of ERα and ERβ subtypes in different brain regions may lead to optimal therapies for neurodegenerative and neuropsychiatric diseases.

4.1.2. Testosterone

Prenatal testosterone plays a crucial role in masculinising the developing male brain [219]. Testosterone activates the androgen receptor to mediate the masculinization of the male brain during the perinatal period to induce male-typical behaviours such as aggression and sexual behavior in male adult rodents [262,263]. Thus, exposure to aberrant levels of prenatal testosterone may influence susceptibility to neurodevelopmental disorders, [264,265].

In 2002, Baron-Cohen proposed the so-called "extreme male brain theory" [144] which hypothesizes that abnormally high levels of foetal testosterone may underlie the cognitive and emotional profile of people with autism, as well as the higher prevalence of autism in males compared with females. In one study, researchers found that girls who had been exposed to high levels of foetal testosterone in the womb had a more male-typical play style [266], whilst another study found a positive relationship between fetal testosterone levels and number of autistic traits in children [267]. Along these lines, males diagnosed with ADHD or autism have lower finger-length ratios [35,36], a proxy measure of high levels of prenatal testosterone exposure [268].

On the other hand, testosterone and its metabolites have been shown to possess anxiolytic properties, reducing anxiety behaviors and enhancing cognition in male rodents [269–271]. Testosterone has shown to influence the severity of tics, as anabolic androgens worsen tic severity in males with Tourette's syndrome [272], whilst treatment with the 5α-reductase inhibitor, finasteride, decreased tic severity and compulsive symptoms in adult men with Tourette's syndrome [273]. Depletion of endogenous testosterone levels in male rodents, via castration, induced neuronal cell loss in animal models of MS [274] and PD [275], suggesting that testosterone may be neuroprotective in males.

In summary, oestrogen exerts neuroprotective effects in females, which may underlie the reduced (or increased) incidence of neurological disorders in females throughout development and adulthood. Exposure to prenatal testosterone appears to be critical for the masculinization of the male brain and hence exposure to abnormal levels of testosterone may contribute to susceptibility of males to neurodevelopmental disorders such as autism and Tourette's syndrome.

4.2. Influence of Sex Chromosome Genes

In addition to the well-established actions of sex hormones on brain sex differences, accumulating evidence demonstrate that sex chromosomes genes (X-linked or Y-linked) can directly influence brain function [3,26,37,38]. Whilst small in number, X- and Y-linked genes are proportionally abundant in the brain and have been shown to influence neural development and function [276,277]. For instance, genes on the sex chromosome may influence neurological diseases by altering the basic differentiation process of the neurons [278], encoding proteins [279], neurotransmitter biosynthesis [26,280] and synaptic transmission [281]. Moreover, sex chromosome abnormalities can influence neurodevelopment and often result in impairments in attention, working memory, verbal skills and executive function [282]. Therefore, the study of sex chromosomes in brain disorders may provide a new angle to understand

the sex differences in the pathogeneses of neurodevelopmental and degenerative diseases. To better understand the relative contribution of sex chromosome complement on sex-bias in preponderance to neurological disorders, we will highlight three distinct genetic mechanisms: (i) X-linked dosage effects, (ii) X-linked imprinting effects, and (iii) Y-chromosome effects.

4.2.1. X-Linked Dosage Effects

As females have two X chromosomes, one of the copies of the X chromosome present in females is inactivated in process known as X-inactivation [283], equalizing the gene products of sex chromosomes between the sexes. However, approximately 15–20% of X-linked genes consistently escape X-inactivation [284], and therefore may be expressed higher in females than males. For instance, X-inactivation gene escapees, *Utx* and *Usp9x*, have higher expression in XX mice brains compared to XY, regardless of their gonadal phenotype [285,286]. Whilst the significance of X-inactivation gene escapee expression in brain function remains to be fully elucidated, it may potentially mask any gain or loss of function in females [9]. For instance, females with Turner syndrome (also known as 45, XO), a condition in which a female is partly or completely missing an X chromosome, have increased vulnerability to neurodevelopmental disorders such as ADHD [287], autism [288] and potentially schizophrenia [289]. Similarly, 39, XO mice (female mice with only one X-chromosome) exhibit attention deficits compared to 40, XX mice [290]. These attention deficits were rescued in 40, XY*^{X} mice (39, XO mice with a small number of pseudoautosomal and X-linked genes on the Y*^{X} chromosome), suggesting a protective role for X-chromosome genes in attentional and cognitive processes [290]. Deletions and frameshift mutations of *NLGN4X*, an X-inactivation gene escapee involved in formation and remodeling of synapses, were identified in boys with autism [291–293]. Polymorphism in the promoter region of *MAOA*, a X-linked gene involved in catecholamine metabolism, have been associated with increased risk of males to autism [294–296] and ADHD [297]. Thus, the extra dose of X-chromosome may have a protective effect in females, reducing the vulnerability to neurodevelopmental disorders such as autism and ADHD. On the other hand, individuals with an additional X-chromosome (e.g., 47, XXX and 47, XXY) exhibit global intellectual impairment [298] and show increased risk of ADHD and autism [299,300], suggesting that over dosage of X chromosomes can also be detrimental to brain development.

4.2.2. X-Linked Imprinting Effects

Imprinted genes are solely expressed by one allele in a parent-of-origin dependent manner [9,301]. Although small in number, high proportion of imprinted genes are expressed in the brain and postulated to have role in neurodevelopment, brain function and behaviour [276]. Given the unique inheritance pattern of the X chromosome—maternal X can be inherited to male or female offspring, whilst paternal X only goes to female offspring—imprinted genes on the X chromosome could potentially influence sex-bias in vulnerability to neurodevelopmental disorders. For instance, any protective function of paternally expressed X-linked imprinted genes will be passed only to the female offspring. Indeed, females with Turner's syndrome (45, XO) who inherited the paternal X chromosome (45, X^{P}O) had superior verbal and higher-order executive function skills compared to females that inherited the maternal X chromosome (45, X^{m}O). In view of these findings, they suggested that a genetic locus for social cognition is imprinted on the paternal X-chromosome, which could contribute to the higher incidence of autism seen in 45, X^{m}O females, and males [302]. Bishop, et al. [303] assessed verbal and visuospatial memory in females with a single paternal X chromosome (45, X^{P}O) and those with a single maternal X (45, X^{m}O). Their findings revealed that 45, X^{m}O females showed enhanced verbal forgetting relative to controls, whilst 45, X^{P}O females showed disproportionate visuospatial memory loss relative to controls. In view of their results, Bishop and colleagues postulated the existence of one or more imprinted genes involved in memory function on both the maternal and paternal X chromosome Bishop, Canning, Elgar, Morris, Jacobs and Skuse [303], which could contribute to sex differences seen in memory-associated neurological disorders. To better understand

the role of X-linked genes in cognitive function, Davies, et al. [304] assessed the performance of 39, XO mice, where the X chromosome was either paternally (39, X^{P}O) or maternally (39, X^{m}O) inherited, in various cognitive tasks. 39, X^{m}O mice exhibited deficits in reversal learning, a measure of impulsive and compulsive behaviour [305]. Furthermore, a novel imprinted gene candidate, *Xlr3b*, which is maternally expressed in the 39, X^{m}O mouse prefrontal and orbitofrontal cortex and hippocampus, was identified as mediator of the inflexible reversal learning. Together, these findings indicate that X-linked imprinted genes expressed in a parent of origin-dependent manner could influence sexually dimorphic phenotypes in brain function and may confer vulnerability to neurodevelopmental disorders such as autism and ADHD.

4.2.3. Y-Chromosome Effects

The Y chromosome is passed only from father to son, indicating that any Y-linked traits are only present in males. Males possess genes on the Y chromosome that have no homologous sequences on the X chromosome, suggesting that genes encoded on the Y chromosome may contribute to biological sex differences [306,307]. Whilst the majority of Y chromosome genes are involved in testis development and spermatogenesis [308–310], a significant proportion are expressed in the brain [225,226]. Thus, Y chromosome-specific genes could contribute to sexual differentiation of the brain indirectly, through influencing gonadal hormone production or directly via cell autonomous actions in the brain [277]. Xu and colleagues showed that six Y-chromosome genes, *Ddx3y*, *Ube1y*, *Kdm5d*, *Eif2s3y*, *Uty* and *Usp9y* were expressed in both the developing and adult XY male mouse brain [226]. All six genes were also expressed in the brain of XY female mice that lacked the male-sex determining gene *SRY* and testes [226]. Another study showed that *Dby* and *Eif2s3y* were expressed in the developing male mouse brain at 10.5 days post coitum, prior to the influence of gonadal hormones [38]. Together, these findings indicate that Y-chromosome gene expression in the brain is independent of hormonal influence, suggesting that Y chromosome genes could contribute to sexually dimorphic brain development and function. In support, several clinical studies have reported ADHD diagnosis in 47, XYY and 48, XXYY boys [299,311,312] which suggests that a dosage-effect of Y-chromosome genes may increase the risk of males to neurological disorders. Whilst it is unclear which Y-chromosome gene(s) have a physiological role in the male brain, emerging studies indicate that the male-sex determining gene, *SRY,* is an ideal candidate to investigate [26,280].

4.2.4. Y-Chromosome Gene *SRY*

SRY (Sex-determining Region on the Y chromosome) encodes is a transcription factor that initiates male-sex determination by directing embryonic bipotential gonads to develop into testes rather than ovaries [313,314]. Subsequently the testes secrete testosterone, which can act to masculinise the brain during development ('organisational effects') or at particular time points leading to reversible neural changes ('activational effects'). In addition to its 'indirect' hormonal effects on the brain, emerging evidence demonstrate that *SRY* can directly exert actions in the adult male brain [26]. In the mouse brain, *SRY* expression is developmentally regulated, with the circular (untranslatable) form of *SRY* transcripts expressed from embryonic day 11 (E11) through E19 whilst postnatal brain *SRY* transcripts are of the linear (translatable) form [315]. In the adult brain, *SRY* mRNA is expressed in regions abundant in catecholamine cell bodies or nerve terminals such as the SNc, ventral tegmental area (VTA), locus coeruleus and hypothalamus [26,316]. Immunohistochemical studies in human and rodent midbrain sections reveal that *SRY* protein co-localizes with dopaminergic neurons in the SNc and VTA, and with GABAergic neurons in the substantia nigra pars reticulata [26,280]. In line with the presence of *SRY* in dopamine-neurons, *SRY* regulates dopamine biosynthesis genes in vitro [280,316,317] and dopamine-dependent functions such as voluntary movement [26] and blood pressure [318,319] in male rats. Combined, these results suggest that *SRY* exerts a direct male-specific action on adult DA neurons, independent of circulating gonadal hormones.

Given that *SRY* is expressed in brain regions closely associated with pathophysiology of ADHD and autism, abnormal regulation of *SRY* expression during development, and consequently dopamine machinery genes, may contribute to the hyper- or hypo-function of dopamine levels in these disorders. Considering the expression of *SRY* in the human male SNc [280], a brain region that degenerates in PD, dysregulation of *SRY* in male dopamine neurons may underlie the male preponderance to PD. Indeed, *SRY* expression is aberrantly elevated in a human cell culture model of PD [320]. Whilst other Y-linked genes, such as neuroligin 4, will also need to be examined for their role in male-biased neurodevelopmental disorders [321], these results highlight the need to better understand the molecular regulation, function, and targets of *SRY* in the healthy and diseased male brain. This information, alongside identifying novel *SRY* polymorphisms, will be essential for the development of novel therapeutic strategies (e.g., male-specific therapies) for sex-biased neurological disorders.

5. Future Directions and Conclusions

Given that sex differences in neurological disorders arises from a combination of hormonal and genetic factors, integrated pre-clinical and clinical research efforts will be needed to distinguish the source of these differences. Animal models in which chromosomal and hormonal factors can be systematically varied, such as the four-core genotype model [37] and sex chromosome trisomy model [322], have proven to be useful tools in partitioning the effect of sex chromosome complement from the actions of gonadal sex hormones. The use of transgenic or knockout rat technologies and spatiotemporal-restricted gene expression strategies (e.g., brain *SRY* overexpressing transgenic rat) will be vital in identifying the role of sex-linked genes in the healthy and diseased brain. In parallel, genome-wide association and copy-number variation studies in human populations need to stratify disease-associated genetic variants by sex to identify potential interactions between sex and genetic vulnerability. Together, these studies may highlight novel sex-specific protective and risk factors, which should ultimately facilitate improved diagnosis, prognosis and treatment for males and females.

Evidence from numerous clinical studies indicate a promising role for sex-specific hormonal therapies in neurodegenerative and neuropsychiatric disorders. For instance, female-specific therapeutic benefit of estrogen treatment has been demonstrated for cognitive decline in AD [323–326] and MS [327], as well as improved efficacy of dopamine agonist medications for female PD patients [233]. Similarly, estrogen has shown to be beneficial in women with post-natal depression [328] and schizophrenia [34]. Conversely, testosterone therapy has shown promising effects on cognitive decline in male patients with AD [329] or MS [330], and non-motor symptoms of PD [331]. Moreover, emerging evidence from animal models and clinical observations also suggest a role for targeting X-linked (e.g., steroid sulfatase) or-Y-linked (*SRY*, neuroligin-4) genes [26,38,321] for male-biased neurodevelopmental disorders such as autism or ADHD. Given the potential therapeutic benefit of gonadal hormones in neuropsychiatric and neurodegenerative disorders, better understanding the sex hormone status of patients have the potential to improve diagnosis and inform treatment selection.

Whilst gender specific medicine still faces many challenges in current healthcare, the recognition that sex affects the pathophysiology and expression of human disease is starting to influence governmental and regulatory bodies. Indeed, National Institutes of Health and Food and Drug Administration are acting to ensure that both sexes are represented in pre-clinical and clinical studies, as well as all the drug development phases. In conclusion, better understanding the biological differences between the male and female brain will not only allow better, and even optimal, treatment of neurological disorders and a reduction in health care expenditure due to the elimination of inadequate treatment or adverse events.

Conflicts of Interest: The authors declare no conflict of interest.

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