MONASH University

The Regulation and Function of the Y-Chromosome Gene, *SRY*, in the Healthy and Diseased Male Brain

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A thesis submitted for the degree of Doctor of Philosophy at
Monash University in 2018

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Abstract

Innate sex differences in anatomy, biochemistry, and physiology of the healthy brain are likely to influence sex differences in neurodegenerative and neuropsychiatric disorders. For instance, females are more likely to suffer from Alzheimer's disease, depression, and anxiety, whereas males are more susceptible to deficits in the dopamine system such as Parkinson's disease (PD), attention-deficit hyperactivity disorder (ADHD), and early-onset schizophrenia. Traditionally, these sex differences have been explained solely by the influence of sex hormones. Emerging evidence however indicates that the sex chromosome genes also contribute. The Y-chromosome gene, SRY (Sex-determining Region on the Y chromosome) is an interesting candidate as it is expressed in dopamine-abundant brain regions such as the substantia nigra pars compacta (SNc), where it regulates dopamine biosynthesis and voluntary movement in males. To better understand the contribution of SRY in mediating brain sex differences, this thesis sought to investigate the regulation and function of SRY in the healthy and diseased male brain.

Chapter 2 examined the regulation and function of SRY in the healthy male brain. In human male dopamine M17 cells, SRY protein expression was regulated by a dopamine D2 receptor-mediated mechanism under physiological conditions. However, a robust upregulation of *SRY* expression was observed in response to cellular stress, which was accompanied by an increase in *GADD45*γ expression. Reducing nigral *Sry* expression in male rats, via Sry antisense oligonucleotide (ASO) infusion, revealed a *Sry*-mediated regulation of dopamine and non-dopamine target genes. SRY immunohistochemistry in the human male brain revealed that SRY protein is expressed in both dopaminergic (SNc) and non-dopaminergic regions such as the cerebellum and frontal cortex within neuronal and non-neuronal cells.

Chapter 3 assessed the regulation and function of SRY in experimental models of PD. I found that SRY expression was aberrantly upregulated in both cell culture and animal models of PD. Remarkably, reducing nigral Sry expression in male rats ameliorated the motor deficits and nigral degeneration in toxin-induced rat models of PD. The protective effect of Sry ASO infusion was concomitant with male-specific attenuation of expression of pro-apoptotic, pro-inflammatory, oxidative stress and mitochondrial genes. The

neuroprotective effect of ASO-infusion was absent in females, thus demonstrating for the first-time neuroprotection mediated by a male-specific gene.

Chapter 4 explored the regulation and function of SRY in the spontaneously hypertensive rat (SHR) model of ADHD. I found that brain *Sry* expression was reduced in ADHD-symptomatic male SHRs, which exhibited hyperactivity and cognitive impairment. Furthermore, reducing brain Sry expression, via intracerebroventricular Sry ASO infusion, in male Wistar-Kyoto rats (WKYs) induced cognitive impairments, similar to those observed in male SHRs. The detrimental effect of ASO-infusion was absent in female WKYs. These results indicate that diminished Sry expression directly contributes to cognitive deficits in the SHR model of ADHD.

Overall, my thesis demonstrates that the dysregulation of the male-specific gene, *SRY*, may underlie susceptibility of males to disorders such as PD and ADHD. Considering that the expression and function of SRY in the male brain is more wide-ranging than previously thought, normalising *SRY* expression may represent a novel sex-specific strategy for treatment of other male-biased disorders, such as autism and ALS.

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.

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Date: 30th March 2018

Publications during enrolment

Loke, H., Harley, V. & Lee, J. Biological factors underlying sex differences in neurological disorders. *The international journal of biochemistry & cell biology* **65**, 139-150 (2015).

Schaefer N, Rotermund C, Blumrich EM, Lourenco MV, Joshi P, Hegemann RU, Jamwal S, Ali N, García Romero EM, Sharma S, Ghosh S,Sinha JK, **Loke H**, Jain V, Lepeta K[1], Salamian A, Sharma M, Golpich M, Nawrotek K, Paidi RK, Shahidzadeh SM, Piermartiri T, Amini E, Pastor V, Wilson Y, Adeniyi PA, Datusalia AK, Vafadari B, Saini V, Suárez-Pozos E, Kushwah N, Fontanet P, Turner AJ. The malleable brain: plasticity of neural circuits and behavior – A review from students to students. *Journal of Neurochemistry*, **142**, 790-811 (2017).

Conference proceedings and presentations

Regulation and function of the y-chromosome gene, *Sry,* in an animal model of attention-deficit hyperactivity disorder (ADHD), ***H Loke****, P Pinares-Garcia, V Harley, J Lee*, 37th Annual Scientific Meeting of the Australasian Neuroscience Society, **2017.** (Oral)

Dysregulation Of Sry In The Male Brain: A Genetic Basis For Sex Bias In Neurological Disorders? ***H Loke****, P Pinares-Garcia, V Harley, J Lee*, Lorne Genome meeting, **2017.** (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*, University of California, Los Angeles, **2016.** (Oral)

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*, Society for Neuroscience meeting, **2016.** (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*, ISN-JNC Flagship School, **2016.** (Poster)

Dysregulation of SRY in the Male Brain: A Genetic Basis for Male Susceptibility to Parkinson's Disease, ***H Loke****, P Pinares-Garcia, D Czech, V Harley, J Lee*, IBRO-APRC Associate School, **2016.** (Oral)

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*, Monash Health & MHTP Research Week, 2015**.** (Poster; **won 1st prize for Neurosciences, Basic Science category**)

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)

Dopaminergic regulation of *SRY*: Dysregulation of SRY in the Male Brain: A Genetic Basis for Male-Biased Neurological Disorders? ***H Loke****, V Harley, J Lee,* Students of Brain Research (SOBR) Student Symposium, 2015. (Oral)

Dopaminergic regulation of *SRY* as a basis for sex-biased neurological disorders, ***H Loke****, V Harley, J Lee,* MIMR-PHI Student Symposium, 2014. (Poster; **won 3rd prize**)

Dopaminergic regulation of *SRY*: A genetic basis for male susceptibility to neurological disorders**? *H Loke****, V Harley, J Lee,* Students of Brain Research (SOBR) Student Symposium, 2014. (Oral)

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)

Dopaminergic regulation of *SRY* as a basis for sex-biased neurological disorders in a male neuronal cell line, ***H Loke****, V Harley, J Lee,* Biomed Link, 2014. (Poster)

Dopaminergic regulation of *SRY*: A genetic basis for male susceptibility to neurological disorders? ***H Loke****, V Harley, J Lee,* ECR Neuroscience Showcase, 2014. (Poster)

Awards and prizes

2018: Awarded Monash Postgraduate Publications Award
2017: Australasian Neuroscience Society 2017 Student Travel Award
2016: Awarded IBRO Travel Award
2016: Selected to attend ISN-JNC Flagship School, Austria, 2016 (fully funded)
2016: Selected to attend IBRO-APRC Associate School, Malaysia, 2016 (fully funded)
2016: Finalist, 3-Minute Thesis, School of Clinical Sciences, Monash University
2015: Australasian Neuroscience Society 2015 Student Travel Award
2015: Australia Postgraduate Awards (APA) scholarship
2015: Best Poster Prize (Neurosciences), Monash Health & MHTP Research Week
2015: Finalist, 3-Minute Thesis, School of Clinical Sciences, Monash University
2015: 2nd prize, 3-Minute Thesis, Centre for Reproductive Health, Hudson Institute
2014: 3rd prize (poster presentation), MIMR-PHI Student Symposium

Thesis including published works declaration

I hereby declare that 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.

This thesis includes 1 original paper published in peer reviewed journals and 0 submitted publications. The core theme of the thesis is investigating the regulation and function of SRY in the healthy and diseased male brain. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Hudson Institute of Medical Research, Faculty of Medicine, Nursing and Health Sciences under the supervision of Dr Joohyung Lee and Prof. Vincent Harley.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 1, my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
1	Biological factors underlying sex differences in neurological disorders	Published	Main author of manuscript, 80%	1. Dr Joohyung Lee – provided input and direction, and revised manuscript, 18% 2. Prof Vincent Harley – provided input and revised manuscript, 2%	No

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date:** 30th March 2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date:** 30th March 2018

Acknowledgements

First and foremost I would like to express my sincerest gratitude to my supervisor, Dr Joohyung Lee for his guidance, support and mentorship throughout my PhD. I would like to thank Dr Joohyung Lee for all the opportunities that he has given me, which has enabled me to learn and grow, not only as an independent and capable scientist, but also a confident young woman. I am truly grateful and proud of the partnership we have established and I hope this can continue in years to come.

I would also like to express my gratitude to my co-supervisor, Prof. Vincent Harley for his mentorship and advice throughout my PhD. I am especially grateful to my PhD buddy, Paulo Pinares-Garcia whom I started this journey with. It has been an amazing experience working together and I thank him for all the assistance and support he has given me. I would like to acknowledge and sincerely thank the past and present members of the Molecular Genetics division – especially Janelle Browne, Jeanne Correia and Dr. Daniel Czech for their technical assistance and guidance.

Within the Hudson Institute, I am really grateful for the friendship and support of other students, particularly Harriet Fitzgerald, Aleisha Symon, Brittany Croft, Jimmy Ham, Rajini Sreenivasan, Cherie Au, Heba Zahid, Amy Winship and Justine Olcorn.

To my sister, Sarah Loke, thank you for your patience and support throughout my PhD journey. I am truly grateful for your never-ending encouragement especially during the times that I felt like giving up.

Finally, I cannot express my love and gratitude enough to my parents for always believing in me and supporting my choices. I would not have made it this far without them. Mum and Dad, this thesis is dedicated to you – I hope I have made you proud!

This research was supported by an Australian Government Research Training Program (RTP) Scholarship.

List of abbreviations

6-OHDA	6-hydroxydopamine
^{o}C	degrees Celsius
µg	microgram
µl	microlitres
µM	micromolar
β2M	β2-microglobulin
β-gal	β-galactosidase
a.u.	arbitrary unit
ADHD	attention deficit hyperactivity disorder
ASD	autism spectrum disorder
ASO	antisense oligonucleotide
BAX	Bcl-2 associated X protein
BCL-2	B-cell lymphoma 2
bp	base pair
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
COMT	catechol-O-methyl transferase
CytC	cytochrome c oxidase
D1R	D1 receptor
D2R	D2 receptor
DA	dopamine
DAPI	4', 6'-diamidino-2-phenyldole
DAT	dopamine transporter
DBH	dopamine beta-hydroxylase
DDC	dopa decarboxylase
DMEM	Dulbecco's minimum essential medium
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
DOPAC	3',4'-dhiydroxyphenylacetic acid
dpc	*days post coitum*
DRD1	dopamine receptor D1
DRD2	dopamine receptor D2
DRD3	dopamine receptor D3
DRD4	dopamine receptor D4
DRD5	dopamine receptor D5
DTT	dl-dithiothreitol
FBS	foetal bovine serum
FO	familiar object
GABA	gamma-aminobutyric acid
GAD	glutamate decarboxylase
GAD 1	glutamate decarboxylase 1
GAD 2	glutamate decarboxylase 2

GAD 65	glutamate decarboxylase 65
GAD 67	glutamate decarboxylase 67
GADD45γ	growth arrest and DNA-damage-inducible protein gamma
GLS	glutaminase
h	hours
HMG	high mobility group
ICV	intracerebroventricular
Il-1β	interleukin-1 beta
Il-10	interleukin -10
iNOS	inducible nitric oxide synthase
kb	kilobase
kDa	kiloDalton
L-DOPA	L-3,4-dhihydroxyphenylalanine
M	molar
MAO-A	monoamine oxidase A
ml	millilitre
mM	millimole
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
NCBI	national center for biotechnology information
ND1	NADH-ubiquinone oxidoreductase chain 1
ng	nanogram
NO	novel object
NOR	novel object recognition
Nurr1	nuclear related 1 protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PFC	prefrontal cortex
Pgc1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pitx3	paired-like homeodomain 3
PUMA	p53 up-regulated modulator of apoptosis
qRT-PCR	quantitative reverse transcriptase PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase PCR
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata

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
SRY	sex-determining region on the Y chromosome
STS	steroid sulfatase
TBP	TATA-box-binding protein
TH	tyrosine hydroxylase
TGF-β1	Transforming growth factor beta 1
TNFα	tumour necrosis factor alpha
TXNRD1	thioredoxin reductase 1
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area
WKY	Wistar-Kyoto rat

CHAPTER 1:

Literature Review

The International Journal of Biochemistry & Cell Biology 65 (2015) 139–150

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Review

Biological factors underlying sex differences in neurological disorders

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ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form 25 May 2015
Accepted 26 May 2015
Available online 28 May 2015

Keywords:
Autism spectrum disorders
Parkinson's disease
Attention-deficit hyperactivity disorder
Catecholamines
Y-chromosome

ABSTRACT

The prevalence, age of onset, pathophysiology, and symptomatology of many neurological and neuropsychiatric conditions differ significantly between males and females. 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), schizophrenia, and autism spectrum disorders (ASD). Until recently, these sex differences have been explained solely by the neuroprotective actions of sex hormones in females. Emerging evidence however indicates that the sex chromosome genes (i.e. X- and Y-linked genes) also contribute to brain sex differences. In particular, the Y-chromosome gene, *SRY* (*Sex-determining Region on the Y chromosome*) is an interesting candidate as it is expressed in dopamine-abundant brain regions, where it regulates dopamine biosynthesis and dopamine-mediated functions such as voluntary movement in males. Furthermore, SRY expression is dysregulated in a toxin-induced model of PD, suggesting a role for SRY in the pathogenesis of dopamine cells. Taken together, these studies highlight the importance of understanding the interplay between sex-specific hormones and sex-specific genes in healthy and diseased brain. In particular, better understanding of regulation and function of *SRY* in the male brain could provide entirely novel and important insights into genetic factors involved in the susceptibility of men to neurological disorders, as well as development of novel sex-specific therapies.

Contents

Abbreviations: DA, dopamine; PD, Parkinson's disease; ADHD, attention-deficit hyperactivity disorder; ASD, autism spectrum disorders; SRY, sex-determining region on the Y chromosome; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area; TH, tyrosine hydroxylase; D1R, dopamine D1 receptor; D2R, dopamine D2 receptor; DAT, dopamine transporter; COMT, catechol-*O*-methyl transferase; MAO, monoamine oxidase; DDC, dopa decarboxylase; DBH, dopamine β-hydroxylase; VMAT, vesicular monoamine transporter; NA, noradrenaline; SHR, spontaneously hypertensive rat; 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; GADD45γ, growth arrest and DNA damage gamma; GWAS, genome wide association studies.

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http://dx.doi.org/10.1016/j.biocel.2015.05.024
1357-2725/

1. Introduction

Aside from generating distinct sexual reproductive behaviours, brain sex differences significantly influence brain anatomy, biochemistry, as well as various psychological and cognitive processes. A meta analysis reviewing 20 years of research into brain structural differences revealed that males on average have 8–13% larger brain volumes compared to females (Ruigrok et al., 2014), although sexual dimorphisms of adult brain volumes are not diffusely spread across the brain but rather are region specific (Goldstein et al., 2001). A diffusion tensor imaging (DTI) study showed sex differences in the structural connectome of the human brain, where the male brains are optimized for intra-hemispheric and female brains for inter-hemispheric communication (Ingalhalikar et al., 2014). Furthermore, genome-wide analysis performed on 137 human post mortem brains showed that 2.5% of genes are differentially expressed and spliced between males and females (Trabzuni et al., 2013). These fundamental sex differences in the anatomy and genetic network of the healthy brain are likely to underlie the pronounced sex differences in susceptibility, progression, symptom severity, and pathology of neurological disorders (Cahill, 2006; Cosgrove et al., 2007; Gillies and McArthur, 2010; McCarthy et al., 2012; Ngun et al., 2011). For example, females are more likely than males to develop depression, anxiety (Nolen-Hoeksema, 1987; Weissman et al., 1996) and Alzheimer's disease (Hebert et al., 2013), whilst males are more likely to be diagnosed with Parkinson's disease (PD) (Wooten et al., 2004), attention deficit hyperactivity disorder (ADHD) (Balint et al., 2009), and autism spectrum disorders (ASD) (Gillberg et al., 2006). Hence, better understanding of the biology underlying sex differences in the healthy and diseased brain will be vital for designing novel therapeutic agents that will have optimal effectiveness in each sex. Historically, the sex differences in neurological disorders have been explained by the protective actions of sex hormones in females (Auyeung et al., 2009; Gillies and McArthur, 2010; Riecher Rossler, 1994). However, emerging evidence suggests that genetic factors, in particular sex chromosome genes, also contribute to brain sex differences (Arnold et al., 2004; Beyer et al., 1992; Carruth et al., 2002; Dewing et al., 2003). Here we review studies of hormonal and genetic factors underlying the sex dimorphism in neurological disorders. We will contend that genetic factors play a far more important role than previously suspected. In particular, we highlight evidence that the Y-chromosome gene, *SRY*, regulates dopamine biochemistry and function in the male brain. Based on SRY expression in brain regions associated with the symptoms of dopamine (DA)-associated disorders, we speculate upon how dysregulation of *SRY* may be a contributing factor to male-susceptibility in disorders such as PD and ADHD.

2. Male bias in neurological disorders associated with dopamine

2.1. Dopamine mediates important physiological processes in the brain

Dopamine (DA) is a neurotransmitter that mediates a variety of important physiological processes such as voluntary movement, feeding, reward, sleep, attention, working memory and learning (Bjorklund and Dunnett, 2007b; Carlsson, 1987; Iversen, 2007). Maintenance of physiological levels of DA by various components of DA machinery is crucial for regulation of these processes. As summarized in Fig. 1A, DA is synthesized by a series of enzymatic reactions. L-Tyrosine, is converted into L-DOPA by tyrosine hydroxylase (TH). L-DOPA is converted to DA by dopa decarboxylase (DDC). In the presynaptic nerve terminal, the vesicular monoamine transporter (VMAT) sequesters DA into synaptic vesicles where DA is stored until an action potential occurs which releases DA into the synapse. DA itself is also used as precursor in the synthesis of the neurotransmitter noradrenaline (NA), as DA is converted into NA by dopamine β-hydroxylase (DBH). DA exerts its action via the dopamine D1 receptor (D1R) or dopamine D2 receptor (D2R) on the postsynaptic target cell. The D1R family includes the DRD1 and DRD5 subtypes whilst the D2R family consists of the DRD2, DRD3, and DRD4 subtypes. The dopamine transporter (DAT), located at the presynaptic nerve terminal, controls the concentration of DA in the extracellular space by actively clearing extra-synaptic DA by re-uptake. Unbound extra-synaptic DA can also bind to presynaptic dopamine DRD2/DRD3 autoreceptors. These autoreceptors maintain normal levels of synaptic DA by inhibition of DA synthesis and release. Cytosolic DA is directly broken down into inactive metabolites by the actions of catechol-*O*-methyl transferase (COMT) and monoamine oxidase (MAO), which has two isoforms MAO-A and MAO-B.

There are four major dopaminergic pathways in the brain – i.e. nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular pathway – which regulate various central functions. The nigrostriatal pathway transmits DA from the substantia nigra pars compacta (SNc) to the striatum for control of voluntary movement (Bjorklund and Dunnett, 2007a; Tsui and Isacson, 2011). Dopaminergic projections from the ventral tegmental area (VTA) to the limbic system (i.e. hypothalamus, hippocampus and amygdala), via the nucleus accumbens, form the mesolimbic pathway which is important for motivation and reward-based learning (Bjorklund and Dunnett, 2007a; Gonzales et al., 2004). The mesocortical system originates from the VTA and transmits DA to the prefrontal cortex for executive functions such as decision-making, cognitive and social behaviour (Bjorklund and Dunnett, 2007a; Robbins, 2000).

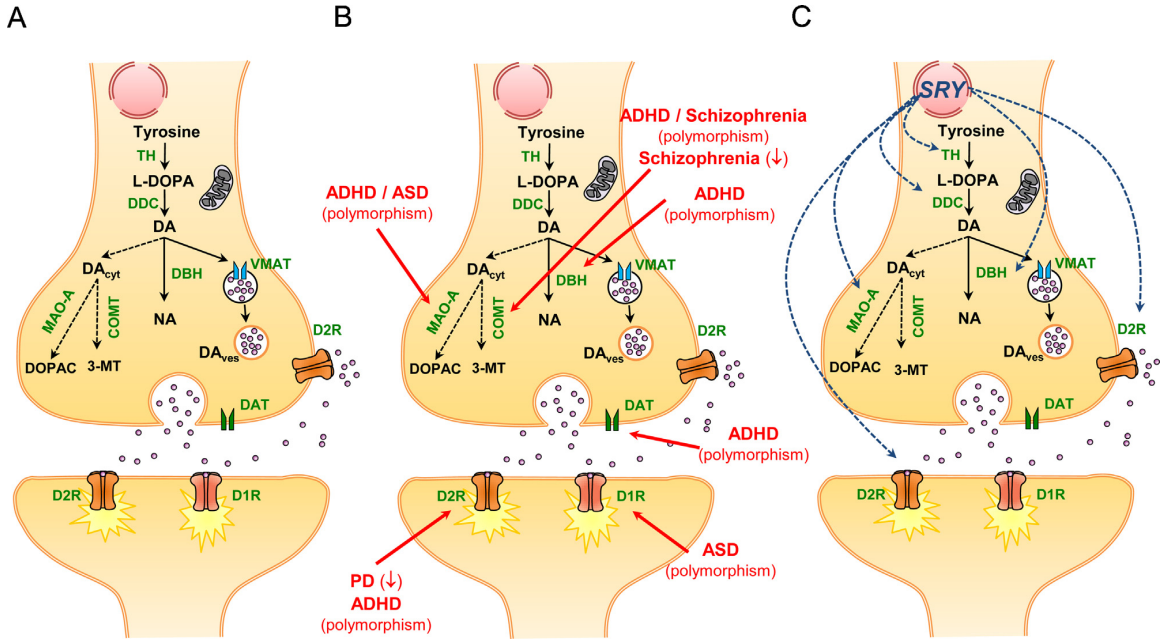

Fig. 1. (A) Schematic diagram of the DA biosynthesis and metabolic pathway. Physiological levels of synaptic DA are maintained by components of DA synthesis, release, re-uptake, and metabolic machinery. (B) Sex differences in DA machinery affected in PD, ADHD, ASD and schizophrenia. Decreased striatal density D2R levels in aged males compared to females could be associated with increased risk of PD in males. Polymorphisms of MAO-A, COMT, DAT and D2R (DRD4) are associated with increased risk of ADHD in males. Polymorphisms of MAO-A are associated with the severity of ASD symptoms whilst polymorphisms of D1R (DRD1) are associated with increased risk of ASD in males. Deficiency and polymorphism of COMT is associated with schizophrenia in males. (C) DA machinery positively regulated by SRY. SRY positively regulates the transcription of TH, DDC, MAO-A and D2R. Note: D1R includes DRD1 and DRD5, D2R includes DRD2, DRD3 and DRD4 (post-synaptic only). Abbreviations **TH** tyrosine hydroxylase; **L-DOPA** L-3,4-dihydroxyphenylalanine; **DDC** DOPA decarboxylase; **DOPAC** 3,4-dihydroxyphenylacetic acid; **D1R** dopamine D1-class receptor; **D2R dopamine** D2-class receptor; **DAT** dopamine transporter; **VMAT** vesicular monoamine transporter; **COMT** catechol-O-methyl transferase; **3-MT** 3-methoxytyramine; **DBH** dopamine-β-hydroxylase; **NA** noradrenaline.

Projections from the hypothalamus to the pituitary gland form the tuberoinfundilar system which is involved in secretion of certain hormones such as prolactin (Weiner and Ganong, 1978). Thus dysregulation of the brain DA system can lead to disturbances in a variety of physiological processes including motor, cognitive, emotional and somatosensory processes, as observed in neurological and neuropsychiatric disorders such as PD, ADHD, ASD and schizophrenia.

2.2. Parkinson's disease

Parkinson's disease (PD) is a common neurodegenerative disorder among the ageing population where it affects approximately 1% of 65 years olds increasing to 5% in patients over the age of 85 (Lang and Lozano, 1998a,b). Hallmark symptoms of PD include slowness in movement, tremor, rigidity and postural instability (Lang and Lozano, 1998a,b). Motor symptoms of PD are associated with the loss of DA producing neurons in the SNc (Dauer and Przedborski, 2003), when more than 70% of the DA neurons in the SNc are lost. Whilst initially levodopa (L-DOPA) therapy alleviates the symptoms of PD, its therapeutic benefit is marred by the development of debilitating side effects known as dyskinesias (Huot et al., 2013).

Whilst the cause of PD is unclear, the male sex is a strong risk factor for PD. Men have on average a 2-fold higher incidence of developing PD and 1.3–3.7-fold higher prevalence of PD compared to women, at all ages and for all nationalities studied (Baldereschi et al., 2000; Haaxma et al., 2007; Van Den Eeden et al., 2003; Wooten et al., 2004). Men also have an earlier age of onset and a faster rate of disease progression in PD than women in a Caucasian study (Haaxma et al., 2007) but the reverse was demonstrated in a Japanese study (Sato et al., 2006). Evidence from animal models of PD reproduce the sex differences observed in humans, as administration of equal doses of DA toxins (e.g. 6-hydroxydopamine) produce greater motor deficits and nigrostriatal DA loss in male rodents and primates than their female counterpart (Leranth et al., 2000; Murray et al., 2003). Studies from human and animal models of PD indicate that the male bias in PD may be explained by sex differences in nigral gene expression. Microarray analysis of single SNc DA 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 (Cantuti-Castelvetri et al., 2007). Another study utilizing normal and PD post-mortem SNc tissues reported a significant down-regulation of genes associated with oxidative phosphorylation, and synaptic transmission in the male population, compared to females (Simunovic et al., 2010). Thus, DA cells in the SNc 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 DA machinery genes between the sexes. For instance, striatal D2R density and binding potential (combined measure of receptor density and affinity) decline twice as fast with age in males compared to females (Pohjalainen et al., 1998), which is likely to reflect sex differences in symptom severity and response to medication in PD. A positron emission tomography (PET) study using the DA tracer fluorodopa revealed that presynaptic DA synthesis declines more rapidly with age in males (Laakso et al., 2002), whilst a single photon emission computed tomography (SPECT) imaging showed that male PD patients have 16% fewer DA transmitters bound onto the striatum than females (Haaxma et al., 2007). The greater bioavailability of DA in females could explain

the lower dosage of levodopa required by females (Kompoliti et al., 2002; Martinelli et al., 2003; Nyholm et al., 2010; Shulman, 2007). Together, evidence from animal models and clinical studies suggests that sex differences in PD pathogenesis and DA machinery genes are likely to contribute to sex differences in prevalence, severity of symptoms and medication response in PD.

2.3. Attention-deficit hyperactivity disorder

Attention-deficit hyperactivity disorder (ADHD) is the most commonly diagnosed psychiatric and behavioural disorder in children, affecting approximately 5% of children worldwide, particularly boys (Polanczyk et al., 2007). ADHD is characterised by symptoms of inattention-disorganization and/or hyperactivity–impulsivity (Biederman, 2005). Symptoms of ADHD have been primarily associated with the hypofunction of catecholamines DA and noradrenaline (NA) in the frontal–subcortical circuit (i.e. prefrontal cortex and striatum), which are involved in attention, reward and motor activity (Arnsten and Pliszka, 2011; Biederman, 2005; Pliszka, 2005; Sharma and Couture, 2014). Thus, the most effective drugs used to treat ADHD, such as methylphenidate (RITALIN®) and d-amphetamine, are stimulants which increase levels of DA and NA to enhance catecholamine signalling in the brain (Biederman et al., 2006; Solanto et al., 2010).

ADHD occurs with a sex ratio of three males to every female (Balint et al., 2009; Biederman et al., 2004; Gaub and Carlson, 1997; Graetz et al., 2005; Rucklidge, 2008). Symptomatically, males with ADHD appear more hyperactive whereas female ADHD patients are more inattentive (Davies, 2014; Lahey et al., 1994). These differences are also reflected in animal models of ADHD, such as the spontaneously hypertensive rat (SHR) model (Berger and Sagvolden, 1998). Genome-wide association studies (GWAS) in ADHD patients have yet to successfully establish any common polymorphisms (besides copy number variants) that might be a risk factor for ADHD and/or male ADHD (Davies, 2014; Stergiakouli et al., 2012; Williams et al., 2012; Yang et al., 2013). However, GWAS and family-based association studies have reported an association between ADHD and catecholamine machinery genes such as *DBH*, *DRD2*, *DDC*, *COMT* and *DRD4* (Lasky-Su et al., 2007, 2008a,b). Association studies in ADHD patients of different ethnicity indicate that catecholamine machinery gene variants *DRD4*, *DAT1*, *MAO-A*, *COMT* and *DBH* (Fig. 1B) are sexually dimorphic and associated with increased risk of developing ADHD in males (Biederman et al., 2008; Das Bhowmik et al., 2013; Das et al., 2006; Qian et al., 2003, 2004). Further GWAS in ADHD populations are necessary to confirm whether polymorphisms within catecholamine machinery genes could be risk factors in males developing ADHD.

2.4. Autism spectrum disorders

Autism spectrum disorders (ASD) are paediatric neurodevelopmental conditions that affect nearly 1 in 100 children (Schaafsma and Pfaff, 2014). Symptoms manifest prior to the age of three and like ADHD, boys are diagnosed with ASD more frequently than girls (Geschwind, 2008; Kim et al., 2011; Lai et al., 2014; Saemundsen et al., 2013). Individuals with ASD have impaired core domains of language and communication, abnormal repetitive and restrictive behaviours and impaired social relationships (Geschwind, 2008; Kelleher, 2008; Lai et al., 2014). Whilst the pathophysiology of ASD remains unclear, studies demonstrate an imbalance in catecholamines, such as serotonin and DA. Several studies have reported that platelet serotonin levels are elevated in ASD patients (Gabriele et al., 2014; Schain and Freedman, 1961), whilst others have reported lower serotonin levels in the frontal cortex and thalamus and lower serotonin synthesis in boys with ASD (Chugani et al., 1999, 1997). These serotonergic abnormalities in the brain may underlie impaired language production and sensory integration symptoms in ASD patients (Chugani et al., 1997). ASD is a hyperdopaminergic condition likely due to the atypical neural network between the amygdala and prefrontal cortex, which could underlie the social deficits in ASD children (Bachevalier and Loveland, 2006; Baron et al., 2000). Although anti-dopaminergic drugs (e.g. risperidone, aripiprazole) and stimulants (e.g. methylphenidate) are currently used to treat ASD, these drugs are associated with adverse side effects such as weight gain, sedation, insomnia, and irritability (Lai et al., 2014).

ASD is a neurological disorder with strong male bias. There are four males to every female diagnosed with ASD (Fombonne, 2003) and this ratio is further increased to eleven to every one female in severe autism (Gillberg et al., 2006). Similar to ADHD, functional polymorphisms and promoter alleles of *MAO-A* have also been associated with ASD severity and risk in males (Fig. 1B) (Cohen et al., 2011, 2003; Tassone et al., 2011). Family-based association studies in families with at least two affected males have shown that over-transmission of a *DRD1* haplotype occurs from mother-to-son and genotype–phenotype assessments revealed significant associations with *DRD1* polymorphisms (Hettinger et al., 2008). However, GWAS from ASD patients have mostly failed to identify candidate causative genes (Chaste et al., 2015; Woodbury-Smith et al., 2015), thereby warranting further studies to identify novel targets underlying the male bias in ASD.

2.5. Schizophrenia

Schizophrenia is a neuropsychiatric disorder that affects 1% of the population (Goldstein et al., 2013; Seeman, 2000), mostly adults between 25 and 50 years old (Whiteford et al., 2013). Schizophrenia is characterised by a mixture of debilitating positive symptoms (hallucinations and delusions) and negative symptoms (depression, cognitive impairment, social withdrawal) (Harrison, 1999). The positive symptoms are likely to be associated with upregulation of presynaptic dopaminergic activity, leading to excessive DA release (Laruelle, 1996; Miyake et al., 2011) in the prefrontal cortex originating from the VTA (Joyce and Meador-Woodruff, 1997; Laruelle, 1996). Therefore, DA antagonists such as haloperidol are typically used to alleviate the hallucinations and delusions in schizophrenic patients (Kapur et al., 2005; Seeman and Seeman, 2014). Glutamate, a major excitatory neurotransmitter has also been associated with the pathophysiology of schizophrenia. The psychotic symptoms exhibited by schizophrenic patients have been attributed to hypofunction of the glutamate N-methyl-D-aspartate (NMDA) receptor, which reduces gamma-amino–butyric-acid (GABA) ergic interneuron-mediated inhibition of DA neurons, leading to excessive firing of DA neurons in the mesolimbic pathway (Coyle, 2006; Schwartz et al., 2012). In support, NMDA receptor antagonists, ketamine and phencyclidine cause psychotic and cognitive abnormalities that mimic schizophrenic symptoms (Ross et al., 2006).

Although on average schizophrenia has a weak male bias (7 males:5 females), this ratio is increased in younger males (<20 years old) where two males to every female are affected (Castle et al., 1993; McGrath et al., 2004, 2008; van der Werf et al., 2014). Considering that dopaminergic dysfunction in the prefrontal cortex is involved in the pathophysiology of schizophrenia, DA machinery genes such as *COMT* have been implicated in a convergent functional genomics approach (integration of GWAS data with other gene expression studies), copy number variant analysis and systematic meta-analysis (Allen et al., 2008; Ayalew et al., 2012; Saus et al., 2010). The interaction between the functional Val158Met polymorphism, a hypomorphic variant (reduced DA degradation) on the *COMT* gene, and the *DRD1* gene was found to be associated with schizophrenia solely in males (Fig. 1B) (Hoenicka et al., 2010a,b). In support, *COMT* homozygous null or heterozygous mice

display sexual dimorphism in DA levels, as well as increased aggression in males (Fig. 1B) (Gogos et al., 1998). Thus, sex dimorphism of catecholamine machinery genes might underlie sex differences observed in the psychopathology between males and females with schizophrenia.

Whilst these neurological disorders may vary in their prevalence, age of onset, symptom presentation and pathophysiology, they are all associated with a dysregulated DA system and are more common in males. Although the biology underlying this male bias is not entirely clear, contributions by protective factors in females (e.g. sex hormones) and/or susceptibility factors in males (e.g. sex chromosome genes) are likely to underlie these sex differences. In the next section, we will discuss the evidence for the influence of sex hormones or sex-chromosome genes in the healthy and diseased DA pathway.

3. Sex hormones and sex chromosome genes

3.1. Influence of sex hormones

A wide range of clinical and animal studies has provided compelling evidence that oestrogen regulates the normal and diseased female DA pathway. In females, oestrogen positively regulates components of the DA pathway (Pasqualini et al., 1995), modulating 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). Physiological levels of oestradiol act directly on DA terminals to stimulate DA levels by enhancing TH activity (Pasqualini et al., 1995) and increasing basal striatal DA release (McDermott et al., 1994). In addition to its role in regulation of the healthy DA pathway, oestrogen exerts neuroprotective actions in females. In female patients with PD, symptoms worsen with the onset of menopause or withdrawal of hormone replacement therapy – i.e. when endogenous oestrogen levels are low (Quinn and Marsden, 1986). Conversely, oestrogen treatment in post-menopausal female PD patients improves motor symptoms (Shulman and Bhat, 2006). Studies in animal models of PD recapitulate these clinical findings as oestrogen treatment attenuated motor and nigrostriatal DA deficits in rodent and primate models of PD (Gillies et al., 2004; Leranth et al., 2000; Murray et al., 2003). In contrast, the effect of exogenous or circulating oestrogen on adult male DA neurons is minimal, if not harmful (Dluzen, 2005; McArthur et al., 2007; Murray et al., 2003). Similar to PD, the incidence of schizophrenia in post-menopausal females is higher compared to pre-menopausal females (Riecher Rossler, 1994). Increased level of oestrogen during the menstrual cycle is associated with an improvement of schizophrenic symptoms and therapeutic response to treatments (Gattaz et al., 1994; Hallonquist et al., 1993). In support, a clinical study of female schizophrenic patients demonstrated that adjunctive oestrogen treatment with antipsychotic drugs led to significant improvements in acute and severe psychotic symptoms when compared to antipsychotic drugs given alone (Kulkarni et al., 2001).

Prenatal testosterone plays a crucial role in masculinising the developing male brain (Morris et al., 2004), and thus abnormal regulation of prenatal testosterone levels may increase susceptibility to neurodevelopmental disorders. Testosterone activates the androgen receptor to masculinise the male brain during the perinatal period to induce male-typical behaviours such as aggression and sexual behaviour in male adult rodents (Matsumoto et al., 2003; Sato et al., 2004). Studies in healthy and clinical populations suggest that the level of exposure to prenatal testosterone influences susceptibility to neurodevelopmental disorders, especially ADHD and ASD (Baron-Cohen et al., 2011; Hines, 2008). Right hand digit ratios (length of the index finger to the length of the ring finger) are a proxy measure of foetal testosterone exposure, where smaller ratios are associated with high levels of prenatal testosterone exposure (Manning et al., 1998). Despite this measure of testosterone being somewhat controversial (Berenbaum et al., 2009), multiple studies reported that males diagnosed with ADHD or ASD have lower finger-length ratios, indicating an abnormally higher level of prenatal testosterone exposure (de Bruin et al., 2006; Martel et al., 2008). Similarly, a significant positive relationship was found between foetal testosterone levels and number of autistic traits in children by comparing foetal testosterone levels in the mother's amniotic fluid with their respective children's cognitive assessment results (Auyeung et al., 2009).

In summary, oestrogen plays a key role in regulating the normal functioning of the healthy DA system and exerts neuroprotective effects in females, which may underlie the reduced incidence of neurological disorders in females. However, the role of oestrogen in males is less clear as oestrogen treatment appears to have little effect in males. On the other hand, exposure to prenatal testosterone appears to be critical for the masculinisation of the male brain, however, exposure to abnormal levels of testosterone could be detrimental and may underlie male susceptibility to neurodevelopmental disorders.

3.2. Influence of sex chromosome genes

In addition to the influence of sex hormones on the healthy and diseased brain, evidence indicates that genetic factors also contribute, in particular sex chromosome genes (Arnold et al., 2004; Beyer et al., 1992; Carruth et al., 2002; Dewing et al., 2003; McCarthy and Arnold, 2011; McCarthy et al., 2012; Ngun et al., 2011). Various animal studies provide evidence that sexually dimorphic gene expression in the brain occurs prior to hormonal influence (Beyer et al., 1992; Dewing et al., 2003). Embryonic brain tissue cultured prior to expression of gonadal hormones developed more TH-positive neurons in XX female cultures than XY male cultures, suggesting a role for sex chromosome genes in regulating DA cell numbers (Beyer et al., 1992, 1991). Similarly, a microarray analysis of male and female mouse brains at E10.5 days *post coitum* – i.e. prior to gonadal hormonal influences – revealed sex-specific differential expression of seven genes involved in neural development and behaviour (*Xist*, *Eif2s3x*, *EST AW121876*, *Dby*, *Eif2s3y*, *Cyp7b* and *Rorα4*) (Dewing et al., 2003).

The "four core genotype" mouse model – consisting of XY male (XYM), XY female (XYF), XX male (XXM) and XX female (XXF) – has proven to be a useful tool in partitioning the effect of sex chromosome genes from the actions of gonadal sex hormones (Arnold, 2009; De Vries et al., 2002). A number of studies have utilised the model to reveal significant physiological and behavioural differences between the XY mice (i.e. XYM and XYF) and the XX mice (i.e. XXM and XXF) – such as DA cell numbers (Carruth et al., 2002), nociception (Gioiosa et al., 2008), habit formation (Quinn et al., 2007), social behaviour (McPhie-Lalmansingh et al., 2008) and susceptibility to autoimmune disease (Smith-Bouvier et al., 2008) – which were independent of the gonadal phenotype. However the "four core genotype" model still has its limitations, as it does not account for the influence of prenatal hormones, fluctuations in hormonal levels and/or the influence of adult circulating hormones secreted from the gonads or other tissues (Ngun et al., 2011). Despite these potential limitations, these findings suggest that the "four core genotype" model provides an avenue to define the effects of sex chromosome genes on various physiological processes.

To better understand the effects of the X- and Y-chromosome in isolation, the following section will highlight three distinct genetic mechanisms that could underlie sex-specific gene expression, (i) X-linked dosage effects, (ii) X-linked imprinting and (iii) the presence of the Y-chromosome.

3.2.1. X-linked dosage effects

To equalise the gene products of sex chromosomes between males and females, most genes on one of the female X-chromosomes are silenced by a dosage compensation process known as X-inactivation (Lyon, 1961). However, 15% of X-linked genes consistently escape X-inactivation (Carrel and Willard, 2005), and therefore may be expressed higher in females than males. For instance, X-inactivation gene escapees, *Utx* (involved in histone modification) and *Usp9x* (associated with neuronal differentiation and synaptic plasticity) have higher expression in XX mice brains compared to XY regardless of their gonadal phenotype (Xu et al., 2008, 2005). Although the consequence of X-inactivation gene escapee expression has yet to be fully understood, it could potentially mask any gain or loss of function in females (Davies and Wilkinson, 2006). For instance, deletions and frameshift mutations of *NLGN4X*, an X-inactivation gene escapee that encodes a cell adhesion molecule associated with the formation of functional synapses, were identified in ASD boys (Jamain et al., 2003; Laumonnier et al., 2004; Schaafsma and Pfaff, 2014). The extra dose of *NLGN4X* in females might therefore have a protective function (Schaafsma and Pfaff, 2014).

Turner syndrome, a female developmental disorder where subjects possess a 45, XO karyotype (i.e. only one X-chromosome), has been associated with increased vulnerability to disorders associated with impaired memory, attention and social interaction such as ADHD (Russell et al., 2006), ASD (Skuse, 2000) and potentially schizophrenia (Prior et al., 2000) indicating that under dosage of the X-chromosome could be a risk factor. A neuroimaging study showed that both 45, XO females and 46, XY males have larger amygdala (involved in emotion and social learning) volumes than 46, XX females. Similarly, the X-monosomy animal model, 39,XO mice (female mice with only one X-chromosome), have provided additional insight into the role that X-chromosome genes play on neurodevelopment. Behavioural studies revealed that 39,XO mice exhibit attention deficits compared to 40,XX mice. 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), indicating a role for an X-inactivation gene escapee in attention processes (Davies et al., 2007). Together, these studies indicate that the additional X-chromosome may have a protective effect in females from male-biased disorders such as ADHD and ASD.

Conversely, human brain imaging studies have shown that 47, XXY and 47, XXX individuals (i.e. additional X-chromosome) have smaller brain volumes and display global intellectual impairment with lower IQ in comparison to 46, XX and 46, XY controls (Warwick et al., 1999). Individuals with 47, XXY and 47, XXX also show increased risk of ADHD and ASD (Lynn and Davies, 2007; Tartaglia et al., 2012), suggesting that over dosage of X chromosomes can also affect brain development. Population studies have shown that schizophrenia is more common among subjects with sex chromosome anomalies than the general population (DeLisi et al., 1994). Together, these studies indicate that both under and over-dosage of X-chromosomes could affect brain development and cognition and potentially increase the risk of developing ASD or ADHD.

3.2.2. X-linked imprinting effects

Imprinted genes are inherited in duplicates like other genes, however they are epigenetically marked and solely (or predominantly) expressed from one of the parental inherited alleles (DeLisi et al., 1994). This monoallelic expression is parent-of-origin dependent, as some genes are preferentially paternally inherited, whereas others are maternally inherited (Davies, 2010). Imprinted genes are highly expressed in the brain and postulated to affect neurodevelopment and ongoing brain function (Davies et al., 2005b). Considering only females inherit the paternal X chromosome, any protective function of paternally expressed X-chromosome genes would not be expressed in males. Thus, expression of paternal X-linked genes (i.e. maternal X-linked imprinting), which has a protective role will reduce the susceptibility of neurological disorders in females whilst males will lack the protective effect.

Skuse et al. (1997) reported that females with Turner's syndrome who inherited the maternal X chromosome (45, X^{m}O) had poorer social cognitive skills, with inferior verbal and higher-order executive function skills compared to their counterparts that inherited the paternal X chromosome (45, X^{p}O). The authors speculated that a genetic locus for social cognition is imprinted on the paternal X-chromosome, which could explain the higher incidence of ASD in 45, X^{m}O females, as well as in males, since they lack the paternal X-chromosome (Skuse et al., 1997). A novel maternally expressed imprinted gene candidate (i.e. paternal X-linked gene silenced), *Xlr3b* was identified in 39, XO mouse models as a mediator of inflexible reversal learning. *Xlr3b* was predominantly expressed amongst its paralogs with a maternal bias throughout the brain with an approximately two-fold expression in male (XY^{SRY-}) brains compared to female (XX) brains, independent of gonadal hormones. The lower expression in females could be explained by X-inactivation, however this has yet to be proven (Davies et al., 2005a). These findings indicate that X-linked imprinted genes expressed in a parent-of-origin dependent manner could influence sexually dimorphic neurobiology phenotypes and underlie male susceptibility to neurological disorders.

3.2.3. Y-chromosome effects

The Y-chromosome passed on from father to son and therefore only present in males, could be contributing to sex differences in the male brain and encode risk/protective factors specifically in males. Xu and colleagues (2002) demonstrated that six Y-chromosome genes, *Ddx3y*, *Ube1y*, *Kdm5d*, *Eif2s3y*, *Uty* and *Usp9y*, were expressed in the XY male mouse brain at one or more ages – developmental (13.5 days *post coitum*), day of birth (P1) and adult ages. These six genes were also expressed in the brain of XY female mice that lack *SRY* and testes, indicating that expression of these genes was independent of hormonal influences (Xu et al., 2002). Furthermore, Y-chromosome genes, *Dby* and *Eif2s3y* expressed in developing mouse brains at 10.5 days *post coitum* before any influence of gonadal hormones could have significant effects in male neurodevelopment (Dewing et al., 2003). These findings provide evidence that regulation of Y-chromosome gene expression in the brain was independent of hormonal influence and may participate in the sexually dimorphic brain development and function. Several cases of 47, XYY and 48, XXYY boys were associated with ADHD (Ross et al., 2009; Ruud et al., 2005; Tartaglia et al., 2012) suggesting that over dosage of Y-chromosomes may increase the risk for neurological disorders. Whilst it is unclear which Y-chromosome gene(s) is involved in the physiology and pathophysiology of the male brain, emerging studies indicate that the testis-determining gene, *SRY*, is an ideal candidate to investigate (Czech et al., 2012; Dewing et al., 2006).

4. The Y chromosome gene *SRY* hardwires the male brain

SRY (sex-determining region on the Y chromosome) is a key transcription factor that switches on male-sex determination by directing embryonic bipotential gonads to develop into testes rather than ovaries (Koopman et al., 1990; Sinclair et al., 1990). *SRY* is passed from father to son via the Y chromosome and is not present in females. If the *SRY* gene is absent or mutated, the testes do not form, and the female phenotype develops

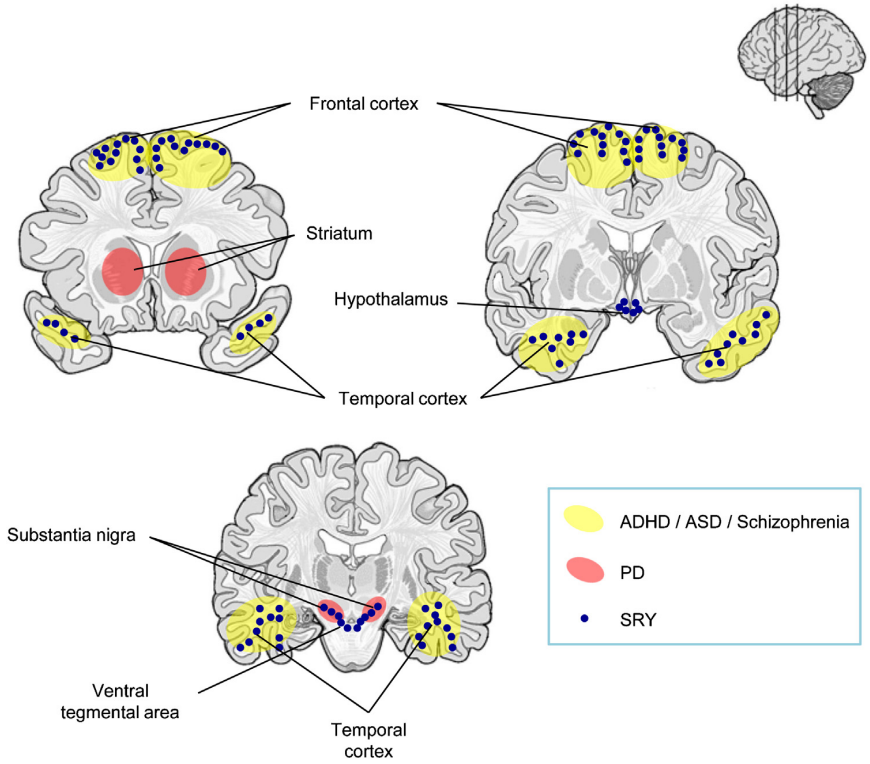

Fig. 2. *SRY* is expressed in brain regions associated with male-biased neurological disorders. Line drawings of coronal sections of the human brain, depicting brain regions expressing SRY (blue dot), regions associated with the neuropsychiatric disorders such as ADHD, ASD and schizophrenia (yellow highlight), and regions associated with neurodegenerative disorders, such as PD (red highlight).

(Harley et al., 1992). Human *SRY* is an intronless gene that encodes a transcription factor with 204 amino acids (Su and Lau, 1993), which binds to the minor groove of DNA and intercalates, causing the DNA to bend (Ferrari, 1992; Harley et al., 1994; King and Weiss, 1993). SRY up-regulates the *Sox9* gene by binding to the testis-specific enhancer of *Sox9* core (TESCO), enabling Sertoli cell differentiation, thereby facilitating testis formation (Sekido et al., 2004; Sekido and Lovell-Badge, 2008). *SRY* expression in the testis is regulated by the growth arrest and DNA damage gamma (GADD45γ) protein, a stress-response protein that mediates a variety of cellular processes specifically expressed in gonadal somatic cells (Gierl et al., 2012; Warr et al., 2012). The signalling cascade involves GADD45γ and MAP3K4 transducing the p38 MAPK pathway that activates GATA4 which in turn binds to the *SRY* promoter to initiate the male sex determining pathway (Gierl et al., 2012). This pathway is also a feature of *SRY* regulation in the injured DA cells, which will be discussed in section 4.4.

Besides being expressed in the testis, *SRY* is expressed in non-gonadal tissues such as the adrenal glands, kidneys, lungs heart and brain (Clepet et al., 1993; Dewing et al., 2006), suggesting that *SRY* might have functions other than testis determination. In the mouse brain, *SRY* is developmentally regulated, as the non-translatable (i.e. non-functional) circular *SRY* transcript expression is restricted to the prenatal brain, whilst the active linear *SRY* transcripts are detected in the postnatal brain (Mayer et al., 2000). This is in contrast to the gonads where *SRY* expression begins at E10.5 days and ceases at E12.5 days in the mouse (Koopman et al., 1990), suggesting that there is a clear temporal segregation in the functionality of gonadal and brain SRY.

4.1. *SRY co-localizes with midbrain dopamine neurons in males*

Initial studies in rodent and human brains revealed *SRY* expression in several brain regions, such as the hypothalamus, frontal and temporal cortex (Lahr et al., 1995; Mayer et al., 1998) (Fig. 2). Subsequent quantitative PCR and in situ hybridization studies demonstrated that *SRY* mRNA is expressed in brain regions abundant in catecholaminergic cell bodies or nerve terminals such as the SNc, VTA, locus coeruleus and hypothalamus in rodents (Dewing et al., 2006; Milsted et al., 2004) (Fig. 2). In rat and human immunofluorescence studies of the SNc, SRY protein co-localized within a subpopulation of TH-positive neurons of male, but not female, brain sections (Czech et al., 2012; Dewing et al., 2006). SRY protein also co-localized with TH-positive neurons in the VTA, which is adjacent to the SNc. VTA is crucial for mediating reward and addictive behaviours (Wise, 2009) and the expression of SRY in the VTA may explain sex differences originating in this region (Gillies et al., 2014). Together these studies demonstrate that SRY is expressed in various regions of the male brain, in particular DA and catecholamine-abundant regions, which may reflect a conserved regulation of DA-dependent function in males, such as the control of movement, attention, and reward.

4.2. *SRY regulates multiple components of the dopamine biosynthesis pathway in males*

In line with the presence of SRY in DA-abundant brain regions, various studies have demonstrated a role for SRY in regulating the DA pathway in vitro and in vivo in males (Czech et al., 2012;

Dewing et al., 2006; Milsted et al., 2004; Wu et al., 2009). In a male rat neuronal cell line, Milsted and colleagues (2004) showed that SRY regulates transcription of the DA synthesis enzyme, TH via AP-1 binding sites on the *TH* promoter (Fig. 1C) (Milsted et al., 2004). Similarly, *SRY* regulates the common functional variants of the proximal promoter of *TH* in humans (Zhang et al., 2010). The gene encoding MAO-A, an enzyme that inactivates DA or NA via removal of an amine group, was identified as another neural target of *SRY* (Wu et al., 2009). Promoter analysis identified that *SRY* binds directly to the *MAO-A* promoter in vitro and in vivo and increases MAO-A catalytic activity (Fig. 1C) (Wu et al., 2009). Furthermore, a study in a human male neuronal cell line revealed that SRY positively regulates multiple components of the DA biosynthesis machinery, including *TH*, *MAO-A*, *DDC*, *DBH*, and *D2R*, which was associated increased extracellular DA levels (Fig. 1C) (Czech et al., 2012). Combined, these results suggest that SRY positively regulates of DA synthesis and metabolism in adult male DA neurons, allowing for a mechanism of sexual dimorphism independent of circulating gonadal hormones in males.

4.3. SRY regulates catecholamine-dependent functions in males

Direct in vivo actions of *SRY* in the brain were first demonstrated by the Vilain and Harley laboratories (Dewing et al., 2006), which assessed the anatomical and behavioural consequences of down-regulating *SRY* expression in the rat SNc. Knockdown of *SRY* in the rat SNc, via repeated antisense *SRY* oligonucleotides (ODNs) injections, resulted in a significant reduction in TH-positive neurons in male rats (Dewing et al., 2006). The reduction in nigral TH expression was associated with reduced motor performance in the akinesia and limb-use asymmetry tests, indicating that *SRY* exerts direct actions on nigral DA neurons and voluntary movement in males. *SRY* also has a functional role in peripheral tissues in males, as transfection of *SRY* into the adrenal medulla or kidneys of male rats increased TH content and plasma NA levels, resulting in an increase in blood pressure (Ely et al., 2007, 2009). Together, these studies demonstrate that SRY exerts direct actions in the adult male brain and peripheral tissues to regulate catecholamine-dependent functions, such as voluntary movement and blood pressure, and potentially fight-or-flight response to stress (Lee and Harley, 2012).

4.4. SRY: a male susceptibility gene for neurological disorders?

In summary, the Y-chromosome gene, *SRY*, is expressed in the adult male brain, where it directly regulates DA biosynthesis, and DA-dependent functions, such as voluntary movement. In view of these findings, abnormal regulation and/or functioning of SRY in the male brain may contribute to sex-bias in neurological disorders, such as PD and ADHD.

PD, which results from the progressive loss of nigral DA neurons, is more common in males, who also have an earlier age of onset and faster rate of disease progression compared to females (Haaxma et al., 2007; Wooten et al., 2004). Considering the expression of SRY in the human male SNc (Czech et al., 2012) (Fig. 2), dysregulation of SRY in male DA neurons may underlie the male-bias in PD. Indeed, recent work from group demonstrated that SRY expression is dysregulated in a toxin-induced model of PD in vitro (Czech et al., 2014). Treatment with the dopaminergic toxin, 6-OHDA, significantly elevated SRY expression in human male DA cells, via a DNA-damage induced activation of the *GADD45*γ signalling pathway (Czech et al., 2014). These initial findings suggest a role for SRY in the cell death process of male DA neurons, and warrant further studies in clinical and animal models of PD to better understand the regulation and function of SRY in male PD.

Although little is known about the role of SRY in male-biased neuropsychatric disorders, SRY is expressed in the frontal and temporal cortex, locus coeruleus, and VTA (Czech et al., 2012; Lahr et al., 1995; Mayer et al., 1998; Milsted et al., 2004), brain regions closely associated with pathophysiology of ADHD, ASD and schizophrenia (Fig. 2). Given that MAO-A dysfunctions can cause numerous neuropsychiatric disorders such as ADHD, ASD, depression, and schizophrenia (Bortolato and Shih, 2011; Shih et al., 1999), abnormal regulation of SRY during development in boys, and consequently MAO-A, may contribute to the hyper- or hypo-function of DA in these disorders. For instance, dysregulation of *SRY* expression in the VTA may contribute to the hyperactive DA release in the mesolimbic pathway in schizophrenia. Alternatively, a regulatory mutation in human *SRY* gene or a SRY binding site mutation in its target gene may also increase the risk of males in developing these brain disorders. Indeed, SRY polymorphism was shown to be responsible for the hypertensive component of male SHRs (Ely et al., 2010; Turner et al., 2009), which have higher blood pressure compare to the female SHRs. Since SHRs are also a rat model of ADHD (Russell, 2002; Sagvolden et al., 2005), it would be of interest to determine whether manipulating *SRY* expression can alter ADHD-like symptoms in the SHRs.

SRY is unlikely to be the only male-specific factor underlying male-biased neurological disorders as other Y-linked genes, such as neuroligin 4, has been associated with increased risk of ASD (Ross et al., 2015). However, these findings highlight the need to better understand the molecular regulation, function, and targets of SRY in the male brain, using both clinical and pre-clinical approaches. This information, alongside identifying novel SRY polymorphisms, will be essential for the development of novel therapeutic strategies (e.g. sex-specific therapies) for sex-biased neurological disorders.

5. Conclusion

Compelling evidence from both clinical and pre-clinical studies demonstrate robust differences between the male and female brain, in both healthy and pathological conditions. In particular, structure and functioning of the dopaminergic systems are intrinsically different between males and females, which are likely to underlie the sex-bias in DA-associated disorders such as PD, ADHD, and ASD. These sex differences may be driven by combined actions of gonadal sex hormones and sex chromosome effects, in particular the Y-chromosome gene, *SRY*, in males. In addition to the well established role of SRY as the male sex-determining gene, emerging evidence indicates that SRY is expressed in a sub-population of adult midbrain dopaminergic neurons, regulates the DA biosynthesis and its associated functions such as movement in males. Given the localization and function of SRY in male DA neurons, abnormal functioning and/or regulation of SRY could increase the risk of males to DA-associated disorders. Although SRY is unlikely to be the only sex-specific factor underlying brain sex differences, a better understanding of the regulation and function of brain SRY will likely to improve strategies for the prevention and treatment of debilitating sex-biased neurological disorders.

Acknowledgements

We acknowledge the Victorian Government's Operational Infra-structure Support Program, Australian NHMRC grants 334314(VRH) and 1029401 (JL) and CASS foundation grant SM/08/2053 (JL). We thank Mrs Janelle Browne and Miss Rajini Sreenivasan for their critical appraisal of the manuscript. There are no conflicts of interest to report.

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CHAPTER 2:

Regulation and function of *SRY* in the healthy male brain

2.1 INTRODUCTION

The influence of sex on the brain is becoming more apparent and is wide-ranging, influencing not only reproductive functions, but also anatomy, biochemistry, and physiology, as well as a range of behavioural and emotional processes. Whilst male brains are on average 8-13% larger than females (Ruigrok et al., 2014), the grey to white matter ratio is higher across structures in the female cerebrum (Allen et al., 2003). Furthermore, sex differences in volume are present in several brain structures, particularly; the hippocampus and caudate nucleus are larger in females (Giedd et al., 1997, Filipek et al., 1994, Murphy et al., 1996) whilst the amygdala and parietal cortex are larger in males (Giedd et al., 1997, Koscik et al., 2009). These brain sex differences are thought to underlie the enhanced motor and spatial skills in males (Linn and Petersen, 1985, Moreno-Briseno et al., 2010), and the superior memory and social cognition skills in females (Saykin et al., 1995, Williams et al., 2009). In addition, brain structure connectivity vastly differs between the sexes whereby structural connectome in the males are predominantly intra-hemispheric whereas the females are mostly inter-hemispheric (Ingalhalikar et al., 2014).

A widespread of clinical and pre-clinical studies has revealed sex differences in neurotransmitter systems, notably in the DA and GABA systems. Clinical studies have revealed that striatal DA levels are similar between males and females, however, females have greater DA availability and synthesis capacity (Mozley et al., 2001, Laakso et al., 2002), whereas males have higher DA release in the striatum (Munro et al., 2006). Furthermore, females have a greater density of striatal DA reuptake transporters (DAT) capacity compared to males (Lavalaye et al., 2000, Mozley et al., 2001). Whilst sex differences in the number

of DA neurons in humans remains elusive, a pre-clinical study has provided an indication, whereby male rats have more DA neurons compared to females in the substantia nigra pars compacta (SNc) (Dewing et al., 2006). Similar to human studies, female rats possess a higher number (Morissette and Di Paolo, 1993, Rivest et al., 1995) and functional activity (Bhatt and Dluzen, 2005, Walker et al., 2000) of striatal DAT relative to male rats. On the other hand, affinity of striatal D2-class receptors was higher in males compared to females (Pohjalainen et al., 1998), whilst D2 receptor density declined at a slower rate compared to males. Similarly, male rats have a higher density and production of striatal D2-class receptors relative to females (Andersen and Teicher, 2000). In parallel, GABA levels are significantly higher in the prefrontal cortex of males compared to females (O'Gorman et al., 2011). In contrast, GABA content in the frontal cortex and substantia nigra pars reticulata was higher in female compared to male rats (Davis et al., 1999, Ravizza et al., 2003). Furthermore, the number of parvalbumin neurons (a subset of GABAergic interneurons) are higher in the female striatum whilst lower in the amygdala compared to male rats (Ravenelle et al., 2014).

These pronounced sex differences in the brain have a significant impact on the sex bias in various neurological disorders. For instance, females are more likely to develop depression, anxiety (Weissman et al., 1996, Nolen-Hoeksema, 1987) and Alzheimer's disease (Hebert et al., 2013). On the other hand, males are more likely to be diagnosed with Parkinson's disease (PD) (Wooten et al., 2004), attention deficit hyperactivity disorder (ADHD) (Balint et al., 2009), and autism spectrum disorders (ASD) (Gillberg et al., 2006). Whilst these male-biased disorders significantly differ in their symptoms, pathology, age of onset and treatment, these male-biased disorders are associated with abnormal functioning

or deficits in both the DA and GABA neurotransmitter systems. Hence, better understanding of the biology underlying the sexual dimorphism in these neurotransmitter systems is essential for designing novel therapeutic agents that will have optimal effectiveness in male-biased neurological disorders such as PD and ADHD.

The biological basis underlying sex differences in DA and GABA neurotransmission systems has predominantly been attributed to the prevailing levels of sex hormone oestrogen. In ovariectomized female rodents, physiological levels of oestradiol treatment act directly on DA terminals to stimulate DA levels by enhancing TH activity (Pasqualini et al., 1995) and increasing basal striatal DA release (McDermott et al., 1994), as well as increase DA turnover (Di Paolo et al., 1985). In contrast, oestrogen had no effect on striatal DA concentrations and release in males, suggesting that oestrogen specifically regulates the female DA pathway (McDermott et al., 1994). Furthermore, oestradiol treatment increases the number of D2-class receptors and quantitatively modulates the ratio between D1 and D2-class receptors in the female rat striatum (Di Paolo et al., 1981, Demotes-Mainard et al., 1990). In parallel, ovariectomy in female rats elevated GABA content in the cerebral hemispheres (Saad, 1970), whilst oestradiol treatment suppressed GABA neurotransmission in the hippocampus (Murphy et al., 1998). Likewise, oestradiol decreased GAD activity in the substantia nigra (SN) and ventral tegmental region when implanted into the caudate nucleus and nucleus accumbens, respectively (McGinnis et al., 1980). Moreover, oestradiol treatment in ovariectomized female rats induced GABA receptor binding in the striatum, frontal cortex and cerebellum (Perez et al., 1988).

In addition to the influence of sex hormones on the healthy brain, evidence indicates that genetic factors also contribute, in particular sex chromosome genes. (Arnold, 2004, Carruth et al., 2002, Dewing et al., 2003, Beyer et al., 1992, Ngun et al., 2011, McCarthy and Arnold, 2011, McCarthy et al., 2012). Various animal studies provide evidence that sexually dimorphic gene expression in the brain occurs prior to hormonal influence (Dewing et al., 2003, Beyer et al., 1992). Embryonic brain tissue cultured prior to expression of gonadal hormones developed more TH-positive neurons in XX female cultures than XY male cultures, suggesting a role for sex chromosome genes in regulating DA cell numbers (Beyer et al., 1992, Beyer et al., 1991). Using the "four core genotype" mouse model, a useful tool in partitioning the effect of sex chromosome genes from the actions of gonadal sex hormones (De Vries et al., 2002, Arnold, 2009), XX cells was shown to have higher numbers of DA cells compared to XY (Carruth et al., 2002). Similarly, XX mice had a higher expression of *Gad67* and *Gad65* (synthesis enzymes of GABA) mRNA in the frontal cortex compared to XY mice (Seney et al., 2013). These findings indicate that sex chromosome genes independent of gonadal hormones can modulate DA and GABA neurons. Whilst it is unclear which sex-specific genes are crucial for driving these sex differences, emerging studies indicate that the Y-chromosome gene, *SRY,* represents an excellent candidate (Dewing et al., 2006, Czech et al., 2012).

SRY (sex-determining region on the Y chromosome) is a key transcription factor that switches on male-sex determination by directing embryonic bipotential gonads to develop into testes rather than ovaries (Sinclair et al., 1990, Koopman et al., 1990). *SRY* expression in the testes is regulated by the growth arrest and DNA damage gamma (GADD45γ) protein, a stress-response protein that mediates

a variety of cellular processes specifically expressed in gonadal somatic cells (Gierl et al., 2012, Warr et al., 2012). The signalling cascade involves GADD45γ and MAP3K4 transducing the p38 MAPK pathway that activates GATA4 which in turn binds to the *SRY* promoter to initiate the male sex determining pathway (Gierl et al., 2012). In the mouse brain, *SRY* is developmentally regulated, as the non-translatable (i.e.. non-functional) circular *SRY* transcript expression is restricted to the prenatal brain, whilst the active linear *SRY* transcripts are detected in the postnatal brain (Mayer et al., 2000). This is in contrast to the gonads where *SRY* expression begins at E10.5 days and ceases at E12.5 days in the mouse (Koopman et al., 1990), suggesting that there is a clear temporal segregation in the functionality of gonadal and brain SRY.

Brain mapping studies in rodents demonstrated that *Sry* mRNA or protein is expressed in DA-abundant brain regions such as the SNc, ventral tegmental area (VTA), locus coeruleus and hypothalamus (Dewing et al., 2006, Milsted et al., 2004). Similarly, human post-mortem brain sections demonstrated that *SRY* mRNA is expressed in the hypothalamus, as well as the frontal and temporal cortex (Mayer et al., 1998). In the human and rodent male SNc, SRY protein co-localised with TH-positive cells in both the cytoplasm and nucleus (Czech et al., 2012, Dewing et al., 2006), indicating a functional role for SRY in male DA neurons. In addition to DA neurons, SRY co-localised with GAD-positive neurons (marker for GABAergic neurons) in both the human and rodent male substantia nigra pars reticulata (SNr) (Czech et al., 2012, Dewing et al., 2006), indicating a role for SRY in non-dopaminergic systems in the male brain. However, further work is required in mapping SRY distribution in other brain regions to better understand the genetic basis of brain sex differences.

Consistent with the expression of SRY in male DA neurons, various studies have demonstrated that *SRY* controls transcription of *TH, dopa decarboxylase (DDC), dopamine β-hydroxylase (DBH), MAO-A, and D2R* (Czech et al., 2012, Wu et al., 2009) and consequently DA levels (Czech et al., 2012). Moreover, lowering nigral *Sry* expression in male rats reduces nigral TH expression and consequently voluntary movement (Dewing et al., 2006), supporting a role for SRY in the control of midbrain DA biosynthesis and consequently voluntary movement in males. However, much work is still needed in understanding the molecular mechanisms underlying the regulation and function of SRY in healthy and injured male DA neurons. Given the need to better understand regulation, function, and distribution of SRY in the male brain, the specific aims of Chapter 2 are:

1) To determine the molecular mechanism of SRY regulation in a human male neuronal cell line.
2) To assess the effect of reducing nigral SRY levels on target gene expression in healthy male rats.
3) To assess the distribution of SRY protein in the human male brain.

2.2 MATERIALS AND METHODS

Assessment of SRY expression in a human male neuronal cell line

Cell culture

Human neuroblastoma BE(2)-M17 (M17) (CRL-2267; ATCC, Manassas, VA, USA) cells, a human male dopaminergic cell line was used to carry out the experiments. The M17 cell line was selected because (i) it is a male cell line that expresses endogenous SRY and (ii) these cells contain the cellular machinery typical of dopamine neurons, including tyrosine hydroxylase, monoamine oxidase and dopamine reuptake transporter.

Subculturing cells

M17 cells were cultured using Dulbecco's modified Eagle's medium with Ham's F12 nutrient in a 50:50 ratio supplemented with Glutamax, 10% Foetal Bovine Serum, 1% penicillin/streptomycin and maintained at 5% CO_2 and 37°C in a Galaxy 170S incubator (New Brunswick, CT). M17 cells were subcultured when a confluence of greater than 70% was reached twice per week. Cells were washed with 1% PBS and aspirated, released with 0.01% Versene (Gibco), and resuspended in a 1:4 dilution in fresh medium. The cell line was maintained in sterile 75 cm^2 cell culture flasks.

Dopamine treatment in human M17 cells

To determine the time-course of dopaminergic regulation of *SRY* mRNA and protein expression *in vitro*, M17 cells were treated with DA (Sigma Aldrich, solubilized in sterile 0.1% ascorbic acid saline) at varying concentrations (0 to 1000μM) and incubated for 0 to 12 hours. M17 cells were seeded 24 hours prior to treatment. When cells were at a confluence level greater than 80%, cells were

treated with 0, 1, 10, 100, or 1000μM DA. RNA was extracted from the cells at 1, 3, and 6 hours post-treatment whilst cells were fixed at 3, 6 and 12 hours post-treatment. To determine the receptor subtype involved in regulation of SRY protein *in vitro*, the M17 cells were cultured and treated with either D1R selective agonist, SKF38393 (1 and 10μM) or the D2R selective agonist, bromocriptine mesylate (2 and 20μM) and incubated for 6 hours and fixed for immunocytochemistry.

Haemocytometry

In brief, M17 cell suspension were mixed at a 1:1 ratio with trypan blue and counted using a 0.0025mm^2 haemocytometer and light microscope (Olympus CK30). The cells were then plated with 100,000 or 500,000 cells (depending on experiment) in six-well plates, corresponding to the different time points (ie. 1, 3, 6 and 12 hours post treatment)

Quantitative RT-PCR (in vitro)

Total RNA (250ng) isolated using RNeasy Mini RNA Isolation Kit (Ambion) was reverse-transcribed into cDNA (Go Script, Promega) and equal amount of cDNA template was added to SensiMix™ SYBR Hi-ROX Mastermix (Bioline) using primers listed in Supplementary Table 1. The relative level of mRNA was interpolated from a standard curve prepared by serially diluting the cDNA reaction. Specificity of PCR product formation was confirmed by monitoring melting peaks. All quantitative PCR reactions were conducted in triplicates. Final values represent fold change of gene expression relative to the housekeeping gene β2-Microglobulin (β2M).

Immunocytochemistry

M17 cells were seeded on glass cover slips in 35 mm tissue culture wells 24 hours prior to treatment. Cells were treated with DA or DA receptor agonist drugs as mentioned above. Treated cells were washed with PBS and fixed with formalin for 7 min. After washing, cells were blocked with CAS block (Thermo Fisher) for 1 h at 37^{o}C. Cells were then incubated with primary antibody against human SRY (1:100, rabbit polyclonal; AVIVA Systems Biology) overnight at 4^{o}C, washed and incubated with fluorescent secondary antibodies (1:1000, Alexa Fluor 594 anti-rabbit; Thermo Fisher) in block for 1 h at 37^{o}C. After washing, coverslips containing the stained cells were mounted on slides with fluorescent nucleic acid stain, Hoechst 33342 (Thermo Fisher). Cells were imaged using a 60x oil immersion objective on a confocal microscope (Olympus FV1200) and quantitative scoring of cells positive for SRY protein immunofluorescence was made using ImageJ software. The intensity was calculated from the average values of the nuclear, cytoplasmic or whole cell fluorescence in 8 different fields of at least 3 independent experiments.

Assessment of nigral *Sry* target genes in male rats

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). 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 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 Sry antisense oligonucleotide (ASO) infusions in healthy male rats

To determine the effect of reducing SRY levels on target gene expression, *SRY antisense* (*ASO*) or *sense* oligonucleotides (SO) was infused daily via an implanted unilateral guide cannula into the right SNc (2µg) for 10 days in male rats. Nigral Sry expression in male rats was reduced by repeated intranigral infusions of SRY ASO (Supplementary Table 2). The Sry ASO used was a cocktail of three distinct ASOs directed against rat *Sry* mRNA added in equal proportions, as described previously (Dewing et al., 2006). ASOs were HPLC-purified (Invitrogen, Australia) and dissolved in artificial cerebrospinal fluid (aCSF) vehicle to a final concentration of 2µg/µL. 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. Following the last antisense or *sense SRY* ODN injection, the rats were culled and brains were fresh isolated and frozen in isopentane at -20^{o}C.

Limb-use asymmetry test

The limb-use asymmetry test assessed spontaneous forelimb usage during vertical explorations in rats, where motor impairment is indicated by a reduction in

limb-use contralateral (i.e. left) to the site of drug injection. 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.

Histology

Rat brains were isolated fresh and processed for qRT-PCR. Coronal sections of 10μm thickness were cut serially through the SNc and stored at -80°C. Between each series, a 200μm slab was collected in order to isolate tissue for RNA processing.

Quantitative RT-PCR (in vivo)

Total RNA (100-300ng) was isolated from the left and right rat nigra using TRI-Reagent (Sigma Aldrich) according to manufacturer's protocol was reverse-transcribed into cDNA (Go Script, Promega) and equal amount of cDNA template was added to SensiMix™ SYBR Hi-ROX Mastermix (Bioline) using primers listed in Supplementary Table 1. The relative level of mRNA was interpolated from a standard curve prepared by serially diluting the cDNA reaction. Specificity of PCR product formation was confirmed by monitoring melting peaks. All quantitative PCR reactions were conducted in triplicates. Final values represent fold change of gene expression relative to the housekeeping genes TATA Box Binding Protein (TBP).

Assessment of SRY expression in the human male brain

Human post-mortem sections

Adult male and female post-mortem brain sections were obtained with informed consent from the Victoria Brain Bank Network (Table 2.1) (under Monash Health Human Research Ethics Committee, Ethics 5073C). Four paraffin-embedded coronal sections (10μm) per brain region from each case were received. Each region was anatomically delineated by the Victorian Brain Bank neuropathologist.

Case	***Sex***	***Age***	***Diagnosis***	***Comments***
1	*M*	*79.6*	*Control*	*NA*
2	*M*	*81*	*Control*	*NA*
3	*M*	*73.6*	*Control*	*NA*
4	*M*	*61.1*	*Control*	*Ischemic heart disease*
5	*F*	*67.3*	*Control*	*Pulmonary thromboembolism*

Table 2.1 Information of post-mortem subjects used for immunohistochemistry.

SRY immunohistochemistry

Paraffin-embedded tissue sections were dewaxed with xylene, ethanol and water, and antigens were retrieved by boiling the tissue in a bath containing 0.01M citric acid buffer (sodium citrate in water, pH 6.0) for 3 hours in a oven at 90°C. Tissues were then cooled at room temperature for 30 minutes before washing in phosphate-buffered saline (PBS) and blocked for 1 hour at room temperature (CAS-block, Thermo Fisher) and incubated with primary antibody against human SRY (1:100, rabbit polyclonal; AVIVA Systems Biology) in block overnight at 4°C. The brain sections were then incubated with Envision DAKO EnVision™+ System, HRP (Agilent) and reacted with cobalt and nickel-intensified diaminobenzidine

(DAB, Sigma-Aldrich). DAB-immunostained sections were counterstained with neutral red or cresyl violet. DAB-immunostained sections were analysed by bright-field microscopy, using an Olympus microscope equipped with Olympus cellSens dimension v1.7.1 image analysis software.

Statistical Analysis

All values are expressed as the mean ± S.E.M. All data was analysed using tools within Graphpad Prism 5. Comparisons of two experimental groups were performed using two-tailed unpaired Student t-test. Multiple comparisons of more than two experimental groups were performed using one-way ANOVA and Bonferroni's *post hoc* tests. 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

SRY mRNA expression is regulated by GADD45γ-independent and GADD45γ-dependent pathways in M17 cells

To determine the effect of DA treatment on SRY mRNA expression *in vitro*, human male neuroblastoma M17 cells were treated with increasing concentrations of DA (0 to 1000μM) and *SRY* mRNA expression was measured at various time points by qRT-PCR. *GADD45γ* mRNA expression was also measured, as it is a known regulator of *SRY* in the developing male gonad (Warr et al., 2012, Gierl et al., 2012) and a sensor of cellular stress (Fornace Jr et al., 1988).

Upregulation of SRY mRNA expression was initially observed at 1 hour post 10μM DA treatment (2.2-fold vs 0μM, $p<0.05$, Figure 2.1A). This initial upregulation of *SRY* was not associated with any change in *GADD45γ* mRNA expression, indicating a *GADD45γ*-independent regulation of *SRY* expression. Increase in SRY mRNA expression was also observed at 6 hours post 1000μM DA treatment (3.0-fold vs 0μM, $p<0.01$, Figure 2.1C), which was accompanied by a robust elevation of *GADD45γ* mRNA expression (5.7-fold vs 0μM, $p<0.0001$, Figure 2.1C). Moreover, pronounced cues of cell death such as clustering of dying cells and loss of neuronal extensions (data not shown) were observed at 6 hours post 1000μM DA treatment, indicating cellular injury resulting from oxidation of DA. Thus, *GADD45γ*-mediated upregulation of *SRY* is likely to reflect a response to cellular stress, as previously shown with 6-OHDA and p-quinone treatment in M17 cells (Czech et al., 2014). *GADD45γ* upregulation was also observed at 3 hours following 1000μM DA treatment (3.7-fold vs 0μM, $p<0.001$, Figure 2.1B), which is consistent with our previous work demonstrating GADD45*γ* upregulation precedes the increase in *SRY* expression in 6-OHDA treated M17 cells (Czech et al., 2014).

In summary, the current results demonstrate that qRT-PCR analysis revealed two distinct stages of *SRY* regulation following DA treatment:

i) an early, modest increase in SRY expression in response to physiological levels of DA, mediated by a GADD45γ-independent mechanism

ii) a later, robust increase in SRY expression in response to supra-physiological levels of DA, likely mediated by a GADD45γ-dependent pathway activated in response to cellular stress, as described previously (Czech et al., 2014).

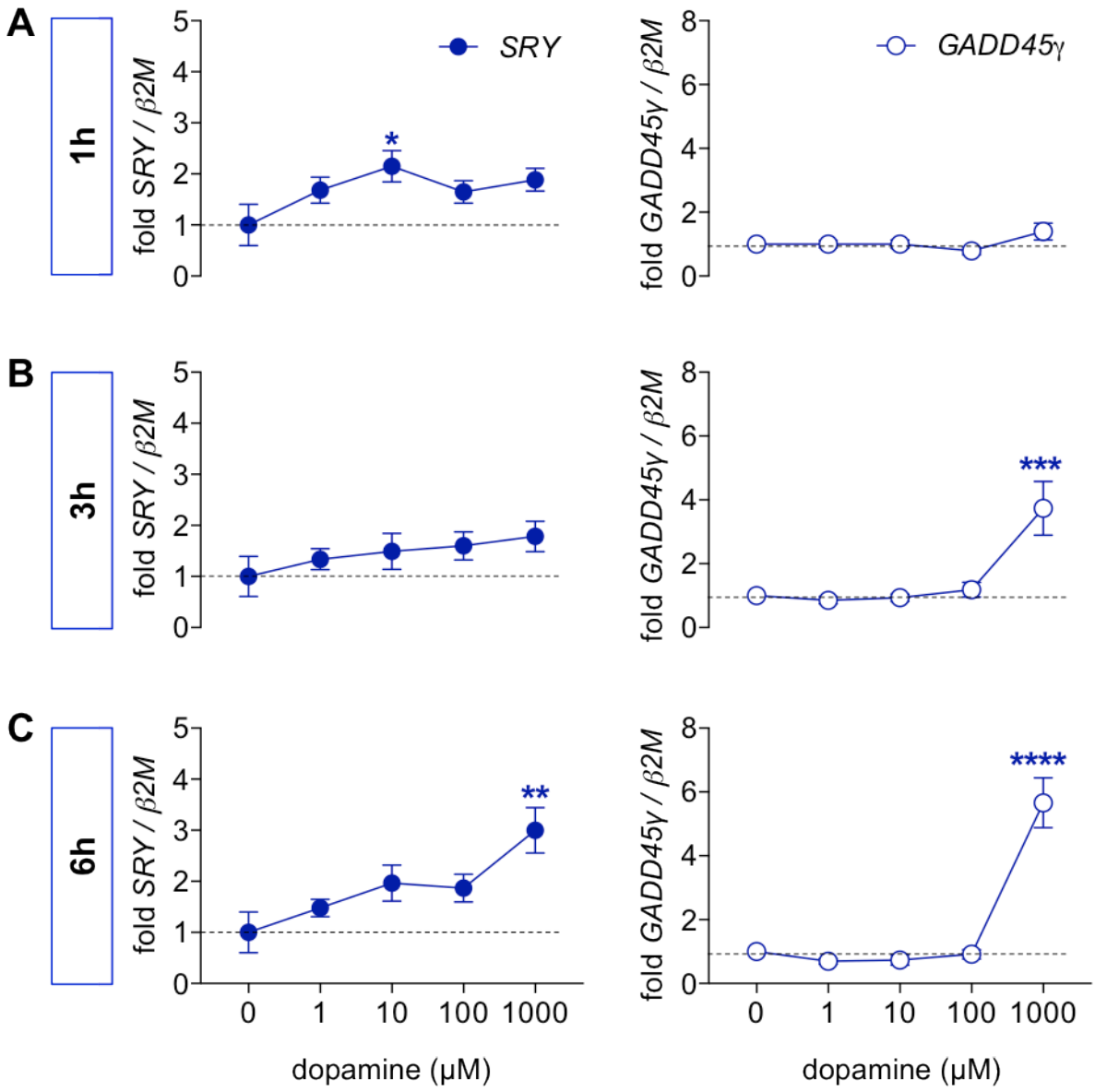


Figure 2.1 Effect of DA treatment (0 to 1000μM) on *SRY* and *GADD45γ* mRNA expression at various time points in M17 cells. *SRY* and *GADD45γ* mRNA expressions were measured at **A)** 1h, **B)** 3h and **C)** 6h following DA treatment at a range of concentrations between 0 to 1000μM. *SRY* and *GADD45γ* mRNA expressions were expressed as a fold change relative to the housekeeping gene, *β2M* (n=5/group, mean ± S.E.M. one-way ANOVA *p<0.05, ***p<0.001, ****p<0.0001, compared to 0μM).

SRY protein expression is regulated by a dopamine D2 receptor-dependent pathway in M17 cells

To determine the effect of DA treatment on SRY protein expression, SRY protein immunoreactivity was assessed at various time points following DA treatment in M17 cells. As observed in Figure 2.2B, treatment with 10μM of DA triggered a marked elevation of SRY immunostaining at 6 hours post treatment. Densitometric quantification revealed a significant increase in total SRY protein immunostaining (1.2-fold vs vehicle, $p<0.05$, Figure 2.2B) as well as nuclear immunostaining (1.2-fold vs vehicle, $p<0.05$, Figure 2.2B). However, there were no significant changes in SRY immunostaining at 3 and 12 hours post DA treatment (Figure 2.2B), indicating a transient upregulation of SRY protein expression in response to DA receptor activation.

To determine the DA receptor involved in the regulation of SRY protein expression, the effect of D1 or D2 receptor agonist treatment on human SRY protein expression was assessed by immunocytochemical analysis. Treatment with D1 receptor agonist, SKF38393 (1 or 10μM) did not affect SRY immunostaining at 6 hours post-treatment, indicating that the D1 receptor was not involved (Figure 2.3A). In contrast, treatment with D2 receptor agonist, bromocriptine mesylate (20μM) induced a significant elevation of nuclear SRY immunostaining in the M17 cells (1.3-fold vs vehicle, $p<0.05$, Figure 2.3B), suggesting that regulation of SRY protein expression in human M17 cells is dependent on the D2 receptor.

Together, these findings demonstrate that SRY expression is regulated by a D2 receptor-dependent mechanism under physiological conditions in the human M17 cell line.

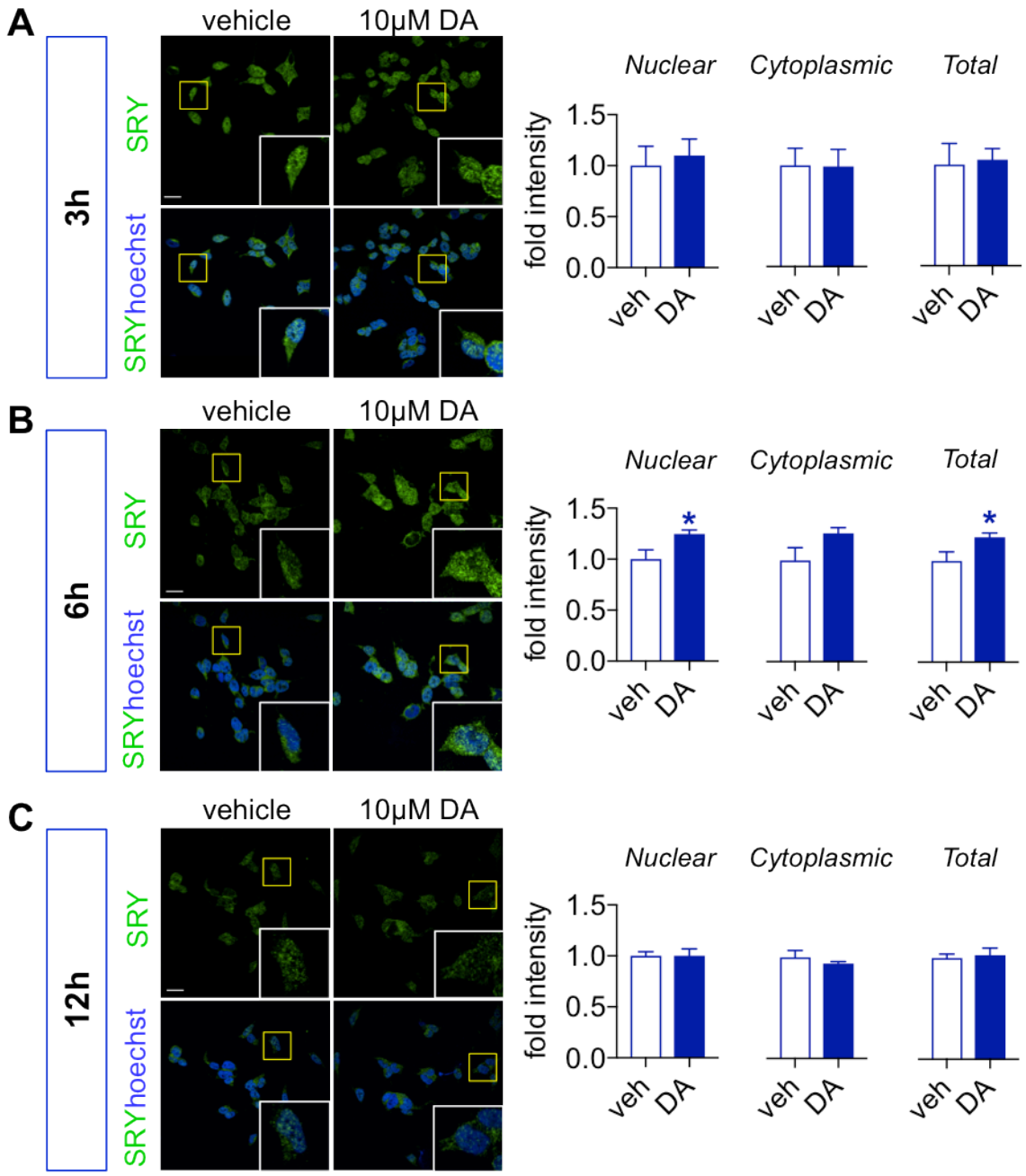


Figure 2.2 Effect of DA treatment on human SRY protein expression in M17 cells. Representative images (left panels) and corresponding quantification of SRY protein expression in the nucleus, cytoplasm and whole cell (right panels) following treatment with vehicle or 10μM DA for **A)** 3h, **B)** 6h or **C)** 12h. SRY protein levels correspond to mean of SRY immunofluorescence in 8 different fields (n=3/group, mean ± S.E.M. student t-test; *P<0.05 compared to vehicle control; scale bar = 10μm).

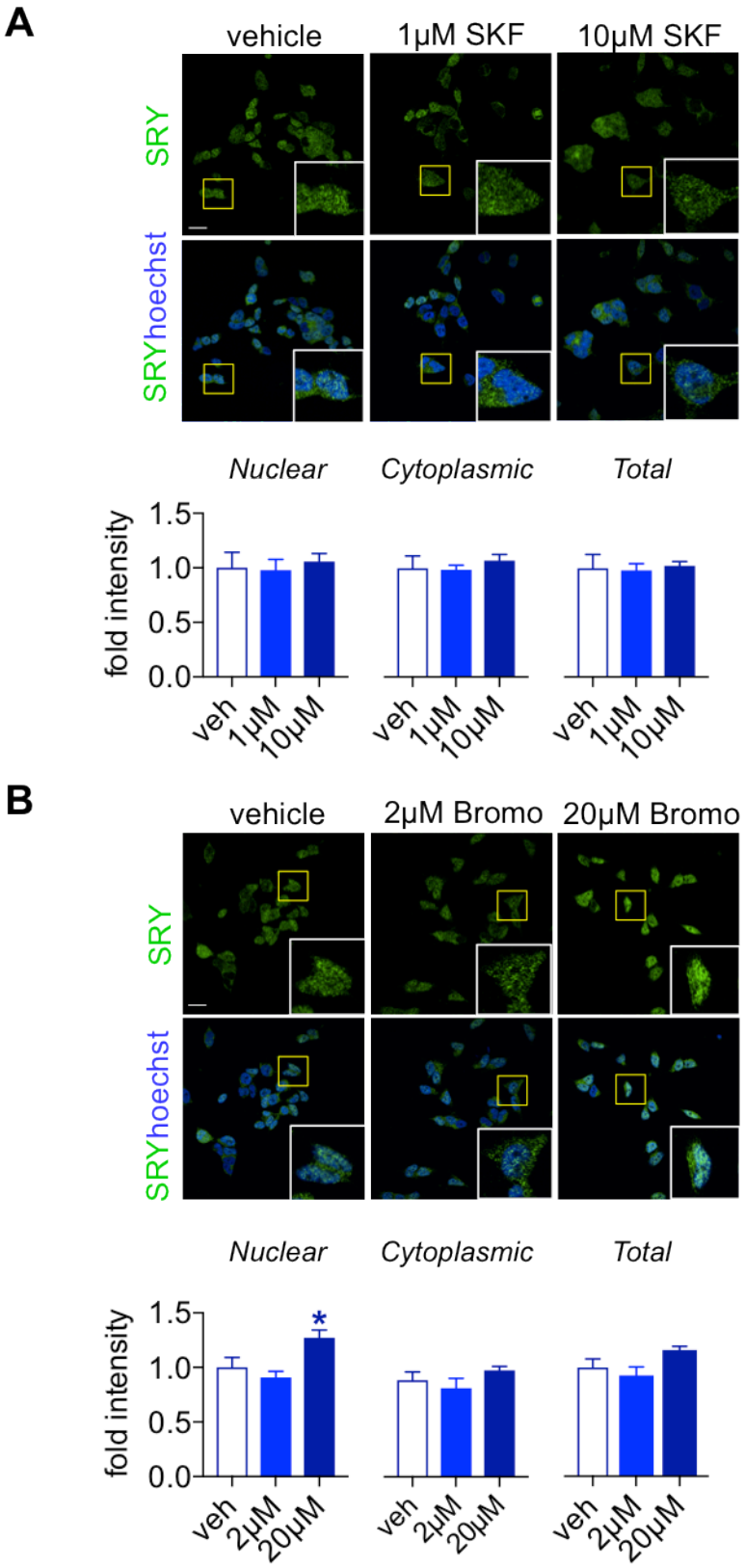

A
vehicle
1μM SKF
10μM SKF
SRY
SRYhoechst
Nuclear
Cytoplasmic
Total
fold intensity
1.5
1.0
0.5
0.0
veh
1μM
10μM
B
vehicle
2μM Bromo
20μM Bromo
SRY
SRYhoechst
Nuclear
Cytoplasmic
Total
fold intensity
1.5
1.0
0.5
0.0
*
veh
2μM
20μM

Figure 2.3 Effect of D1 or D2 receptor agonist treatment on human SRY protein expression in M17 cells. Representative images (left panels) and corresponding quantification of SRY protein expression in the nucleus, cytoplasm and whole cell (right panels) following treatment with **A)** vehicle, 1μM, or 10μM of SKF38393 (D1 receptor agonist) and **B)** vehicle, 2μM or 20μM of bromocriptine mesylate (D2 receptor agonist). SRY protein levels correspond to mean of SRY immunofluorescence in 8 different fields (n=4/group, mean ± S.E.M. one-way ANOVA; *P<0.05 compared to vehicle control; scale bar = 10μm).

Sry regulates dopamine machinery and non-dopamine machinery gene expression in male rats

Previous study by Dewing and colleagues has shown that *Sry* directly regulates DA biosynthesis and consequently motor behaviour in male rats (Dewing et al., 2006). To confirm and extend these findings, the effect of reducing nigral *Sry* expression, via *Sry* antisense oligonucleotide (ASO) infusion, on motor function and nigral gene expression was assessed in healthy male rats.

Motor function assessed prior to *Sry* ASO (or SO) infusion revealed no significant bias in left or right limb use between the two treatment groups (Table 2.2). Repeated ASO-infusion in male rats reduced motor function compared to SO-infused male rats in the limb-use test (47%, SO vs 33%, ASO, $p<0.05$ vs SO, Table 2.2). In contrast, motor function was unaffected by the ASO-infusion in female rats, indicating that the ASO-mediated effect was male specific (Table 2.2)

	Male		**Female**	
	pre-infusion % left limb use	**post-infusion** % left limb use	**pre-infusion** % left limb use	**post-infusion** % left limb use
SO	47.8 ± 1.3	47.4 ± 2.5	46.3 ± 1.0	45.6 ± 2.9
ASO	48.9 ± 1.2	33.4 ± 2.6*	48.9 ± 2.9	46.7 ± 1.9

Table 2.2 Motor function were assessed by limb use test as % of left limb use at pre- and post 10 day *Sry* ASO (or SO) infusion in male and female rats ($n \geq 8$/group, mean ± S.E.M. student t-test, *$p<0.05$, compared to sense)

qRT-PCR analysis revealed that *Sry* ASO-infusion in male rats reduced nigral *Sry* mRNA expression (0.6-fold of intact side vs SO, $p<0.01$, Figure 2.4A), but not that of *SRY* homologs *Sox3* or *Sox6.* In addition, ASO-infusion reduced nigral *tyrosine hydroxylase (Th)* (0.4-fold of intact side vs SO, $p<0.01$, Figure

2.4B)*, monoamine oxidase-a (Mao-a)* (0.6-fold of intact side vs SO, $p<0.05$, Figure 2.4B)*, dopa decarboxylase (Ddc)* (0.5-fold of intact side vs SO, $p<0.05$, Figure 2.4B) and *dopamine-β-hydroxylase (Dbh)* (0.8-fold of intact side vs SO, $p<0.05$, Figure 2.4B) mRNA expression in male rat SNc. Similarly, ASO-infusion reduced *DA reuptake transporter* (*Dat)* (0.4-fold of intact side vs SO, $p<0.05$, Figure 2.4B), as well as DA receptor *Drd1* (0.6-fold of intact side vs SO, $p<0.05$, Figure 2.4B) and DA receptor *Drd2* (0.4-fold of intact side vs SO, $p<0.05$, Figure 2.4B) mRNA expression in male rat SNc. In parallel, previous results in our lab have demonstrated that striatal DA and DOPAC levels were significantly reduced following nigral Sry ASO-infusion (Supplementary figure 1). In addition to the DA machinery genes, nigral Sry ASO-infusion significantly reduced GABA synthesis enzymes, *glutamate decarboxylase 1 (Gad1)* (0.6-fold of intact side vs SO, $p<0.05$, Figure 2.4C), and *glutamate decarboxylase 2 (Gad2)* (0.5-fold of intact side vs SO, $p<0.05$, Figure 2.4C) in male rat nigra.

Given the crucial role of SNc in pathophysiology of PD, the effect of *Sry* ASO-infusion on expression of genes involved in PD pathogenesis, such as apoptosis and oxidative stress were assessed in male rats. However, ASO-infusion in male rats had no significant effects on the expression of genes involved in apoptosis (Figure 2.5A) or oxidative stress (Figure 2.5B) when compared to the SO-infused group.

Together, these results suggest that SRY plays a role beyond the regulation of DA in the nigra, as it not only regulates DA biosynthesis genes but also non-dopaminergic targets such as GABA synthesis genes.

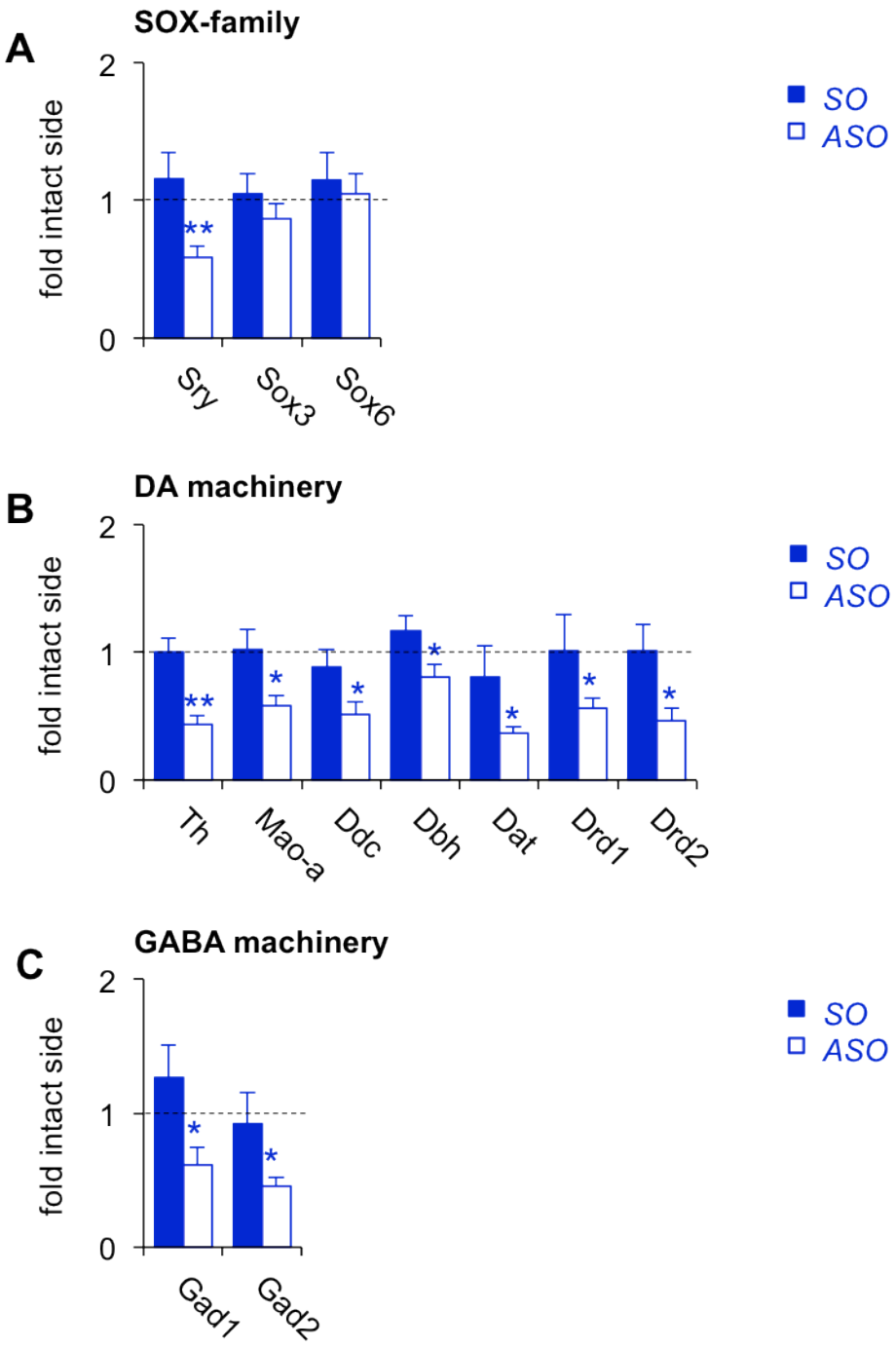


Figure 2.4 Effect of nigral *Sry* ASO infusion on DA and GABA machinery genes in male rats. Following repeated nigral *Sry* antisense or sense ODN injections (2ug/daily for 10 days) brains were processed for nigral **A)** Sry and SOX-family, **B)** DA and **C)** GABA machinery genes (n≥8/group, mean ± S.E.M. student t-test, *p<0.05, ** p<0.01, compared to sense; dashed line = baseline levels)

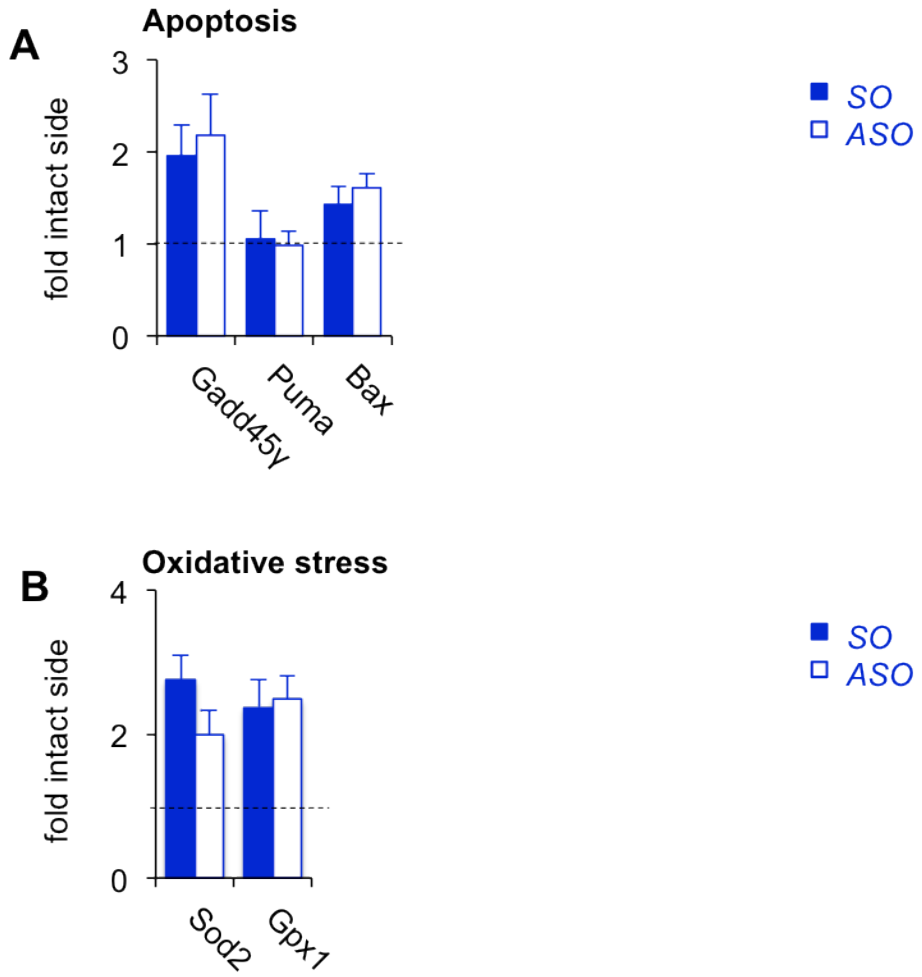


Figure 2.5 Effect of nigral *Sry* ASO infusion on apoptosis and oxidative stress genes in male rats. Following repeated nigral *SRY* antisense or sense ODN injections (2ug/daily for 10 days) brains were processed for nigral **A)** apoptosis and **B)** oxidative stress genes (n≥10/group, student t-test; dashed line = baseline levels)

SRY immunohistochemistry in the human male brain

To further our understanding of the distribution of SRY protein in the human male brain, SRY immunohistochemistry was performed on various forebrain, midbrain and hindbrain sections from human post-mortem male brains (see Table 2.1). Non-specific staining was determined by male brain sections with no primary antibody and female (which do not express SRY) brain sections.

SRY-immunoreactivity was present in the CA1 layer of the human male hippocampus and localised within the nuclei of pyramidal neurons (Figure 2.6A), which are excitatory neurons that release glutamate. In the human male SNc, SRY protein was localised within the cytoplasm of neuromelanin-rich DA neurons (Figure 2.6B), as previously observed by our group (Czech et al., 2012). In the human male cerebellum, SRY protein immunoreactivity was mainly localised in the nuclei of Purkinje neurons, a class of GABAergic neurons, in the cerebellar vermis and dentate nucleus (Figure 2.7A and B). However, SRY protein was not expressed in all Purkinje cells in the cerebellum. Importantly, SRY immunoreactivity in the hippocampus, SN and cerebellum were absent in both the male negative control and female tissues, confirming that staining in the male positive sections were specific for SRY.

SRY immunoreactivity was also observed in non-neuronal cells in the human male brain. In the frontal cortex and thalamus, SRY protein was expressed within activated microglia as indicated by the larger cell bodies as well as the fewer and thicker processes (Figure 2.8A and B). Similarly, SRY-positive immunoreactivity was absent in both the male negative control and female frontal cortex and thalamus sections.

Together, these findings show for the first time that SRY protein is expressed outside the midbrain, and found in areas such as the hippocampus and cerebellum. Furthermore, the presence of SRY protein expression in the microglia of the male frontal cortex and thalamus indicates that SRY could play a role in neuroinflammation.

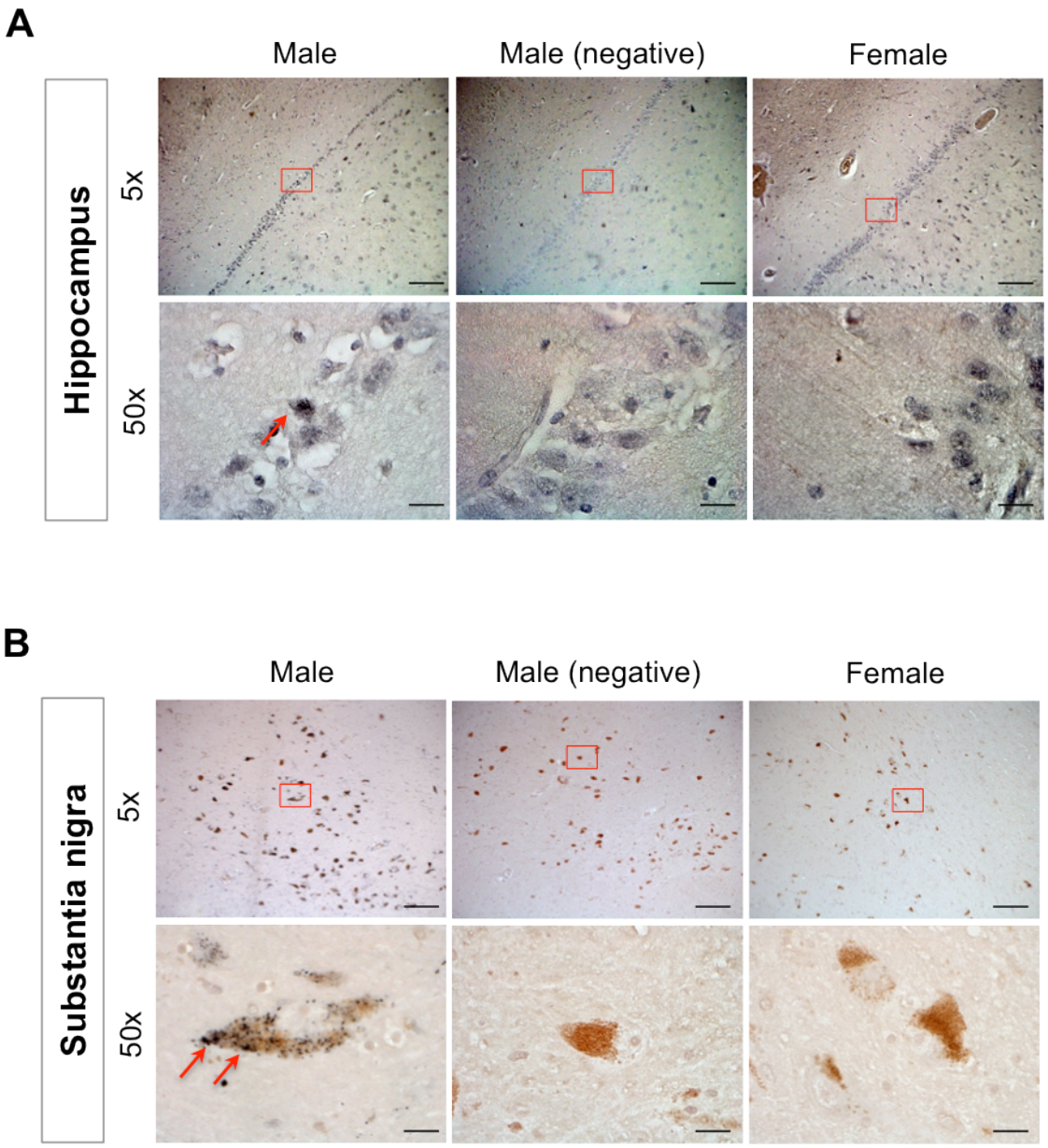


Figure 2.6 SRY expression in human male hippocampus and substantia nigra. SRY-positive expression in **A)** nigral DA neurons and **B)** hippocampal pyramidal neurons are present in human male sections and absent in negative control and female sections. Hippocampus and substantia nigra sections were counterstained with cresyl violet and neutral red, respectively. Light microscopy images were taken at 5x and 50x magnification, scale bar = 100µm and 10µm, respectively. Red boxes indicate magnified area and red arrows indicate SRY-positive neurons.

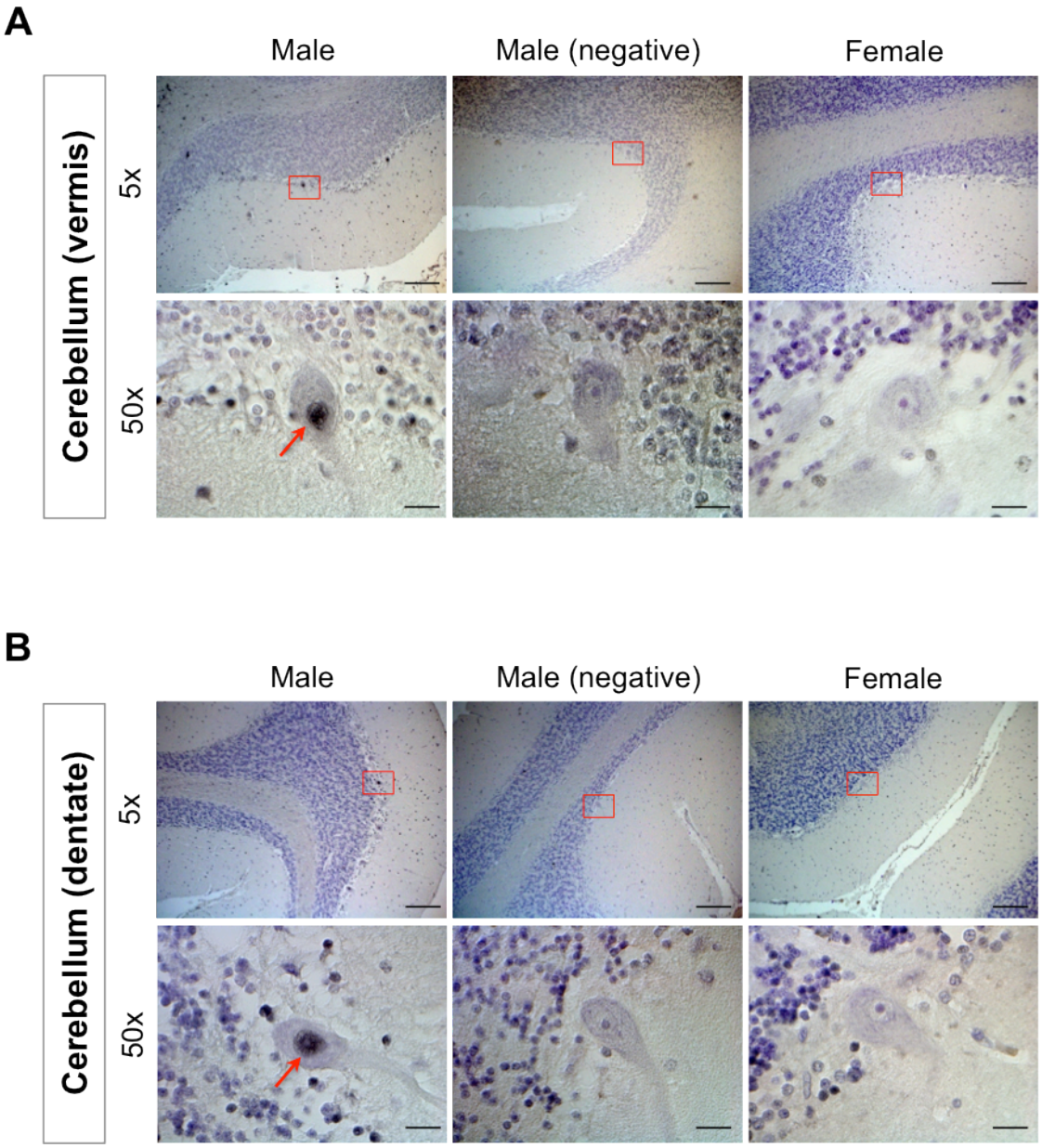


Figure 2.7 SRY expression in human male cerebellum. SRY-positive expression in cerebellar **A)** vermis and **B)** dentate nucleus Purkinje neurons are present in human male sections and absent in negative control and female sections. Sections were counterstained with cresyl violet. Light microscopy images were taken at 5x and 50x magnification, scale bar = 100μm and 10μm, respectively. Red boxes indicate magnified area and red arrows indicate SRY-positive neurons.

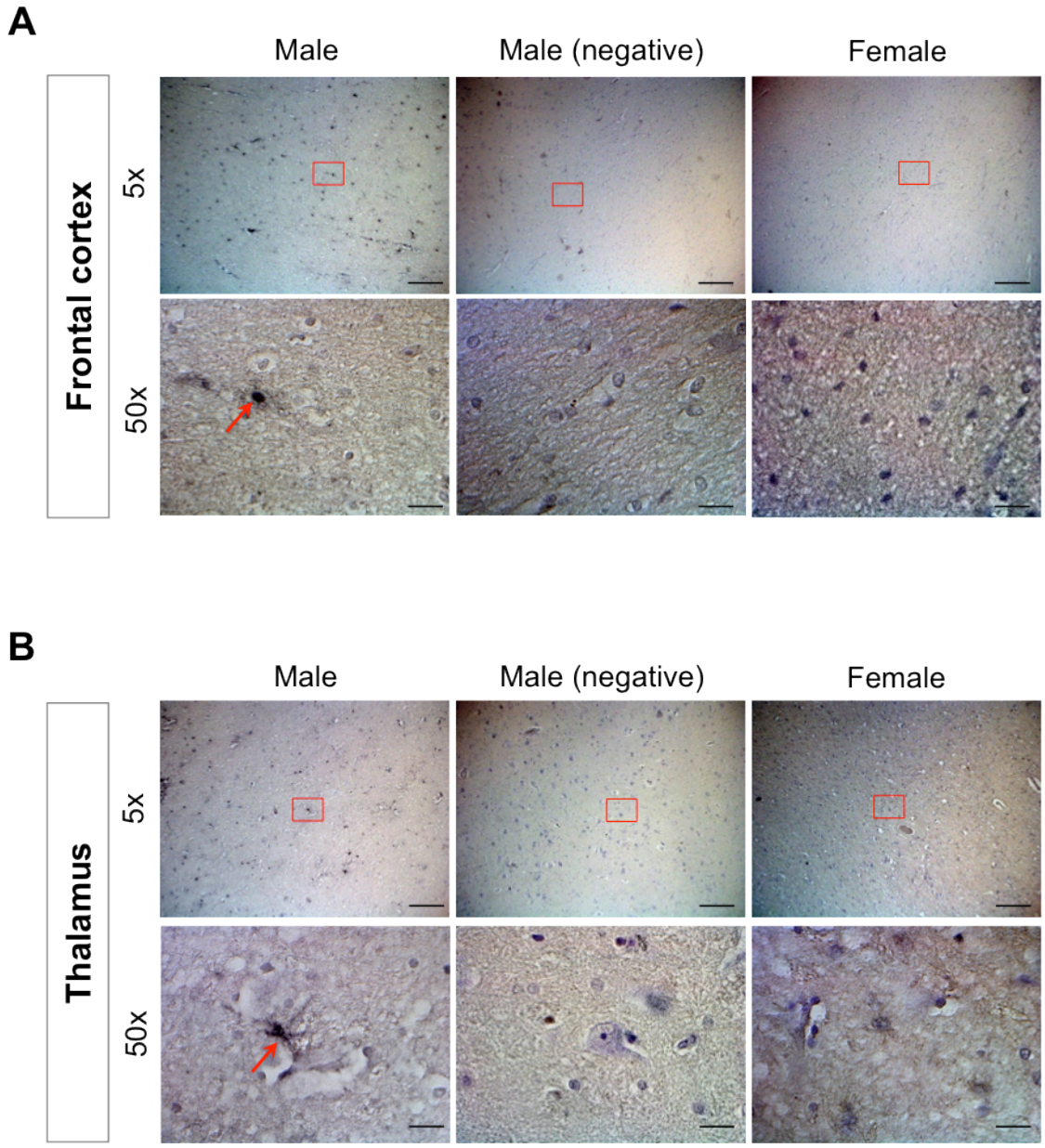


Figure 2.8 SRY expression in human male frontal cortex and thalamus. SRY-positive expression in **A)** frontal cortex and **B)** thalamus microglia are present in human male sections and absent in negative control and female sections. Sections were counterstained with cresyl violet. Light microscopy images were taken at 5x and 50x magnification, scale bar = 100μm and 10μm, respectively. Red boxes indicate magnified area and red arrows indicate SRY-positive neurons.

2.4 DISCUSSION

Our group and others have previously demonstrated that SRY is present in male DA neurons where it regulates DA biosynthesis and DA-dependent functions such as voluntary movement (Czech et al., 2012, Dewing et al., 2006, Wu et al., 2009). The current chapter has not only extended these previous findings on the regulation and function of SRY in DA neurons, but also provided a glimpse into the role of SRY in non-DA neurons. Whilst we have previously identified that the upregulation of SRY expression was mediated by a GADD45γ-dependent mechanism following DA cell injury, this chapter reveals for the first time that a D2 receptor-mediated pathway regulates SRY expression under physiological conditions in male DA cells. Moreover, reducing nigral *Sry* expression in male rats revealed that *Sry* regulates dopaminergic and non-dopaminergic target genes *in vivo*. In line with this, SRY immunohistochemistry in the human male brain revealed for the first time that SRY protein is expressed in both dopaminergic and non-dopaminergic regions such as the cerebellum and frontal cortex within neuronal and non-neuronal cells.

SRY expression is regulated by the D2 receptor under physiological conditions

Previous studies have shown that induction of Sry is dependent on the Gadd45γ pathway in developing male gonads (Gierl et al., 2012, Warr et al., 2012). Moreover, we have previously shown that SRY expression is regulated by a GADD45γ-dependent mechanism in response to treatment with 6-OHDA or its metabolite, p-quinone in the human M17 cell line (Czech et al., 2014). In line with this, expression of *SRY* and *GADD45γ* mRNA were markedly increased in response to cellular injury resulting from oxidization of supra-physiological levels of DA (1000μM) (Figure 2.1). The current results also showed an earlier upregulation

of *SRY* mRNA expression in response to lower physiological concentrations of DA (10μM), without the parallel increase in *GADD45γ* expression. Moreover, expression of the SRY protein in M17 cells was increased following treatment with DA or D2 receptor agonist, but not D1 receptor agonist (Figure 2.2 and 2.3). These results indicate that DA regulates SRY expression via the D2 receptor under physiological conditions. Notably, SRY expression was significantly increased in the nucleus following bromocriptine treatment, indicating increased translocation into the nucleus. In support, bromocriptine treatment has been shown to increase the expression and nuclear translocation of another transcription factor, nuclear factor-E2-related factor-2 (Nrf2), which is involved in regulating antioxidant enzymes, in a cell culture model (Lim et al., 2008).

The D2 receptor-mediated regulation of SRY expression may underlie the sex differences in response to drugs that modulate D2 receptor function. Rodent studies have revealed a greater decline in locomotor activity and stereotypy behaviour in males compared to females following D2 receptor agonist injections particularly, quinpirole or apormorphine (Schindler and Carmona, 2002, Savageau and Beatty, 1981, Van Hartesveldt, 1997). Furthermore, a recent study has revealed that administration of eticlopride, a D2 receptor antagonist, impaired decision-making in male but not female rats (Georgiou et al., 2018). Similarly, decreased D2 receptor availability in the nucleus accumbens is associated with increased impulsivity in male rats (Dalley et al., 2007). The sex differences in behaviour following pharmacologic disruption of the D2 receptor could not be fully explained by gonadal hormones (Savageau and Beatty, 1981), suggesting that SRY could be involved in mediating these differences.

Sry regulates dopaminergic and non-dopaminergic target genes in the male substantia nigra

To confirm and expand upon the existing knowledge of SRY targets in the substantia nigra (Czech et al., 2012, Dewing et al., 2006), the effect of reducing Sry expression on nigral gene expression was assessed in healthy male rats. The present findings demonstrate that reducing nigral Sry expression in male rats decreased the expression of DA biosynthesis genes such as *Th*, *Mao-a*, *Drd1*, *Drd2, Dat, Ddc* and *Dbh* (Figure 2.4), which is consistent with the *in vitro* findings (Wu et al., 2009, Milsted et al., 2004, Czech et al., 2012). Given the potential regulation of Sry expression by the D2 receptor pathway, and reciprocal regulation of the DA pathway by SRY, it is tempting to speculate that SRY and the DA system homeostatically regulate DA and SRY in the male brain. Moreover, disruption to the SRY-DA feedback may contribute to the sex differences in neuropsychiatric disorders such as ADHD and schizophrenia.

In addition to the role of *Sry* in modulating DA biosynthesis genes, the current study shows that Sry regulates *Gad1* and *Gad2* mRNA expression *in vivo* (Figure 2.4). Given the difficulty in segregating the rat SNc from the substantia nigra pars reticulata (SNr), and the abundance of GAD-positive neurons in the SNr, it is likely that the *Gad1* and *Gad2 mRNA* expressions were predominantly SNr in origin. In support, SRY immunoreactivity co-localises with GAD-positive neurons in both the human and rodent male SNr (Czech et al., 2012, Dewing et al., 2006). Considering the presence of Sry in both the SNc and SNr, where it regulates DA and GABA biosynthesis genes, Sry is likely to mediate input and output activity of the basal ganglia to potentially fine tune basal ganglia functions, such as voluntary movement and goal-directed actions in males.

SRY is expressed in dopaminergic and non-dopaminergic regions in the human male brain

To better understand the role of SRY in the male brain, SRY protein expression was assessed in human post-mortem brain sections. Consistent with our previous study (Czech et al., 2012), SRY immunoreactivity was observed in the human male SNc, where it co-localised with neuromelanin-rich DA neurons (Figure 2.7B). SRY protein expression was also observed for the first time outside the midbrain, particularly in the frontal cortex, hippocampus, cerebellum and thalamus, suggesting that SRY has a role beyond the midbrain.

In the male hippocampus, SRY protein expression was expressed in excitatory pyramidal neurons, which serve to transform synaptic inputs into patterned output of action potentials (Figure 2.7A). The presence of Sry in the hippocampus is in keeping with the well-established sex differences in the size of the hippocampus (Giedd et al., 1997, Filipek et al., 1994), dendritic spines, densities of pyramidal neurons (Shors et al., 2001, Gould et al., 1990), and spatial learning (Monfort et al., 2015). Although the sexual dimorphism in the hippocampus has been largely explained by the effects of oestrogen in females (Galea et al., 2013, Duarte-Guterman et al., 2015), the presence of SRY in the hippocampus may also contribute to the sex differences. Given that the hippocampus is implicated in a variety of neuropsychiatric disorders such as autism, early-onset schizophrenia and ADHD, the dysregulation and/or abnormal functioning of SRY in the hippocampus may also contribute to the sex differences in these disorders. SRY immunoreactivity was also present within the cerebellar Purkinje neurons, which are a class of GABAergic neurons - providing additional support for a role for SRY in GABAergic neurons. Thus, preliminary findings of the

current chapter reveal that SRY is found in non-dopaminergic neurons outside the midbrain, highlighting the potential of SRY in mediating other sexually dimorphic brain functions such as spatial memory and impulsivity (Voyer et al., 1995, Lage et al., 2013).

SRY immunoreactivity was also detected in non-neuronal cells in the human male brain, as SRY protein was expressed in the microglia in the human male frontal cortex and thalamus. The presence of SRY in the microglia was surprising as these were control human samples. The neurological basis for the SRY-positive staining in the microglia of the frontal cortex and thalamus remains elusive. However, the presence of SRY in the microglia could be due to random effects of aging. These findings will need to be confirmed by co-immunofluorescence studies using microglia markers such as Iba1. Sex differences have been well established in microglia morphology and densities (Lenz and McCarthy, 2015), which was thought to arise solely from the influence of oestrogen (Vegeto et al., 2001, Loram et al., 2012, Villa et al., 2016). However, the presence of SRY in microglia suggests a role for SRY in neuroinflammation, potentially as a male-specific transcription factor to regulate inflammatory gene networks that are also sex biased (Villa et al., 2016). Along these lines, male susceptibility to neurodevelopmental disorders such as autism spectrum disorders (ASD) and early-onset schizophrenia and neurodegenerative disorders such as PD have been proposed to be associated with sex differences in the microglia and inflammatory molecules (Lenz et al., 2013, Villa et al., 2016).

Together, these findings support the notion that SRY has a role in non-DA neurons, particularly GABAergic neurons, and for the first time indicates that SRY might have a role in non-neuronal cells, specifically in the microglia. Considering

that the cerebellum, hippocampus, frontal cortex and/or thalamus are involved in several male biased neurological disorders, particularly ADHD, ASD and PD, the presence of SRY expression in these regions could explain the male bias in these disorders.

Future studies

Given that SRY is a transcription factor, it could be playing a similar role as other nigral transcription factors such as Nurr1, Pitx3 and Sox6 in adult male DA neurons. Whilst *Nurr1* is involved in maintaining gene expression in developing DA cells, *Pitx3* is required for the development of dopaminergic neurons specifically in the SN and its absence results in an abnormal nigrostriatal circuitry (Nunes et al., 2003). Similarly, *Sry box 6* (*Sox6*), a member of the Sry-related transcription family, is important for the development and maintenance of midbrain DA neurons. Hence, generating mice using the Cre/lox system with Sry floxed and a cre specifically driven by genes in DA neurons such as *TH*, would provide insight into whether Sry is important for the development and survival of DA neurons. Alternatively, Sry floxed and a cre driven by GAD could shed some light on the role of Sry in GABAegic neurons.

Whilst the current chapter has revealed novel aspects of SRY in the male brain, further work is needed to better understand the interactions between the hormonal (i.e. oestrogen and testosterone) and genetic (i.e. SRY) influences. For instance, manipulating sex hormone levels via gonadectomy and/or injecting sex hormone blockers in the Sry knockout mice (Kato et al., 2013) or Sry ASO infused male rats could provide insight into the role of Sry in the absence of oestrogen influences.

This chapter has also provided some early evidence that SRY is expressed in brain regions that has not been previously shown such as the hippocampus, thalamus and cerebellum. Region or cell-specific knockdown of Sry expression in the hippocampus or cerebellum either by pharmacological or genetic approaches, has the potential to reveal previously unrecognized functions of SRY in the male brain.

Conclusion

This chapter provides a better understanding on the role of SRY in healthy male DA neurons as well as insight into other roles of SRY in non-DA neurons. The findings presented revealed that DA regulates SRY expression via a D2 receptor-dependent pathway in healthy DA neurons. On the other hand, *SRY* expression is mediated by a GADD45γ–dependent pathway in injured DA neurons, and likely to be a compensatory response to DNA damage and cell injury. Furthermore, nigral *Sry* is not only involved in regulating DA machinery genes but also GABA machinery genes. Preliminary human mapping studies have revealed that SRY is found in brain regions such as the hippocampus and cerebellum, indicating a wider role for SRY than previously thought. Together, these findings demonstrate that SRY is involved in the functioning of the healthy male brain, thus the dysregulation of SRY could underlie the male bias observed in several neurological disorders such as PD and ADHD.

CHAPTER 3:

Regulation and function of *SRY* in male Parkinson's disease

3.1 INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder, primarily characterized by the inability to initiate and maintain voluntary movement. Motor symptoms of PD emerge when the loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) exceeds 70%. The current hallmark treatment for PD is levodopa (L-DOPA) therapy which initially alleviates the symptoms of PD, however, its therapeutic benefit is eventually marred by the development of debilitating side effects known as dyskinesias (Huot et al., 2013). Thus, there is an urgent need to better understand the biological basis underlying PD to develop effective disease-modifying therapies.

The nigral DA cell loss in PD arises from a complex cascade of interrelated pathogenesis processes including oxidative stress, mitochondrial dysfunction, DNA damage and neuroinflammation (Dexter and Jenner, 2013). Oxidative stress has mostly been implicated as the cornerstone for the mechanisms underpinning DA cell loss in PD. Both increased DA oxidation and the mechanism of action of DA toxins (e.g. 6-hydroxydopamine and rotenone) elevate production of reactive oxygen species (ROS). Furthermore, post-mortem nigral tissues of PD patients consistently displayed increased oxidative damage (Graham, 1978, Jenner, 2003, Jenner and Olanow, 2006, Jenner and Olanow, 1998, Spina and Cohen, 1989). The mitochondria play a dual role as the source and target of ROS to mediate various important physiological roles (Sena and Chandel, 2012). Defective mitochondrial function, particularly the inhibition of mitochondrial complex I (a component of mitochondrial electron transport chain), has been associated with the PD phenotype in toxin-induced animal models of PD (Graham, 1978, Ramsay et al., 1986, Betarbet et al., 2000) and human PD post-mortem tissues (Schapira

et al., 1990, Mizuno et al., 1989). A significant downstream effect of increased oxidative stress and mitochondrial dysfunction include genomic and mitochondrial DNA damage (Sanders et al., 2014). 8-hydroxyguanosine (8-OHdG), a biomarker of nucleic acid oxidative damage, is increased in nigral cytoplasmic and mitochondria DNA of PD patients (Zhang et al., 1999, Alam et al., 1997, Shimura-Miura et al., 1999). Human and animal PD studies have also established a link between neuroinflammation and PD, whereby an increase in reactive microglia accompanied with elevated levels of pro-inflammatory cytokines and ROS occurs in the SNc (McGeer et al., 1988, Mogi et al., 1995, Mogi et al., 1996, Hunot et al., 1999, Kurkowska-Jastrzębska et al., 1999, Cicchetti et al., 2002). Taken together, these lines of evidence suggest 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.

Currently the underlying cause(s) that triggers the pathogenesis of DA neurons in PD is not well understood, with over 85% of PD cases having idiopathic origin, likely to be arising from a combination of environmental and genetic factors. Aging is the most significant risk factor for idiopathic PD, affecting 1% in the population over 60, and rising to almost 5% of the population over 85 (Nussbaum and Ellis 2003, de Lau and Breteler, 2006). Additionally, a range of environmental factors linked with increased risk of developing PD include 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, McCormack et al., 2002, Tanner et al., 2011, Semchuk et al., 1992, Costello et al., 2009, Dick et al., 2007). In the remaining 10-15% of cases, PD is caused by a single gene mutation with either autosomal dominant or recessive inheritance

(Klein and Westenberger, 2012, Trinh and Farrer, 2013, Antony et al., 2013). Mutations in three 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 cause recessive forms of PD.

Aside from aging, the male sex is the most significant risk factor for PD (Baldereschi et al., 2000, Picillo and Fasano, 2015, Wooten et al., 2004, Van Den Eeden et al., 2003). Men have a two-fold higher risk of developing PD and 1.3 to 3.7-fold higher prevalence of PD compared to women (Baldereschi et al., 2000, Wooten et al., 2004, Van Den Eeden et al., 2003, Moisan et al., 2016). Males with PD also have an earlier age of onset (Wooten et al., 2004, Haaxma et al., 2007), faster rate of disease progression (Haaxma et al., 2007), and greater dopaminergic denervation (Kaasinen et al., 2015, Kotagal et al., 2013) than female PD patients. Animal models of PD reproduce these sex differences, as male rodents or non-human primates are more susceptible to the toxin-induced dopaminergic degeneration than females (Murray et al., 2003, Leranth et al., 2000, Ookubo et al., 2009). Better understanding of the biological mechanisms underlying the sex dimorphism in PD may help to identify novel mechanism(s) mediating nigral DA cell loss.

The prevailing view is that sex differences in PD arise solely from the neuroprotective actions of oestrogen in females (Gillies and McArthur, 2010, Benedetti et al., 2001, Litim et al., 2016, Bourque et al., 2012). In female patients with PD, symptoms worsen with the onset of menopause or withdrawal of hormone replacement therapy – i.e. when endogenous oestrogen levels are low (Quinn and Marsden, 1986). Conversely, oestrogen treatment in post-menopausal

female PD patients improves motor symptoms (Shulman and Bhat, 2006). Studies in animal models of PD recapitulate these clinical findings as oestrogen treatment attenuated motor and nigrostriatal DA deficits in rodent and primate models of PD (Murray et al., 2003, Gillies et al., 2004, Leranth et al., 2000). In contrast, the effect of exogenous or circulating oestrogen on adult male DA neurons is minimal, if not harmful (Murray et al., 2003, Dluzen, 2005, McArthur et al., 2007). Unlike oestrogen, studies linking testosterone to male PD have been relatively minimal and inconclusive. Castration in young male mice induced motor deficits as well as DA cell loss and increased levels of α-synuclein and pro-inflammatory markers, although these effects were absent in older mice (Khasnavis et al., 2013). On the other hand, the effect of testosterone in male PD patients is inconclusive due to conflicting results and small sample sizes (Okun et al., 2006, Okun et al., 2002).

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, Beyer et al., 1991). For instance, embryonic midbrain cells developed more DA neurons when the cultures were composed of XY cells, rather than XX cells (Carruth et al., 2002, Beyer et al., 1991). Microarray analysis of single DA neurons from human SNc sections revealed that expression of PD pathogenesis genes (e.g. α-synuclein, PINK-1) was higher in men than women (Cantuti-Castelvetri et al., 2007), whilst 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, male DA cells have intrinsic sex differences that may influence their pattern of gene expression, predisposing the male-sex to developing PD. This chapter describes a novel pathway for male-specific DA cell loss involving the Y- chromosome gene, *SRY*.

The Y-chromosome gene, *SRY* (Sex-determining Region Y), switches on male- sex development by directing the development of the testes (Harley et al., 1992, Sinclair et al., 1990) and subsequent male phenotype. SRY is also expressed in the non-reproductive tissues of males such as the heart, adrenal glands, kidneys and brain (Dewing et al., 2006, Czech et al., 2012). In the male brain, SRY is expressed in DA-abundant regions such as the SNc and ventral tegmental area (Dewing et al., 2006, Czech et al., 2012, Mayer et al., 1998). In the male SNc, SRY protein co-localises with DA neurons (Dewing et al., 2006, Czech et al., 2012), where it regulates transcription of DA machinery genes (Dewing et al., 2006, Czech et al., 2012, Wu et al., 2009) and voluntary movement (Dewing et al., 2006). Given the presence and function of *SRY* in healthy male DA neurons, I hypothesise that:

i) **SRY expression is dysregulated in male PD**

ii) **Correcting SRY dysregulation will diminish DA cell loss and normalize aberrant nigral gene expression in male PD**

To test these hypotheses, the aims of this chapter are:

A) To determine the regulation of SRY expression in experimental models of PD

B) To determine the molecular targets of SRY in experimental models of PD

3.2 MATERIALS AND METHODS

Assessment of human SRY expression in toxin-induced cellular model of PD

Cell culture

Human neuroblastoma BE(2)-M17 (M17) (CRL-2267; ATCC, Manassas, VA, USA) cells, a human male neuronal cell line was used to carry out the experiments. The M17 cell line was selected because (i) it is a male cell line that expresses endogenous SRY and (ii) contain the cellular machinery typical of DA neurons, including tyrosine hydroxylase, monoamine oxidase and DA reuptake transporter.

Subculturing cells

M17 cells were cultured using Dulbecco's modified Eagle's medium with Ham's F12 nutrient in a 50:50 ratio supplemented with Glutamax, 10% Foetal Bovine Serum, 1% penicillin/ streptomycin and maintained at 5% CO_2 and 37°C in a Galaxy 170S incubator (New Brunswick, CT, USA). M17 cells were subcultured when a confluence of greater than 70% was reached twice per week. Cells were washed with 1% PBS and aspirated, released with 0.01% Versene (Gibco), and resuspended in a 1:4 dilution in fresh medium. The cell line was maintained in sterile 75 cm^2 cell culture flasks.

Dopamine toxin treatment in M17 cells

To determine the effect of DA toxin treatment on SRY expression *in vitro*, M17 cells were treated with DA toxins, 6-hydroxydopamine hydrobromide (6-OHDA, Sigma-Aldrich, solubilized in sterile 0.1% ascorbic acid saline) or rotenone (Sigma-Aldrich, solubilized in 1% DMSO/saline) and incubated for either 0, 6, 24, 48 or 72 hours. M17 cells were seeded 24 hours prior to treatment. When cells were at a confluence level greater than 80%, cells were treated with 0 or 20μM 6-OHDA or,

0 or 100μM rotenone. RNA was extracted from the cells at 0, 6, 24, 48 and 72 hours post-treatment. The cell confluence was observed and *SRY* mRNA and protein were measured for each time point (except mRNA was only measured at 6 hours post treatment).

Haemocytometry

In brief, M17 cell suspension was mixed at a 1:1 ratio with trypan blue and counted using a 0.0025mm^2 haemocytometer and light microscope (Olympus CK30). The cells were then plated with 500,000 cells in 5 different six-well plates, corresponding to the 5 different time points (i.e. 0, 6, 24, 48 and 72 hours post toxin treatment).

Quantitative RT-PCR (in vitro)

Total RNA (250ng) isolated from M17 cells following 0, 6, 24, 48 or 72 hours of 6-OHDA or rotenone treatment using the RNeasy Mini RNA Isolation Kit (Ambion) according to manufacturer's protocol was reverse-transcribed into cDNA (Go Script, Promega) and equal amount of cDNA template was added to SensiMix™ SYBR Hi-ROX Mastermix (Bioline) using primers listed in Supplementary Table 1. The relative level of mRNA was interpolated from a standard curve prepared by serially diluting the cDNA reaction. Specificity of PCR product formation was confirmed by monitoring melting peaks. All quantitative PCR reactions were conducted in triplicates. Final values represent fold change of gene expression relative to the housekeeping gene, β2-Microglobulin (β2M).

Western blot

A quantity of 10μg of protein was run on a 12% acrylamide gel. After transfer to a polyvinylidene difluoride membrane (Millipore, USA), bound proteins were blocked

with 5% bovine serum albumin powder in TBS-Tween20 for one hour. Primary antibodies, SRY (1:50; purified by Harley lab) and β-tubulin (1:1000; Milipore) were applied overnight then washed in TBS-Tween20. Secondary antibodies raised against the species of the primary antibody (1:1000, Alexa Fluor 488 anti-sheep and 1:1000, Alexa Fluor 594 anti-mouse; Thermo Fisher), were incubated with the membrane for 1 hour at 37°C, washed in TBS and processed on a Typhoon9400 variable mode imager (GE Healthcare, Little Chalfont, UK). Quantitation of three independent experiments was performed by measuring the optical density (intensity) of the SRY immunoreactive bands (protein blots) relative to the β-tubulin loading control bands.

Immunocytochemistry

M17 cells were seeded on glass cover slips in 35 mm tissue culture wells 24 hours prior to treatment. Cells were treated with either 6-OHDA (20μM) or rotenone (100μM) for 24 hours. Treated cells were washed with PBS and fixed with formalin for 7 minutes. After washing, cells were blocked with CAS block (Thermo Fisher) for 1 hour at 37^{o}C. Cells were then incubated with primary antibody against human SRY (1:100, rabbit polyclonal; AVIVA Systems Biology) overnight at 4^{o}C, washed and incubated with a fluorescent secondary antibody (1:1000, Alexa Fluor 594 anti-rabbit; Thermo Fisher) in block for 1 hour at 37^{o}C. After washing, coverslips containing the stained cells were mounted on slides with fluorescent nucleic acid stain, Hoechst 33342 (Thermo Fisher). Cells were imaged using a 60x oil immersion objective on a confocal microscope (Olympus FV1200) and quantitative scoring of cells positive for SRY protein immunofluorescence was made using MetaMorph software (Molecular Devices). The intensity was calculated from the

average values of the nuclear, cytoplasmic or whole cell fluorescence in 6 different fields from 4 independent experiments.

Assessment of SRY expression in toxin-induced rat models of PD

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 in the proposal. Animals were housed in a 12 hour light:dark cycle room and had access to food and water *ad libitum.*

Acute intranigral injection of DA toxins in male rats

To assess the effect of nigral injury on *SRY* expression, a unilateral lesion of the right SNc was made by a single injection of DA toxins 6-hydroxydopamine hydrobromide (6-OHDA, 30µg dissolved in 1.5µL of 0.1% ascorbic acid saline) or rotenone (30µg in dissolved 1.5µL of 1% DMSO/saline) 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, or 21 days post 6-OHDA or at 0 or 7 days post rotenone). At the end of the test, rats were culled and the brains were processed for measurement of nigral mRNA levels.

Limb-use asymmetry test

The limb-use asymmetry test assessed spontaneous forelimb usage during vertical explorations in rats, where motor impairment is indicated by a reduction in limb-use contralateral (i.e. left) to the site of drug injection. 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. The behavioural assessor was blinded to the animal groups.

Histology

Rat brains were isolated fresh and processed for qRT-PCR. Coronal sections of 16µm and 10µm thickness were cut serially through the striatum and SNc respectively and stored at -80°C. Between each series, a 200µm slab was collected in order to isolate tissue for RNA processing.

Quantitative RT-PCR (in vivo)

Total RNA (100-300ng) isolated from the left and right rat nigra using TRI-Reagent (Sigma Aldrich) according to manufacturer's protocol was reverse-transcribed into cDNA (Go Script, Promega) and equal amount of cDNA template was added to SensiMix™ SYBR Hi-ROX Mastermix (Bioline) using primers listed in Supplementary Table 1. The relative level of mRNA was interpolated from a standard curve prepared by serially diluting the cDNA reaction. Specificity of PCR product formation was confirmed by monitoring melting peaks. All quantitative PCR reactions were conducted in triplicates. Final values represent fold change of gene expression relative to the housekeeping genes TATA Box Binding Protein (TBP).

Assessment of *Sry* target genes in toxin-induced rat models of PD

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 Sry antisense oligonucleotide (ASO) infusions in toxin-induced rat models of PD

Nigral Sry expression in male rats was reduced by repeated intranigral infusions of SRY ASO as per Chapter 2. To assess the targets of *Sry* in PD, rats were infused unilaterally with ASO or SO daily (2µg in 1µL in aCSF) for 10 consecutive days followed by a single unilateral intranigral injection of 6-OHDA (30µg) or rotenone (30µg) at 4 hours following the last ASO-infusion. Motor function was assessed by the limb-use asymmetry test two days post 6-OHDA or rotenone injection. At the end of the behavioural test, the rats were culled and the brains were processed for measurement of nigral mRNA levels (as explained above).

Measurement of nigral mRNA expression

Rat brains were isolated fresh and processed for qRT-PCR. Coronal sections of 10μm thickness were cut serially through the SNc and stored at -80°C. Between each series, a 200μm slab was collected in order to isolate tissue for RNA processing.

Statistical Analysis

All values are expressed as the mean ± S.E.M. All data was analysed using tools within Graphpad Prism 5. Comparisons of two experimental groups were performed using two-tailed unpaired Student t-test. Multiple comparisons of more than two experimental groups were performed using one-way ANOVA and Bonferroni's *post hoc* tests. 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.

3.3 RESULTS

SRY mRNA and protein expression is aberrantly upregulated in toxin-induced *in vitro* models of PD

To determine the regulation of SRY expression in *in vitro* models of PD, the effect of 6-OHDA or rotenone treatment on human *SRY* expression was assessed in the human male neuroblastoma cell line, M17 (Figures 3.1 to 3.3). 6-hydroxydopamine (6-OHDA) and rotenone are both widely used DA toxins to model PD as it induces key symptoms and pathophysiological features such as motor deficits, and nigral DA cell loss (Blum et al., 2004). 6-OHDA is structurally similar to DA and enters DA neurons via the DA reuptake transporter (DAT). 6-OHDA is auto-oxidised in DA neurons to generate toxic metabolites such as quinones, hydrogen peroxide and superoxide radicals (Jellinger et al., 1995, Blum et al., 2004). 6-OHDA also mediates its actions by inhibiting the mitochondrial respiratory chain complexes I and IV (Glinka et al., 1997). Rotenone is a DA toxin which acts as a mitochondria inhibitor by binding to the complex 1 of the electron transport chain to impair oxidative phosphorylation (Bove et al., 2005, Blum et al., 2004). Unlike 6-OHDA, rotenone is lipophilic and can cross the blood brain barrier (BBB) following peripheral administration. Despite the different chemical structure and route of administration, both DA toxins mediate cell death by similar DNA damage, neuroinflammation, mitochondrial-dependent, oxidative stress and apoptotic pathways, ultimately causing DA cell loss (Bove et al., 2005, Blum et al., 2004).

Treatment with 20μM 6-OHDA increased human *SRY* mRNA expression at 6 (2.5-fold vs 0h, $p<0.05$, Figure 3.1B) and 24 hours (4.4-fold vs 0h, $p<0.01$, Figure 3.1B), post treatment in M17 cells. *SRY* mRNA expression diminished to basal levels at 48 and 72 hours post 6-OHDA treatment (Figure 3.1B). The

elevation of *SRY* mRNA expression at 6 and 24 hours was accompanied by an increase in *GADD45γ* mRNA expression (2.3 and 4.6-fold vs 0h, $p<0.05$, $p<0.01$ respectively, Figure 3.1B), a marker of DNA-damage and regulator of SRY expression (Warr et al., 2012, Gierl et al., 2012). Similarly, treatment with 100μM rotenone increased human *SRY* and *GADD45γ* mRNA expression at 6, 24, and 48 hours post-treatment (Figure 3.1C), with maximal increase observed at 6 hours post treatment (4.3 and 3.5-fold vs 0h, $p<0.01$ respectively, Figure 3.1B). Thus, the data shows that the aberrant upregulation of *SRY* is accompanied by the upregulation of *GADD45γ* following 6-OHDA or rotenone treatment in M17 cells.

To determine whether the SRY protein was also dysregulated in response to DA cell injury, the effect of 6-OHDA treatment on human SRY protein in M17 cells was assessed by western blot and immunocytochemical analysis (Figure 3.2 and 3.3). Western blot analysis revealed a rise in SRY protein expression at 24 hours post 6-OHDA treatment, reaching its maximal expression at 48 hours (2.2-fold vs 0h, $p<0.01$, Figures 3.2B and C). As observed in Figure 3.3, treatment with 20μM of 6-OHDA triggered a marked elevation of SRY immunostaining in both the nucleus and cytoplasm at 24 hours post treatment. Densitometric quantification revealed a significant increase in total SRY protein immunostaining (14.5 ± 1.8 a.u., veh, vs 29.6 ± 5.6 a.u., 6-OHDA, $p<0.05$, Figure 3.3B) as well as nuclear (14.8 ± 1.6 a.u., veh vs 27.0 ± 5.5 a.u., 6-OHDA, $p<0.05$, Figure 3.3B) and cytoplasmic (13.5 ± 1.8 a.u., veh vs 25.7 ± 6.0 a.u., 6-OHDA, $p<0.05$, Figure 3.3B) immunostaining. Similarly, treatment with 100μM of rotenone induced an upregulation of nuclear and cytoplasmic SRY immunostaining (Figure 3.3C), with significant increases in total (13.6 ± 2.4 a.u., veh vs 25.6 ± 4.0 a.u., rotenone, $p<0.05$, Figure 3.3C) and cytoplasmic (14.1 ± 3.5 a.u., veh vs 26.7 ± 2.6 a.u.,

rotenone, $p<0.05$, Figure 3.3C) SRY protein, as well as an increase in nuclear SRY protein, although not significant.

In summary, human SRY and protein mRNA expression is highly and persistently elevated in cellular models of PD. The increase in *SRY* expression was paralleled by an elevation in expression of *GADD45*γ mRNA, suggesting that the upregulation of SRY expression is *GADD45*γ-dependent, as described previously (Czech et al., 2014).

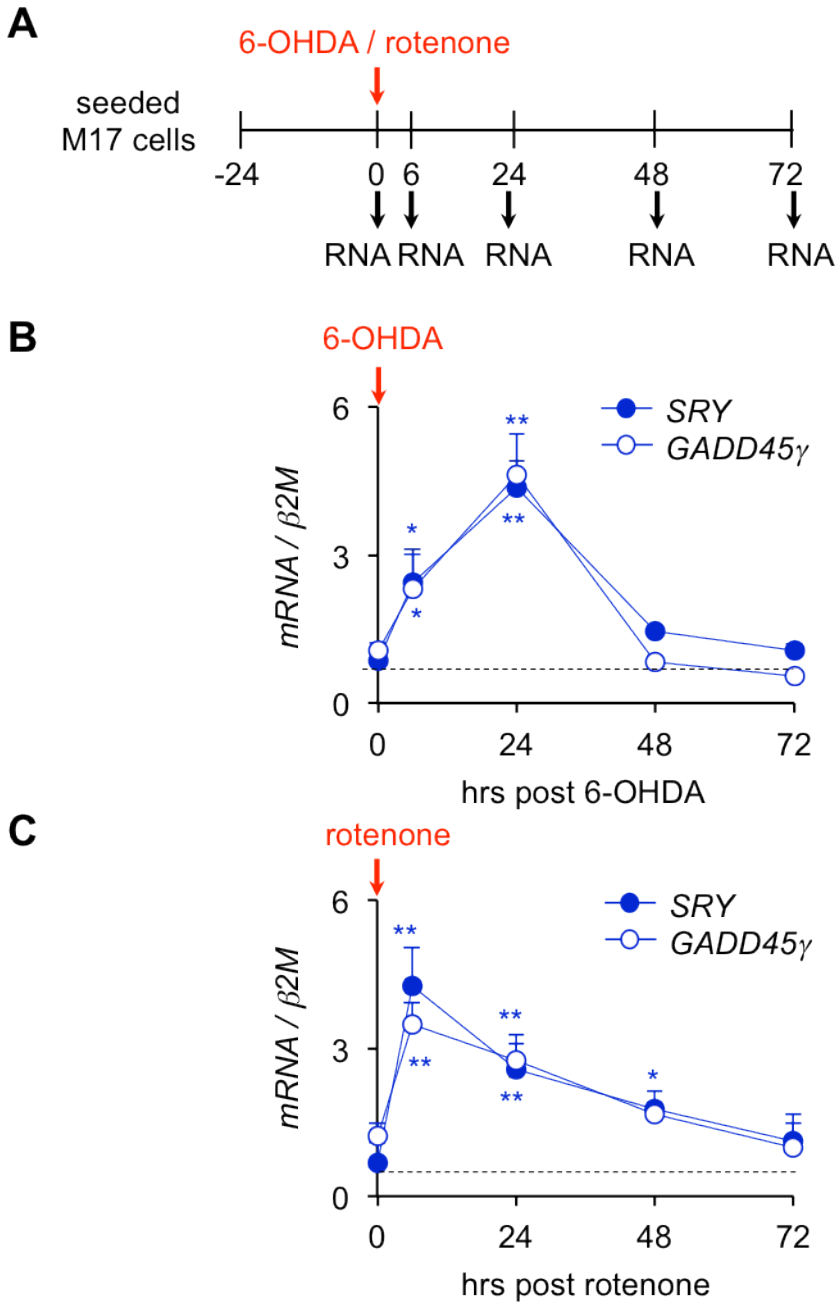


Figure 3.1 Effect of 6-OHDA or rotenone treatment on human *SRY* mRNA expression in M17 cells. A) Human M17 cells were seeded 24h prior to 6-OHDA or rotenone treatment and RNA was extracted at 6, 24, 48 and 72h post treatment. Effect of **B)** 6-OHDA or **C)** rotenone treatment on human *SRY* mRNA expression. (n=3/group; mean ± S.E.M. one-way ANOVA; *P<0.05, **P<0.01 compared to 0h)

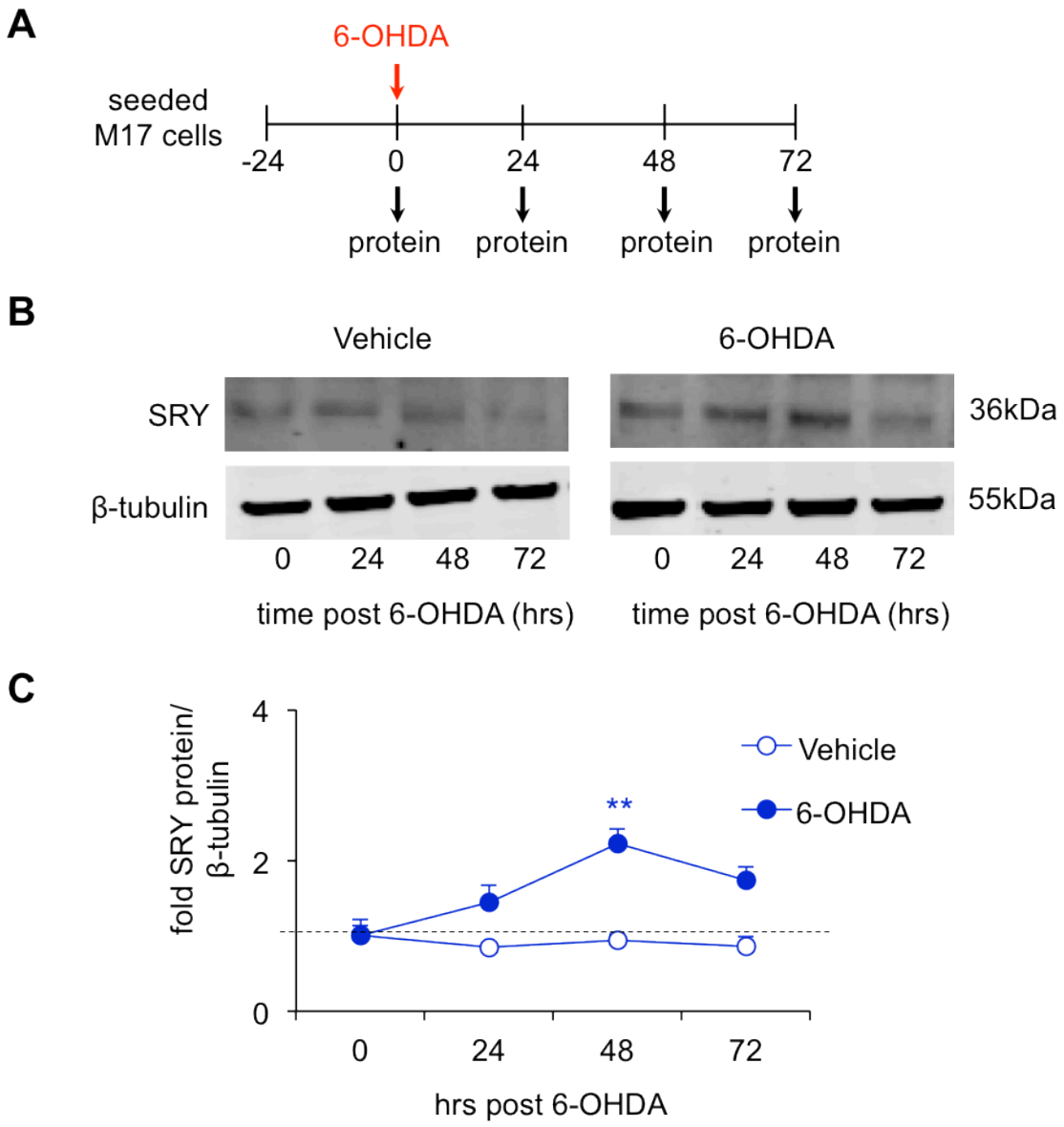


Figure 3.2 Effect of 6-OHDA or rotenone treatment on human SRY protein expression in M17 cells. A) M17 cells were seeded 24h prior to 6-OHDA treatment and protein was extracted at 24, 48 and 72h post treatment. **B)** Representative images of SRY and β-tubulin protein expression following 6-OHDA or vehicle treatment, with observed molecular weight based on migration of molecular weight standards. **C)** Densitometric analyses of the SRY/β-tubulin protein expression ratios (n=3/group, mean ± S.E.M. one-way ANOVA; *P<0.05 **P<0.01, compared to vehicle control).

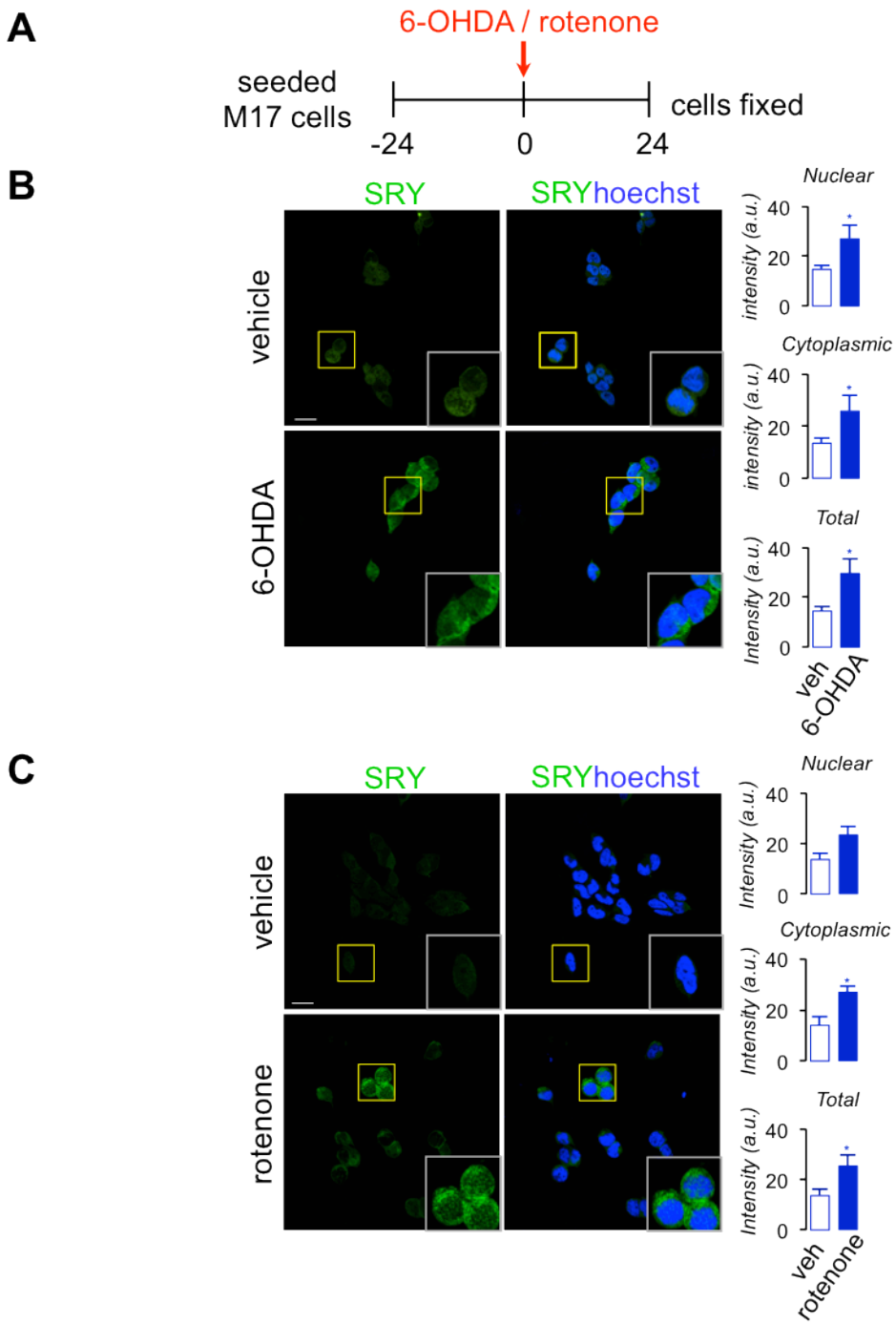


Figure 3.3 Effect of 6-OHDA or rotenone treatment on human SRY protein expression in M17 cells. A) M17 cells were seeded 24h prior to 6-OHDA or rotenone treatment and cells were fixed at 24h post treatment. Representative images (left panels) and corresponding quantification of SRY protein expression in the nucleus, cytoplasm and whole cell (right panels) following treatment with **B)** vehicle or 6-OHDA and **C)** vehicle or rotenone for 24h. SRY protein levels correspond to mean of SRY immunofluorescence in 6 different fields (n=4/group, mean ± S.E.M. student t-test; *P<0.05 compared to vehicle control; a.u. = arbitrary unit).

Sry mRNA expression is upregulated in toxin-induced *in vivo* rat models of PD

To determine the regulation of *Sry* expression in *in vivo* models of PD, the effect of acute 6-OHDA or rotenone injection on nigral Sry mRNA expression was assessed at various time points following toxin injection. In the 6-OHDA rat model of PD, nigral *Sry* mRNA was significantly elevated from days 2 to 14 post 6-OHDA injection in male rats, with maximal expression at day 7 (2.9-fold vs day 0, $p<0.001$, Figure 3.4A lower graph). Similar to the *in vitro* 6-OHDA model of PD, the increase in nigral *Sry* expression was paralleled by increase in nigral *Gadd45y* mRNA expression (Figure 3.4A lower graph). 6-OHDA injection significantly reduced motor function and *Th* mRNA expression at 7 days post 6-OHDA injection (Figure 3.4A upper and lower graphs), indicating that *Sry* and *Gadd45y* mRNA up-regulation occurs prior to and during DA cell death. In the rotenone-induced rat model of PD, nigral *Sry* (2.6-fold vs day 0, $p<0.01$, Figure 3.4B lower graph) and *Gadd45y* (1.8-fold vs day 0, $p<0.05$, Figure 3.4B lower graph) mRNA was significantly elevated at 7 days post rotenone injection in male rats.

Together, these results show that the highly and persistently upregulation of *Sry* expression is accompanied by the elevation of *Gadd45y* levels in multiple *in vivo* and *in vitro* models of PD, and that this induction precedes DA cell death.

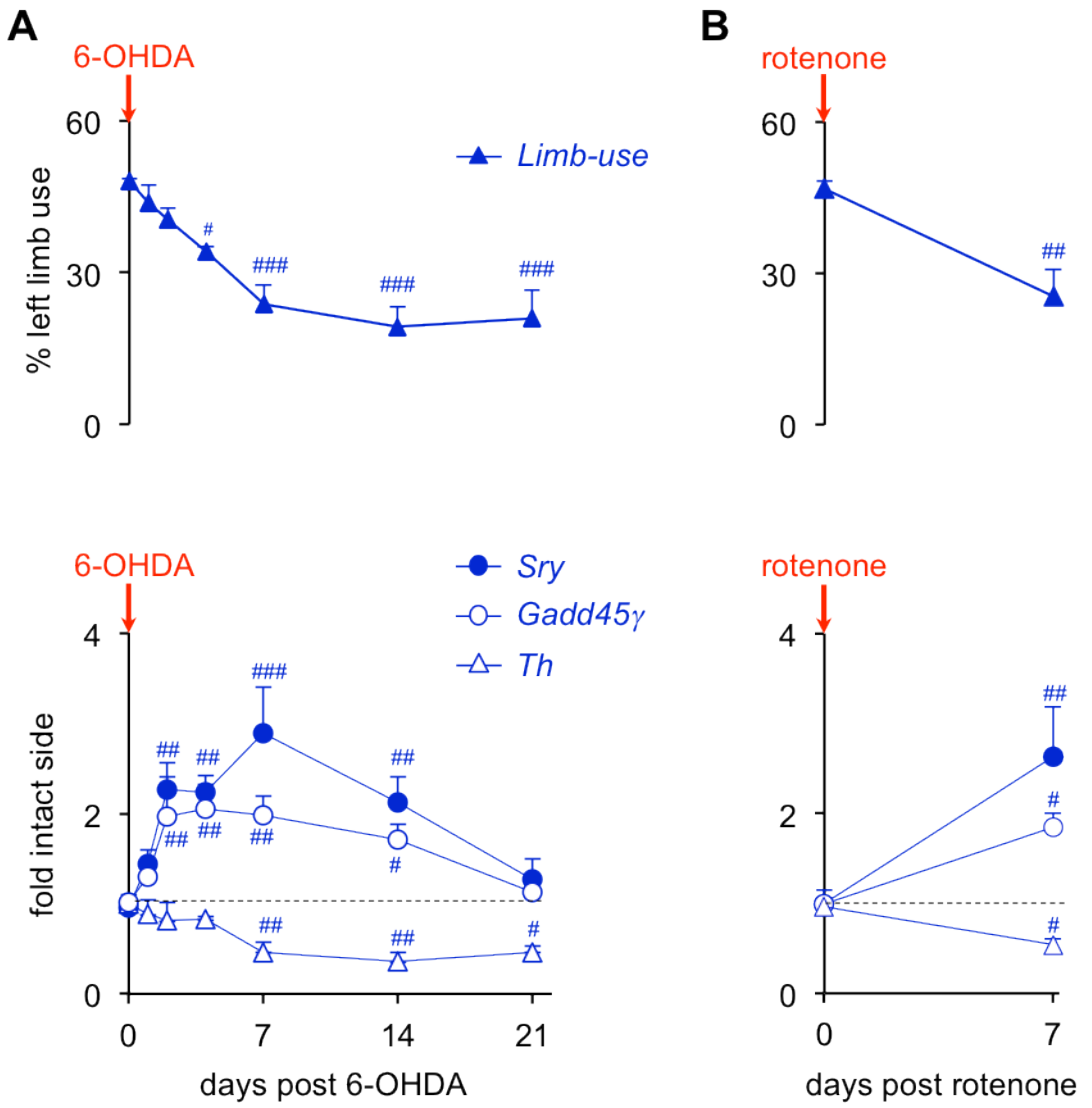


Figure 3.4 Effect of 6-OHDA or rotenone injection on nigral *Sry* mRNA expression in male rats. Male rats received a single intranigral injection of 6-OHDA or rotenone (30µg) and limb-use was assessed and *Sry* mRNA was measured at various time points post injection. Effect of a single intranigral injection of **A)** 6-OHDA or **B)** rotenone on limb use (top) and nigral *Sry, Gadd45*γ, or *Th* mRNA expression (bottom) at various days post-injection in male rats (n=5/group; mean ± S.E.M. one-way ANOVA, $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ compared to day 0; dashed line = baseline levels)

Reducing *Sry* expression exerts male-specific neuroprotection in toxin-induced rat models of PD

To determine the role of Sry upregulation in injured male DA neurons, the effect of reducing Sry expression, via repeated nigral Sry ASO-infusion, was assessed in the 6-OHDA or rotenone induced rat models of PD. In the 6-OHDA rat model of PD, prior ASO infusion in male rats diminished the 6-OHDA induced deficits in limb use (18%, SO vs 32%, ASO, $p<0.01$, Figure 3.5A) at 21 days post 6-OHDA injection. In line with motor behavior, post-mortem analysis revealed that the protective effect of ASO-infusion on motor function was associated with the alleviation of 6-OHDA losses in nigral TH-positive cells (26%, SO vs 53%, ASO, $p<0.01$, Figure 3.5B). These results indicated that SRY ASO-infusion prior to 6-OHDA injection diminished 6-OHDA-induced motor deficits via alleviation of 6-OHDA-induced nigral degeneration in male rats. Similarly, prior Sry ASO-infusion in male rats attenuated limb use deficits in the rotenone-induced rat model of PD (27%, SO vs 40%, ASO, $p<0.05$, Figure 3.5C). In line with the motor behaviour, prior ASO-infusion in male rats also prevented rotenone-induced loss of nigral TH-positive cells (41%, SO vs 72%, ASO, $p<0.01$, Figure 3.5D). In contrast, 6-OHDA-injected female rats (which do not express Sry) showed no differences between the ASO and SO-infused groups in limb use or total TH-positive cells at 21 days post 6-OHDA injection (Figure 3.5E and F). These results indicate that the protective effect of SRY ASO-infusion in the rat models of PD was male-specific. Taken together, these results show that reducing nigral SRY expression in male rats mitigates 6-OHDA or rotenone-induced nigral degeneration and consequent motor deficits, perhaps through a common downstream mechanism shared by both toxins (Study and analysis performed by Paulo Pinares-Garcia).

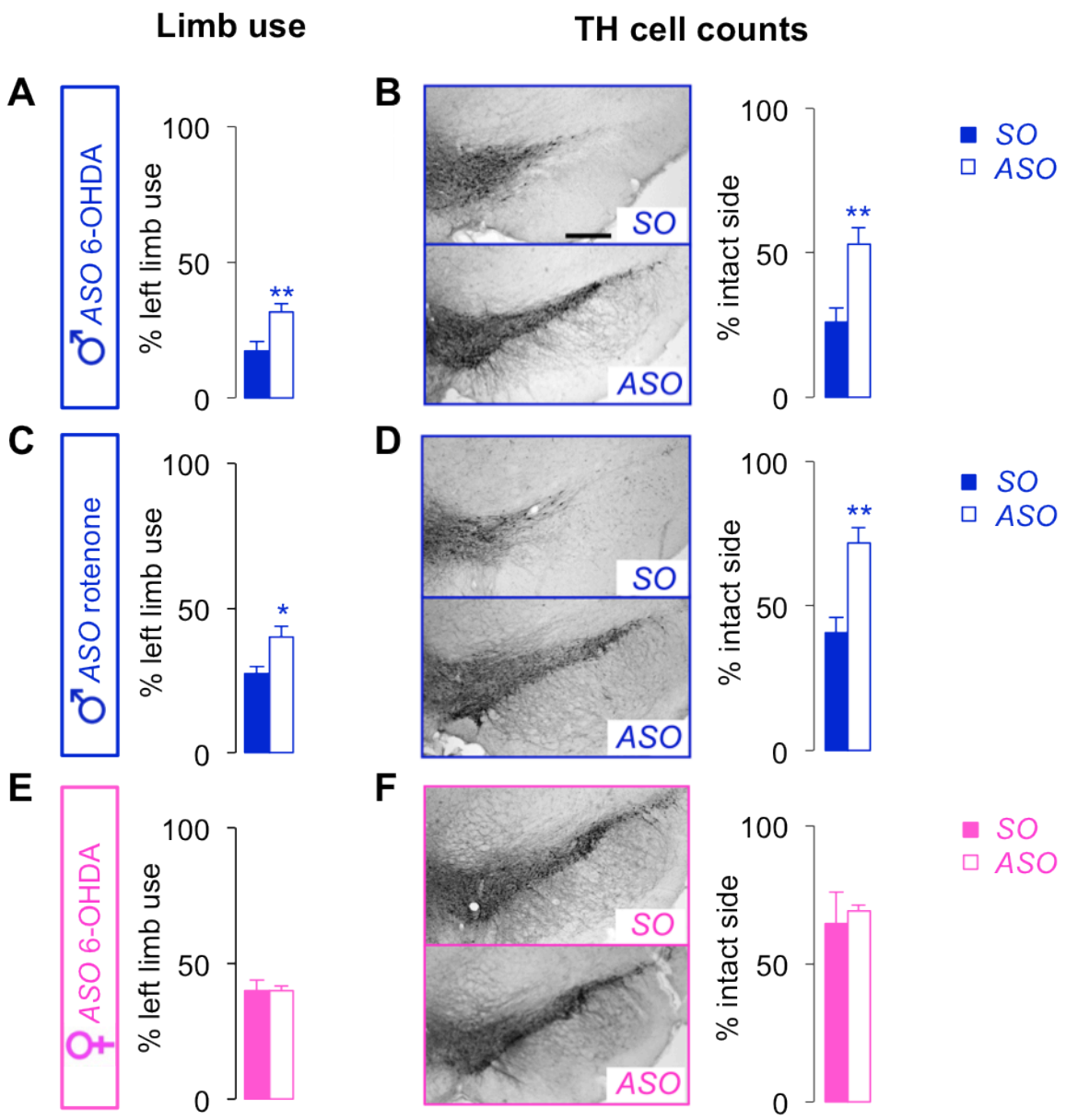


Figure 3.5 Reducing nigral Sry synthesis ameliorates toxin-induced motor deficits and nigral degeneration in male rats. ASO (or SO) was infused prior to toxin injection in male rats. Motor deficits were assessed by limb use test as % of left limb use and nigral degeneration was assessed by TH cell counts (scale=400µm) as % of intact side **A and B)** male 6-OHDA, **C and D)** male rotenone and **E and F)** female 6-OHDA models, 21 days post toxin injections (n=5/group, mean ± S.E.M. unpaired t-test; **P<0.01 compared to sense) *(Work by Paulo Pinares-Garcia)*

Reducing *Sry* expression exerts male-specific attenuation of PD pathogenesis gene expression in toxin-induced rat models of PD

To identify potential downstream targets of Sry in toxin-induced rat models of PD, we assessed the effect of Sry ASO-infusion on nigral gene expression toxin-induced rat model of PD. Nigral gene expression was assessed at two days post 6-OHDA or rotenone injection – the optimal time point for measurement of key cellular events underlying DA cell loss and nigral *Sry* knockdown (Hebert et al., 2003, Mandel et al., 2002). Moreover, we focused on PD pathogenesis pathways that are sex-biased, such as mitochondrial function and oxidative stress (Pomatto et al., 2017, Misiak et al., 2010), apoptosis (Rodriguez-Navarro et al., 2008), and neuroinflammation (Khasnavis et al., 2013, Villa et al., 2016, Joniec et al., 2009).

Quantitative PCR analysis revealed that 6-OHDA or rotenone-induced up-regulation of nigral *Sry* mRNA at two days post injection in male rats (2.9 and 2.7-fold of intact side, respectively, Figure. 3.6A and B), which were suppressed with ASO-infusion (1.3 and 1.4-fold of intact side, respectively, $p<0.05$ vs SO, Figure. 3.6A and B). As expected, *Sry* mRNA expression was not detected in the 6-OHDA treated female SNc (Figure 3.6C). Work from others (Czech et al., 2012, Wu et al., 2009, Dewing et al., 2006) and my chapter 2 have shown that *SRY* regulates DA machinery genes such as *TH*, *MAO-A* and *DRD2* mRNA. In the 6-OHDA rat model of PD, nigral *Mao-a, Ddc* and *Drd2* mRNA expression (0.7, 0.3 and 0.2-fold of intact side, respectively, SO, Figure 3.6A), were reduced at two days post 6-OHDA injection in male rats. Remarkably, ASO-infusion restored the 6-OHDA-induced decreases in *Mao-a* and *Drd2* mRNA expression in male rats (1.0 and 1.6-fold of intact side, respectively, ASO, $p<0.01$ vs SO, Figure 3.6A). However, no significant changes between the ASO- and SO-infused groups were

observed in the rotenone-injected male rats (Figure 3.6B).

In parallel, 6-OHDA or rotenone-induced increases in DNA damage marker, *Gadd45y* (3.5 and 3.5-fold of intact side, respectively, SO, Figure 3.7A and B), was attenuated with ASO-infusion in male rats (1.8 and 2.2-fold of intact side, respectively, ASO, $p<0.05$ vs. SO, Figure 3.7A and B). However, the apoptosis regulator, *Bcl-2-associated X* (*Bax*) mRNA expression (1.7-fold of intact side, SO, Figure 3.7A) was attenuated solely in the ASO-infused 6-OHDA-lesioned male rats (1.1-fold of intact side, ASO, $p<0.05$ vs SO, Figure 3.7A). Similarly, the 6-OHDA or rotenone-induced upregulation of the mitochondrial and oxidative stress marker, *superoxide dismutase 2* (*Sod2*) mRNA expression (2.8 and 2.2-fold of intact side, respectively, SO, Figure 3.8 A and B) was diminished in the ASO-infused male rats (0.9 and 1.2-fold of intact side, respectively, ASO, $p<0.05$ vs SO, Figure 3.8A and B). Likewise, the 6-OHDA or rotenone-induced elevation of *glutathione peroxidase 1* (*Gpx1*) (5.8 and 4.4-fold of intact side, respectively, SO, Figure 3.8A and B) was reduced with ASO-infusion in male rats (2.1 and 1.7-fold of intact side, respectively, ASO, $p<0.01$ vs SO, Figure 3.8A and B). In particular, the 6-OHDA or rotenone-induced upregulation of the neuroinflammation marker, *inducible nitric oxide synthase* (*iNos*) (10.3 and 8.5-fold of intact side, respectively, SO, Figure 3.9A and B) was abolished in the ASO-infused male rats (1.8 and 1.9-fold of intact side, ASO, respectively, $p<0.05$, Figure 3.9A and B). Furthermore, the 6-OHDA or rotenone-induced massive increases of *interleukin 1β* (*Il1β*) (34.1 and 56.5-fold of intact side, respectively, SO, Figure 3.9A and B) were markedly reduced in the ASO-infused male rats (3.6 and 15.0-fold of intact side, respectively, ASO, $p<0.05$, Figure 3.9A and B). However, the 6-OHDA or rotenone-induced increase of *tumor necrosis factor α* (*Tnfα*) (16.4 and 21.0-fold of

intact side, respectively, SO, Figure 3.9A and B) was only diminished in the ASO-infused 6-OHDA-induced male rats (1.6-fold of intact side, ASO, $p<0.05$ vs SO, Figure 3.9A). In contrast, there were no significant differences in nigral gene expression between the ASO- and SO-infused groups in 6-OHDA-treated female rats (Figure 3.6 to 3.9).

Together, these results showed that male-specific neuroprotection by Sry-ASO infusion is accompanied by attenuation of nigral genes that detect or promote DNA damage, mitochondrial function, and inflammation.

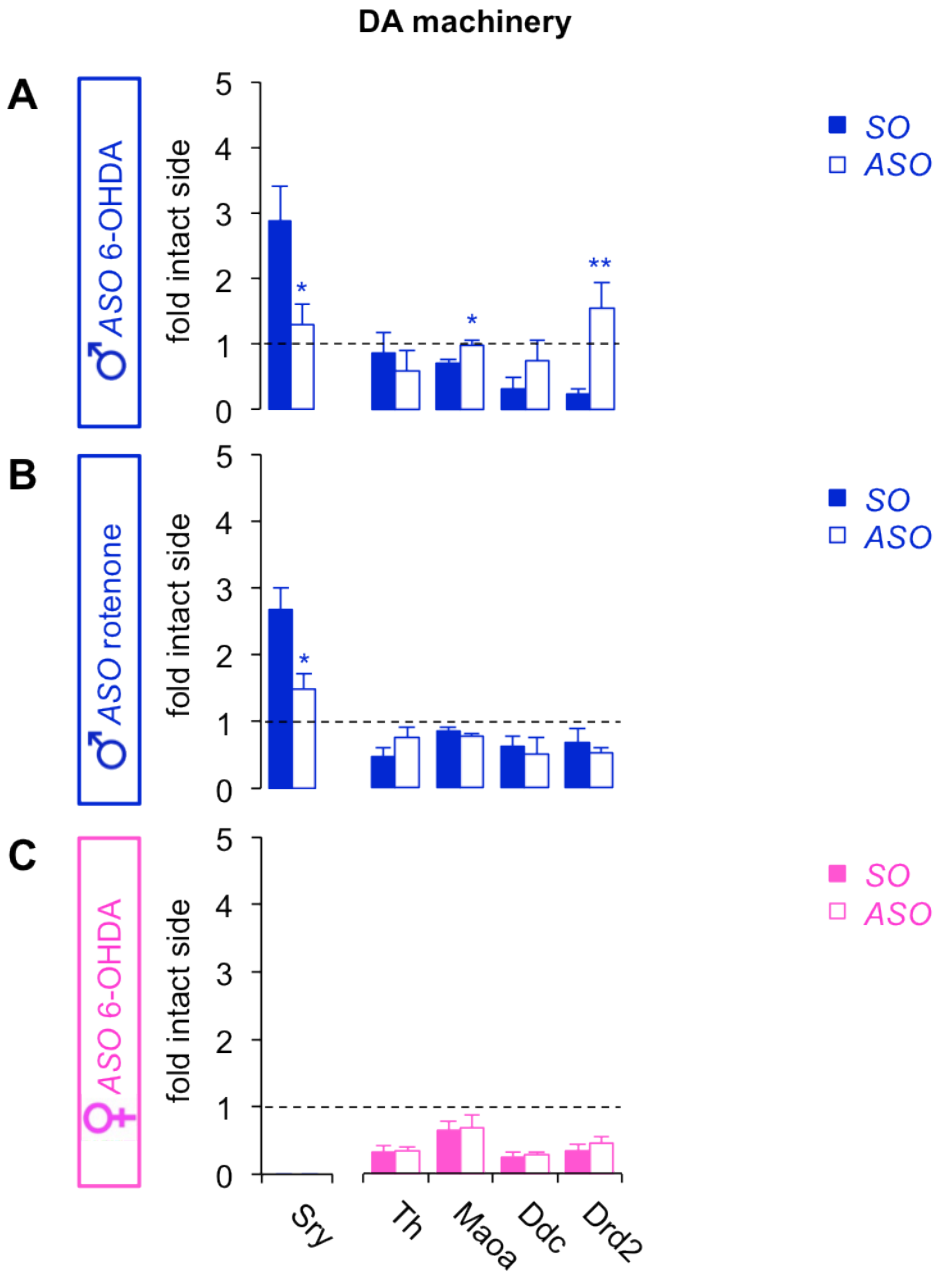


Figure 3.6 Effect of *Sry* ASO in toxin-induced models of PD on nigral *Sry* and DA machinery mRNA expression in A) male 6-OHDA, **B)** male rotenone and **C)** female 6-OHDA models (n≥5/group, mean ± S.E.M. student t-test; *P<0.05 **P<0.01, compared to sense; dashed line = baseline levels).

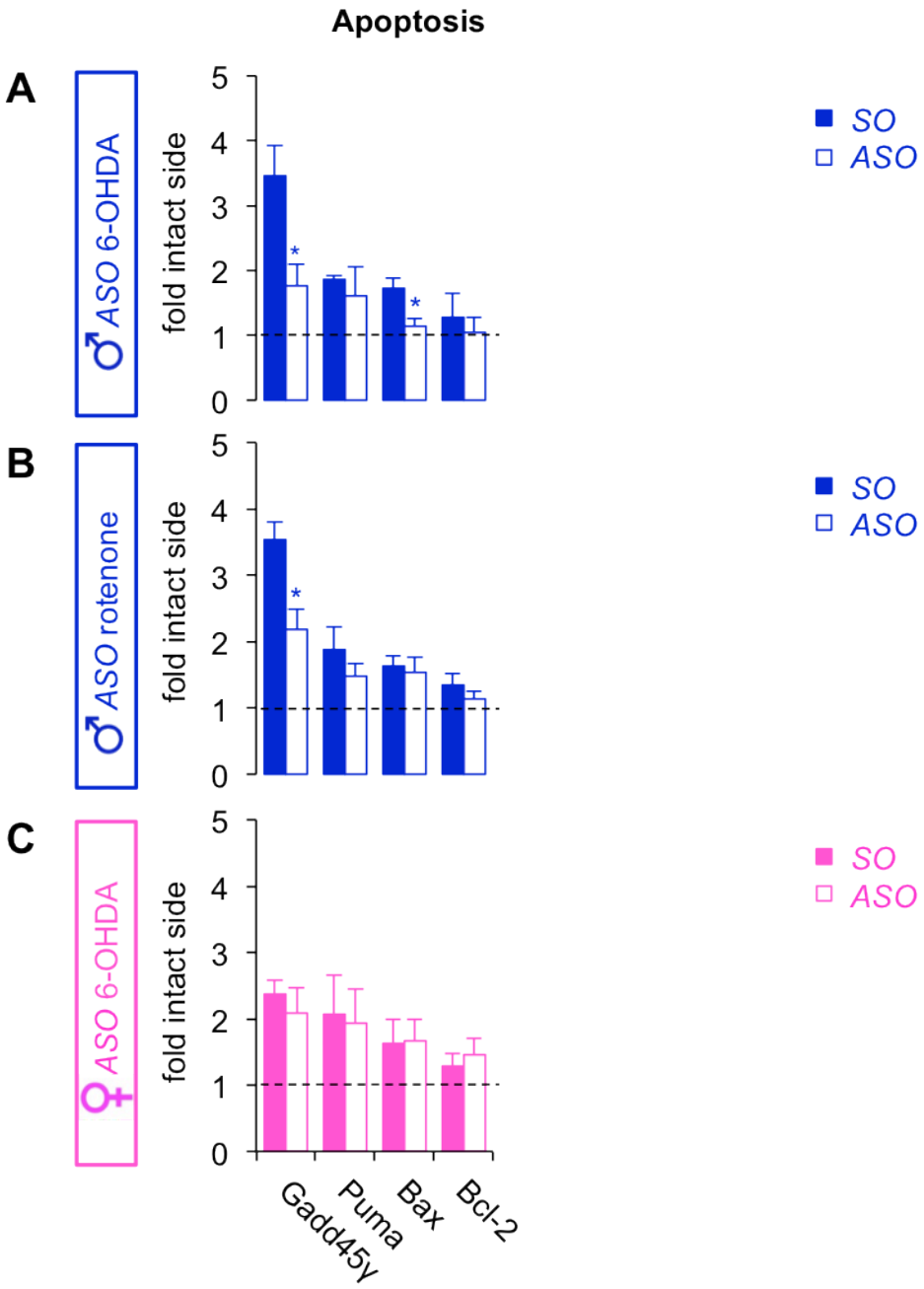


Figure 3.7 Effect of *Sry* ASO in toxin-induced models of PD on mRNA expression of nigral apoptotic markers in A) male 6-OHDA, **B)** male rotenone and **C)** female 6-OHDA models (n≥5/groups, mean ± S.E.M. student t-test; *P<0.05 **P<0.01, compared to sense; dashed line = baseline levels).

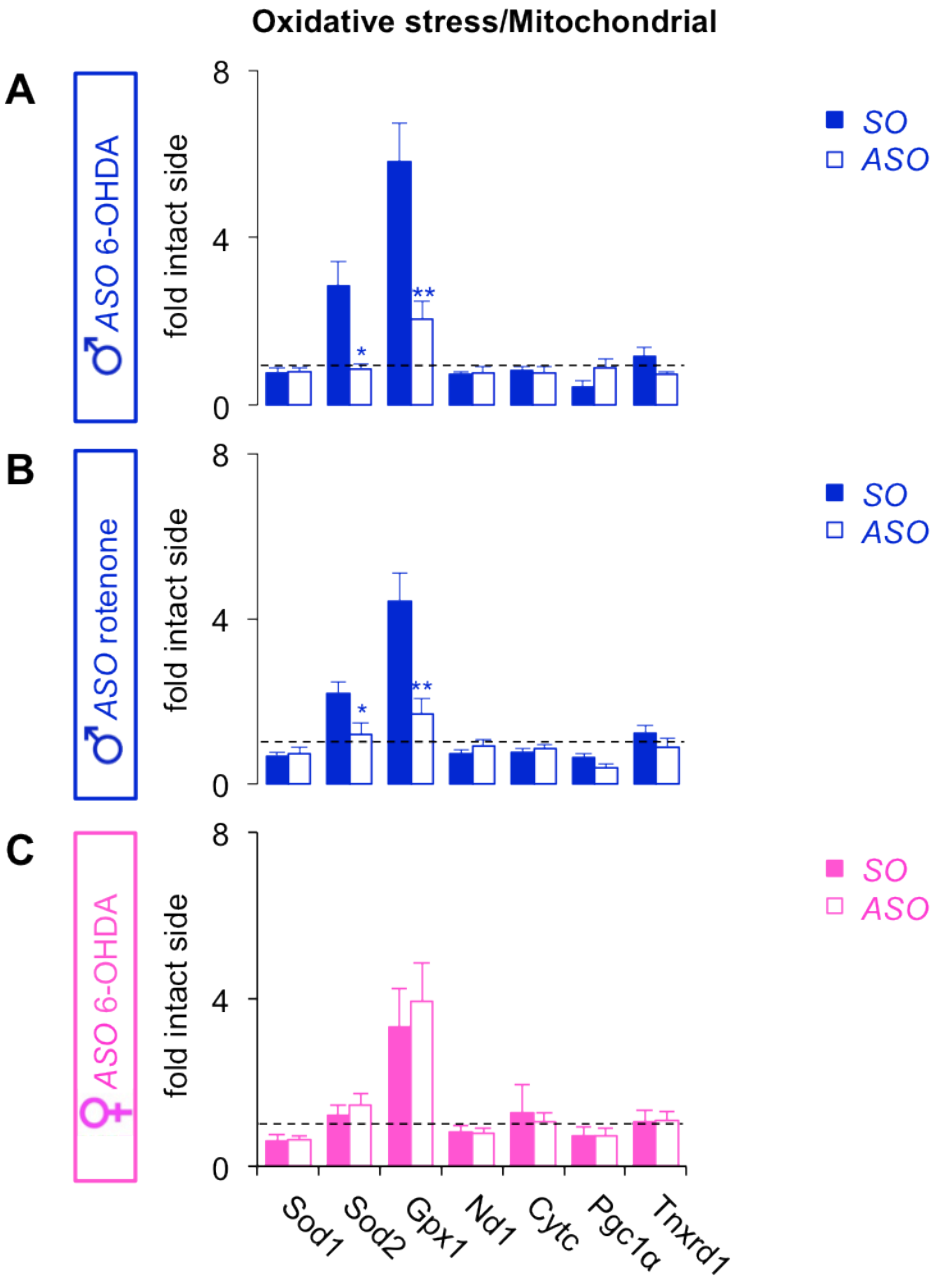


Figure 3.8 Effect of *Sry* ASO in toxin-induced models of PD on mRNA expression of nigral oxidative stress and mitochondrial markers in A) male 6-OHDA, **B)** male rotenone and **C)** female 6-OHDA models (n≥5/groups, mean ± S.E.M. student t-test; *P<0.05 **P<0.01, compared to sense; dashed line = baseline levels).

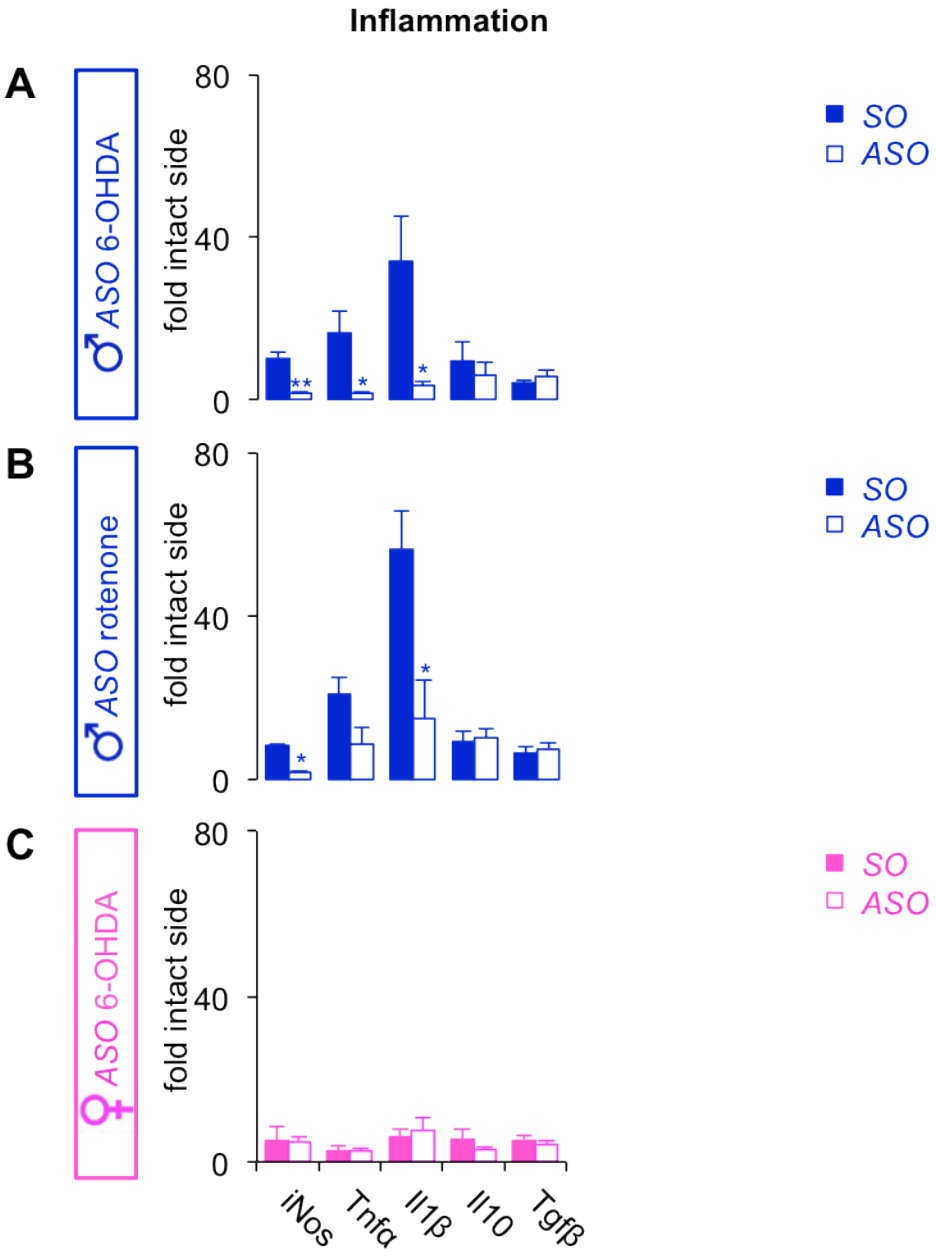


Figure 3.9 Effect of *Sry* ASO in toxin-induced models of PD on mRNA expression of nigral inflammation markers in A) male 6-OHDA, **B)** male rotenone and **C)** female 6-OHDA models (n≥5/group, mean ± S.E.M. student t-test; *P<0.05 **P<0.01, compared to sense).

3.4 DISCUSSION

In this chapter, the findings demonstrate that, *SRY*, a Y-chromosome gene present exclusively in males, directs a novel genetic mechanism for DA cell loss in males. To summarise, SRY expression was aberrantly upregulated in multiple experimental models of PD. Moreover, reducing Sry expression, via Sry ASO infusion, in male rats diminished motor deficits and nigral degeneration in toxin-induced rat models of PD, which was concomitant with attenuated expression of pro-apoptotic, pro-inflammatory, oxidative stress and mitochondrial genes. Importantly, the protective effect of ASO-infusion was absent in female rats (which do not express Sry), demonstrating for the first-time neuroprotection mediated by a sex-specific gene.

SRY expression is aberrantly upregulated in experimental PD

The present study shows that the Y-chromosome gene, *SRY,* is aberrantly upregulated in 6-OHDA or rotenone-induced rat and human cell culture models of PD (Figure 3.1 to 3.4). *Sry* up-regulation occurred prior to and during active DA cell loss, indicating a role for *Sry* in the onset and progression of PD. The elevation in *Sry* expression was paralleled by an increase in *Gadd45*γ expression in both the 6-OHDA and rotenone-induced rat models of PD. This is in line with our previous findings that identified the GADD45γ-p38-MAPK pathway as the upstream regulator of SRY expression in a 6-OHDA cellular PD model (Czech et al., 2014). These findings suggest that both 6-OHDA and rotenone-induced DA cell injury aberrantly upregulate *SRY* expression via a common signaling cascade involving the DNA damage induced GADD45γ-MAPK pathway. In parallel, Sry induction in developing male gonads is also regulated by

the GADD45γ-MAPK pathway (Gierl et al., 2012, Warr et al., 2012), demonstrating that *SRY* expression is mediated by a common pathway in the embryonic gonads and the adult male SNc. In contrast to the embryonic gonads, where the SRY protein is found exclusively in the nucleus, the present chapter revealed that SRY protein is present and upregulated in both the nucleus and cytoplasm of human neuroblastoma M17 cells. However, SRY protein is also detected in the cytoplasm of gonadal cells if there is a defect in SRY nuclear import (Harley et al., 2003). Thus, DA toxin-induced upregulation of SRY may be due to both increased *Sry* transcription and disrupted SRY nuclear import in the human neuroblastoma M17 cells. In support, oxidative stress, which plays a key role in PD pathogenesis, has been associated with impeding nuclear import of various transcription factors (Patel and Chu, 2011). Considering that regulation of *Sry* expression is mediated by a common signaling pathway in both the embryonic gonad and the male brain, increased understanding of Sry-mediated signaling in the gonads could reveal novel insights in the role of Sry in the male brain, and vice versa.

Detrimental effect of SRY upregulation in injured male DA neurons contributes to the male-bias in experimental PD

To determine the role of *Sry* upregulation in the injured male SNc, the effect of reducing nigral *Sry* expression was assessed in rat models of PD. Remarkably, reducing nigral Sry expression (via nigral Sry ASO-infusion) in male rats attenuated both 6-OHDA or rotenone-induced motor deficits and nigral DA cell loss, whilst no effects were seen in females (Figure 3.5). Given that 6-OHDA or rotenone-induced *Sry* up-regulation was suppressed with *Sry* ASO-infusion, these results reveal that toxin-induced *Sry* up-regulation in injured male DA neurons is detrimental. In line with previous studies (Murray et al., 2003, Ookubo et al., 2009),

the present chapter also showed that male rats are more susceptible to 6-OHDA-induced motor deficits and nigral degeneration than their female counterparts. However, reducing nigral *Sry* expression in male rats blocked or diminished the male-bias in motor deficits and nigral degeneration in the 6-OHDA rat model. These results demonstrate that *Sry* upregulation directly contributes to the male-bias in experimental PD, independent of gonadal hormonal influence. Thus, male susceptibility to DA cell loss is unlikely to be solely explained by lower oestrogen levels in males, but it may also reflect an intrinsic susceptibility of the single male DA neuron to injury, potentially via Y-chromsome genes such as *Sry*.

Reducing Sry expression diminishes male-biased increases PD pathogenesis gene expression in rat models of PD

To better understand the molecular mechanisms by which SRY upregulation exacerbates DA cell loss in males, the effect of reducing Sry expression on nigral gene expression profiles of key PD pathogenesis pathways were assessed. Acute 6-OHDA or rotenone injection induced robust elevation of nigral genes involved in DA cell loss such as sensors of DNA damage (*Gadd45y*) and oxidative stress (*Gpx1, Sod2*) and pro-inflammatory mediators (*iNos* and *Il1β*), which were far greater in males compared to females (Figures 3.7 to 3.9). Indeed, previous studies have demonstrated sexually dimorphic expression for antioxidants Sod2 and Gpx1 (Ott et al., 2007, Demarest and McCarthy, 2015), which are higher in males compared to females. Similarly, oxidative damage is higher in males compared to females during aging in rodents (Guevara et al., 2011, Borras et al., 2003), suggesting that the female brain has better mitochondrial efficiency, resulting in increased resilience to ROS-mediated injury. Furthermore, MPTP-

induced rodent models of PD demonstrated an earlier and higher upregulation of *iNos* and *Il-1β* expression in males compared to females (Ciesielska et al., 2007, Joniec et al., 2009, Bian et al., 2009), suggesting an earlier onset of PD in males. The inherent sex differences in oxidative stress, mitochondrial dysfunction and inflammation were thought to be solely attributable to prevailing levels of sex hormones (Arnold and Beyer, 2009, Villa et al., 2016). However, the results from the current chapter demonstrate that reducing *Sry* expression in 6-OHDA-injected male rats blocked or attenuated the male-biased elevations in mitochondrial and pro-inflammatory genes. Thus, sex differences in cellular events underlying PD pathogenesis, such as oxidative stress and neuroinflammation, are likely to result from a combination of the deleterious effects of Y-chromosome genes, such as Sry, in males, in addition to the well-established protective effect of sex hormones in females.

Divergent role of SRY in the healthy and diseased DA neurons

The contrasting effect of reducing Sry expression in healthy and Parkinsonian rats suggests an opposing role for Sry in the healthy 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 (Czech et al., 2012, Dewing et al., 2006) (Figure 3.10, Healthy). Similar to nigral transcription factors such as Nurr1, Pitx3 (Kadkhodaei et al., 2009) and Sox 6 (Panman et al., 2014), *Sry* may be required for the maintenance of adult male DA neurons, potentially as an alternate mechanism to oestrogen in female DA neurons (Gillies and McArthur, 2010). However, following 6-OHDA or rotenone-treatment we demonstrate a GADD45γ-MAPK mediated increase in *SRY* transcription (Czech et al., 2014), accompanied by DNA damage, mitochondrial

dysfunction and neuroinflammation (Figure 3.10, Injured). Moreover, reducing nigral *Sry* expression alleviated nigrostriatal DA cell loss and motor deficits in 6-OHDA or rotenone lesioned male rats (Figure 3.10, Injured + ASO), indicating a detrimental role for *Sry* in the injured male SNc. Thus, our current study significantly expands the understanding of Sry in the male brain, demonstrating a divergent role for Sry in the healthy and injured male SNc.

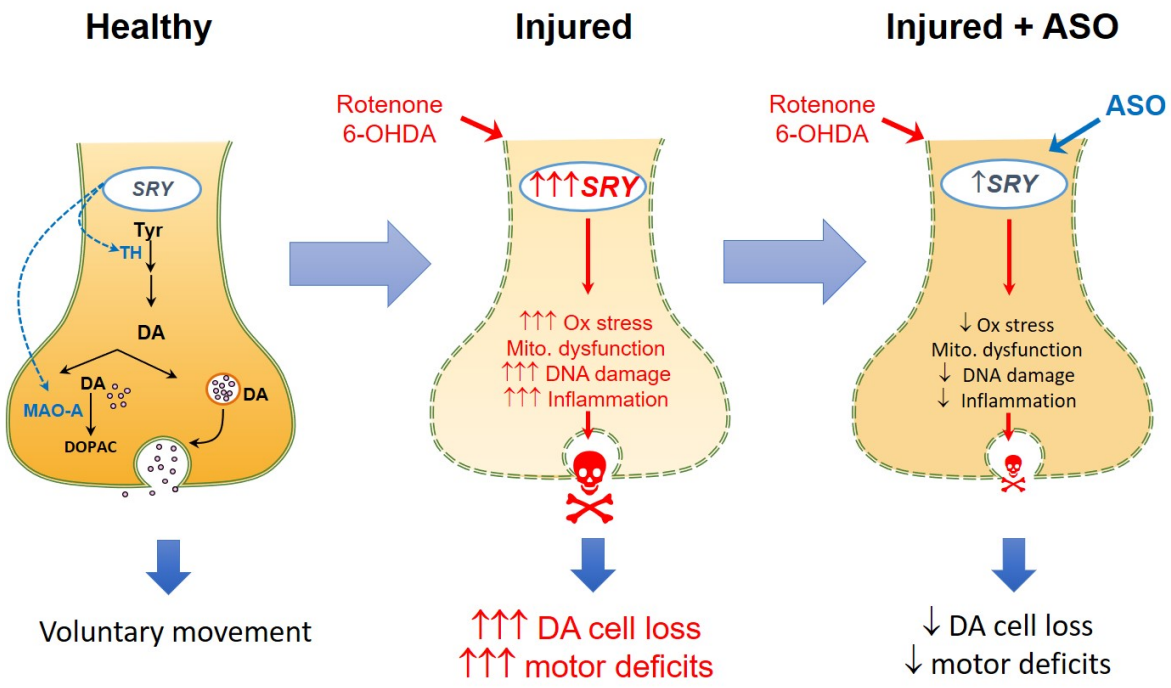


Figure 3.10 The proposed role for Sry in healthy and injured nigral male DA neurons. SRY positively regulates DA biosynthesis in male DA neurons, and consequently regulates voluntary movement in males **(Healthy)**. Nigral SRY is up-regulated in response to acute toxin treatment in the male SNc. Toxin 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 upregulation?

The precise molecular mechanisms by which SRY upregulation exacerbates DA cell loss remains to be elucidated. Whilst *Sry* transcriptionally activates *Sox9* in the embryonic gonads to initiate the development of testes (Sekido and Lovell-Badge, 2008), the lack of *Sox9* expression in the SNc (Pompolo and Harley, 2001) suggests that *Sry* exerts its actions via an alternate down-stream mechanism in the SNc. In human and rodent neuronal cells, SRY transcriptionally activates *Th* (Milsted et al., 2004) and *Maoa* (Wu et al., 2009) and regulates the expression of DA machinery genes including *Drd2*, *Ddc*, and *Dbh* (Milsted et al., 2004). Thus, Sry up-regulation may dysregulate DA machinery genes and consequently increases DA turnover and oxidative stress, processes which are exacerbated in males (Gillies and McArthur, 2010).

Alternatively, the toxin-induced elevation of *Sry* expression may lead to regulation of a much broader range of binding partners, such as genes involved in PD pathogenesis pathways. Indeed, in the E11.5 embryonic gonads (time point at Sry expression is at maximal), *Sry* binds to promoters of target genes involved in oxidative stress (glutathione peroxidase 4, 6, nitric oxide synthase 3), mitochondrial function (mitochondrial membrane proteins 7, 13, 40) and inflammation (interleukin, tumor necrosis factor) (Li et al., 2014). Moreover, overexpression of SRY in male transgenic mice and ectopic expression of SRY in female transgenic mice livers promoted N-nitrosodiethylamine-induced hepatocarcinogenesis and inflammation, adding further support to the notion that aberrant up-regulation of *Sry* expression is detrimental (Liu et al., 2017).

Another potential mechanism is that SRY could be competing with resident SOX transcription factor(s) and impairs those gene regulatory pathways. For

instance, SRY was found to competitively displace SOX10 and repress regulatory functions on the tyrosine kinase receptor RET, thus predisposing males to Hirschsprung disease, a highly male-biased congenital disorder (Li et al., 2015). In parallel, mice with disrupted *Sox10* function exhibits neurodegeneration in several brain regions including the SN (Anderson et al., 2015). Thus, aberrant upregulation of *Sry* expression could interfere with *Sox10* function in the SNc due to increased competition with SRY and resulting in DA cell death. However, further studies that elucidate the interaction(s) between *Sry* and downstream pathways are crucial to better understand the mechanisms underlying the toxic up-regulation of SRY in male PD.

Future studies

Initial analysis of sex-biased PD pathogenesis gene expression in the current chapter has revealed some important insight into molecular mechanisms underlying the detrimental effect of SRY upregulation. However, an unbiased approach such as RNA and *Sry* chromatin-immunoprecipitation sequencing is essential to identify novel downstream targets activated by *Sry* in male DA neurons. 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* during physiological and pathophysiological situations. 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 and/or sex hormones 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.

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 upregulation in males may also contribute to the male-bias in PD, supporting 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). Thus, normalization of SRY expression may be important for the protection of male DA neurons. Future research aimed at better understanding the role of SRY in male PD is necessary for the development of human SRY inhibitors as a potential novel disease-modifying therapy for PD in males.

CHAPTER 4:

Regulation and function of *SRY* in male attention deficit hyperactivity disorder

4.1 INTRODUCTION

Attention-Deficit Hyperactivity Disorder (ADHD) is the most commonly diagnosed neuropsychiatric and behavioural disorder in children, affecting about 5% of children worldwide (Polanczyk et al., 2007). Initially, ADHD was thought to be solely a childhood disorder, but it is now clear that the symptoms persist into adulthood in a large number of cases (Modesto-Lowe et al., 2012). ADHD is a complex disorder and heterogeneous in its presentation, hence patients are typically diagnosed with one of the three clinical subtypes; predominantly hyperactive and impulsive (e.g. 'often fidgeting' or 'difficulty in awaiting turn'), predominantly inattentive (e.g. 'difficulty attending to tasks for extended periods of time' or 'making careless mistakes in schoolwork') and a combination of the first two subtypes (Berger, 2011, Association, 2013). These symptoms can have lifelong adverse effects on an individual's quality of life. Children with ADHD have poor academic performance, familial issues, and impaired social behaviour (Stein et al., 1995, Greene et al., 1996, DiScala et al., 1998). In addition to the childhood issues that continue into adolescent and adulthood, ADHD has been linked with social impairment, drugs/alcohol abuse, emotional problems, low self-esteem, and employment difficulties (Wilens et al., 2002, Barkley et al., 1990, Biederman et al., 1998). The impaired quality of life experienced by ADHD patients is further exacerbated by comorbid psychiatric disorders such as autism spectrum disorders (ASDs), Tourette's syndrome, obsessive compulsive disorders (OCD) and anxiety (Kooij et al., 2012, Taurines et al., 2010, Thapar et al., 2012). Therefore, a better understanding of the biological mechanisms underlying ADHD is important for improved diagnosis and treatment to prevent or reduce these detrimental outcomes.

Numerous brain imaging studies have demostrated structural abnormalities in ADHD patients (Seidman et al., 2005, Castellanos et al., 2002, Greven et al., 2015). Magnetic resonance imaging (MRI) studies have delineated that ADHD patients have 3-5% smaller total brain volume compared to healthy individuals (Castellanos et al., 2002, Greven et al., 2015, Durston et al., 2004). A recent study has revealed that total grey matter volume is smaller whilst total white matter volume was unchanged in ADHD patients relative to healthy controls (Greven et al., 2015). However, other studies have shown that total white matter volumes were smaller, and white matter regions such as the prefrontal-temporal areas were morphologically abnormal in ADHD patients (Castellanos et al., 2002, Silk et al., 2009). Interestingly, the delay in cortical maturation during early childhood in ADHD patients relative to normal could be correlated with the severity of hyperactive and impulsive symptoms (Shaw et al., 2011). Several brain regions within these networks including the prefrontal cortex (PFC), striatum, globus pallidius and cerebellum were significantly smaller whilst the hippocampus was enlarged in ADHD patients (Valera et al., 2007, Ellison-Wright et al., 2008, Nakao et al., 2011, Frodl and Skokauskas, 2012, Seidman et al., 2005, Plessen et al., 2006). In particular, the PFC appears to be a pivotal region, whereby anomalies in the prefrontal circuitry networks including connections with the basal ganglia, thalamus and amygdala have been linked to inattention, hyperactivity and behavioural disinhibition in ADHD patients (Cao et al., 2013, Cubillo et al., 2011, Bailey and Joyce, 2015, Oldehinkel et al., 2016, Burruss et al., 2000, Plessen et al., 2006). As summarised in Figure 4.1, these connections include the attention network that consist of the frontal, parietal and motor cortices, whilst executive function network primarily involves the PFC and regions within the basal ganglia

necessary for effective goals formulation (Makris et al., 2009). On the other hand, motor regulation networks that act to regulate motor and cognitive behaviours predominantly involve the cortico-striatal and cortico-cerebellar systems (Makris et al., 2009). The typical motor deficits in ADHD modulated by these networks include behaving inappropriately (such as excessive moving or talking) and poor fine motor abilities (Makris et al., 2009). Dysfunctional reward circuitry which mainly consists of the amygdala, striatum, thalamus and cortical areas, is also implicated in ADHD and thought to underlie the deficits in reward guided behaviour and memory consolidation (Makris et al., 2009). In parallel, underactivity of the hypothalamic-pituitary-adrenal axis during stress exposure was displayed in ADHD patients, especially those exhibiting severe hyperactivity (Ma et al., 2011). These findings demonstrate that the brain structure and connectivity of individuals with ADHD is altered in a widespread manner, resulting in a spectrum of symptoms.

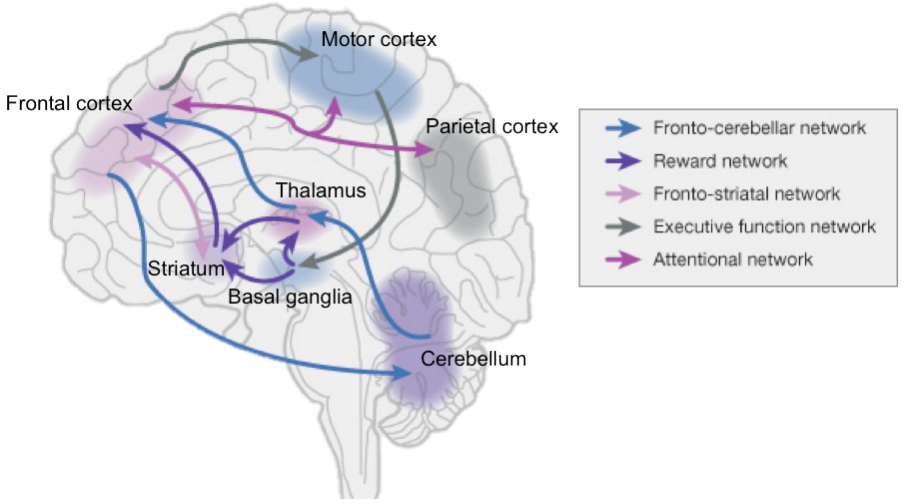


Figure 4.1 Schematic representation of brain regions and functional circuits involved in ADHD (Reproduced from Purper-Ouakil et al, 2011).

Considering the complexity of ADHD, abnormalities in a multiple neurotransmitter system underlie the pathophysiology of ADHD. In particular, symptoms of ADHD has been largely explained by hypo-catecholaminergic, hypo-

GABAergic, as well as hyper-glutamatergic activity in the brain (Arnsten and Pliszka, 2011, Edden et al., 2012, MacMaster et al., 2003). Reduced DA and NA neurotransmission in the PFC and associated subcortical structures such as the striatum have been strongly associated with ADHD (Prince, 2008), in particular with symptoms such as inattention (Ernst et al., 1998, Volkow et al., 2007). The depletion of DA levels is predominantly thought to be a consequence of defects in the pre-synaptic DA machinery such as DA transporter (DAT) and DA autoreceptors. Notably, striatal DAT density and binding in ADHD patients are elevated compared to the healthy controls (Spencer et al., 2007, Fusar-Poli et al., 2012, Dougherty et al., 1999, Krause et al., 2000), although another study has shown that DAT density is reduced (Volkow et al., 2009). Several studies have also shown that pre-synaptic DA receptors, DRD2 and DRD3 have diminished availability in the striatal, midbrain and hypothalamic regions (Volkow et al., 2007, Bellgrove et al., 2006, Volkow et al., 2009, Faraone and Mick, 2010, Gizer et al., 2009). Along these lines, polymorphisms of *DAT1* (Bellgrove et al., 2005, Gizer et al., 2009) and dopamine receptors *DRD4* and *DRD5* (Volkow et al., 2007, Bellgrove et al., 2006, Volkow et al., 2009, Faraone and Mick, 2010, Gizer et al., 2009) have been observed in ADHD patients. Whilst there has been a paucity of functional studies on NA due to the lack of suitable radiotracers (Ding and Fowler, 2005), attention deficits have been associated with a polymorphism of dopamine beta hydroxylase (*DBH*), the principle enzyme that catalyses the conversion of DA to NA (Bellgrove et al., 2006).

GABA and glutamate, which are inhibitory and excitatory neurotransmitters, respectively have been implicated in ADHD particularly in the fronto-striatal circuit (Edden et al., 2012, Bollmann et al., 2015, Naaijen et al., 2017, Maltezos et al.,

2014). Glutamate and glutamine (metabolite of glutamate) ratios are increased in the PFC and striatum of children with ADHD (Ferreira et al., 2009, Courvoisie et al., 2004, Moore et al., 2006, Carrey et al., 2007, MacMaster et al., 2003), whilst decreased within the basal ganglia of adults with ADHD (Maltezos et al., 2014). Furthermore, genetic approaches have revealed that glutamate gene sets were linked to hyperactivity/impulsivity symptoms whilst *GRIN2B*, a glutamate receptor subunit was associated with inattention and hyperactivity symptoms in ADHD (Naaijen et al., 2017, Dorval et al., 2007). In parallel, GABA is found to be reduced in the sensorimotor cortex of ADHD children whilst elevated in the basal ganglia of adults with ADHD (Edden et al., 2012, Bollmann et al., 2015). Morever, GABA levels appeared to be correlated with ADHD symptom scores in adults (Bollmann et al., 2015). Whilst there are substantial evidence linking the catecholamine, GABA and glutamate neurotransmission systems to ADHD, the precise interactions between these systems remain elusive. During physiological conditions, DA regulates glutamatergic cell firing (Surmeier et al., 2007) and GABA release (Tritsch et al., 2012), whilst glutamate and GABA regulates DA release (Whitton, 1997) and DA neuron firing, respectively (Creed et al., 2014). Thus, aberrant levels and/or interactions of these neurotransmitter systems could underlie the pathophysiology of ADHD, and better understanding of which neurotransmitter system(s) is the root cause for dysfunction may lead to better therapies for ADHD.

Given the important role for the catecholamine system in ADHD, the first line therapies for ADHD act to normalize levels of aberrant catecholamine levels in the brain (Pliszka, 2007). In particular, stimulants such as methylphenidate (ie.RITALIN®) and amphetamine, both block the reuptake of DA and NA whilst

amphetamine also promotes DA and NA release into the synapse (Prince, 2008, Sharma and Couture, 2014) (Figure 4.2). Even though these medications are relatively effective in treating the inattention and cognitive deficits (Lakhan and Kirchgessner, 2012), these drugs are associated with side effects such as sleep and appetite disturbances, and the potential for substance abuse following prolonged treatment (Sonuga-Barke et al., 2009, Kollins et al., 2001, Levin and Kleber, 1995).

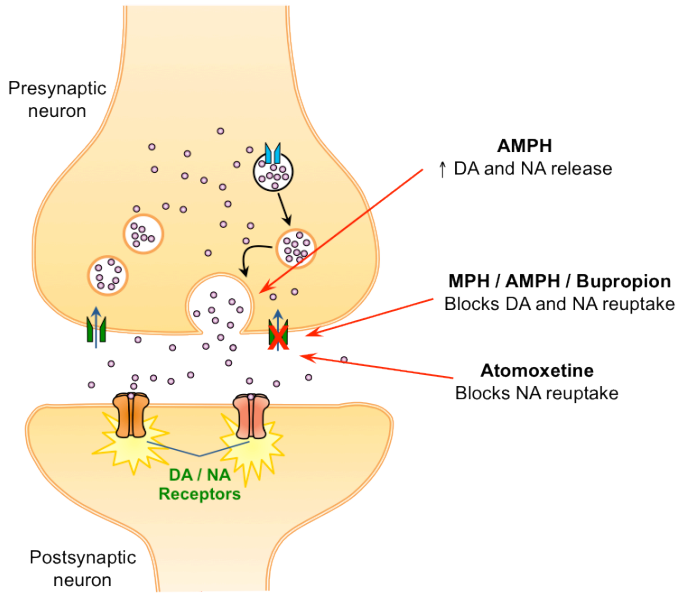


Figure 4.2 Schematic representation of the mechanism of action for ADHD medications. Methylphenidate (MPH), amphetamine (AMPH) and bupropion blocks DA and NA reuptake whilst atomoxetine specifically blocks NA reuptake, leading to increased DA and NA in the synapse. In addition, amphetamine mediates its actions by increasing DA and NA release.

Other drugs used to treat ADHD include non-stimulants (e.g. atomoxetine) and antidepressants (e.g. bupropion). These drugs act to block DA and/or NA reuptake to ameliorate the ADHD symptoms (Sharma and Couture, 2014) (Figure 4.2). However, these drugs are less effective in treating ADHD symptoms compared to

stimulants (Faraone and Glatt, 2010). Thus, it is imperative that novel treatments that better manage or modify the disease progression of ADHD are developed.

Genetic models of ADHD have significantly advanced the understanding of neural mechanisms underlying ADHD. One of the commonly used transgenic models is the DAT-knockout (DAT-KO) mouse. Although the absence of the DAT protein in the DAT-KO mouse is in contrast with the increased striatal DAT density and binding in ADHD patients (Spencer et al., 2007, Fusar-Poli et al., 2012, Dougherty et al., 1999, Krause et al., 2000), this model provides useful information on the consequences of DAT function deficits. For instance, DAT-KO mice displays hyperactivity (Gainetdinov and Caron, 2000, Trinh et al., 2003) as well as cognitive impairment, particular learning and memory deficits (Hironaka et al., 2004, Trinh et al., 2003). Interestingly, DAT-KO mice exhibit the comorbid psychiatric disorders associated with ADHD in humans such as Tourette's syndrome and obsessive compulsive disorder (Berridge et al., 2005). Another popular genetic model of ADHD is the synaptosomal-associated protein of 25kDa (SNAP-25) deficient mutant coloboma mouse that exhibits spontaneous hyperactivity, impulsivity and impaired inhibition (Wilson, 2000, Bruno et al., 2007). Amphetamine was effective in treating the hyperactivity in these coloboma mice as seen in humans, however methylphenidate had no effect (Wilson, 2000, Hess et al., 1996).

However, the most widely used and accepted animal model of ADHD is the spontaneously hypertensive rat (SHR) (Sagvolden et al., 2005, Russell, 2011). Whilst SHR was initially used as a model of hypertension (Okamoto and Aoki, 1963), later studies from Sagvolden et al. showed that SHRs mimic the key symptoms of ADHD i.e. inattention, hyperactivity and impulsivity which are

manifested prior to the onset of hypertension (Knardahl and Sagvolden, 1979, Wultz and Sagvolden, 1992, Wultz et al., 1990, Sagvolden et al., 1992). In addition, the SHRs have a similar pathophysiology to the human ADHD condition, particularly the reduced levels of DA in the PFC and striatum, and GABA in the hippocampus, SN and hypothalamus, as well as increased glutamate in the PFC and striatum (Sterley et al., 2013, Dimatelis et al., 2015, Miller et al., 2014, Czyzewska-Szafran et al., 1989, Russell et al., 1995). Similar to humans, SHRs also exhibit brain structure abnormalities particularly in the PFC and hippocampus (Mignini et al., 2004, Sabbatini et al., 2000). In addition, SHRs have polymorphisms in the *Dat1* gene, as seen in several human studies, however, unlike the human studies, no polymorphisms were found in *Drd4* (Mill et al., 2005). Even though the SHRs might not fully reflect the human condition, this rat model fulfills the most validation criteria compared to other animal models of ADHD (Russell, 2007, Sagvolden et al., 2005). Specifically, the SHR model mimics the most behavioural characteristics, genetics, and neurobiology of ADHD as well as exhibits similarities with ADHD clinical cases (Russell, 2007, Sagvolden et al., 2005). Therefore, it is unsurprising that the SHR model remains the most widely used to study ADHD.

Whilst the cause(s) of ADHD remains elusive, the pronounced male bias in ADHD provides important clues to the aetiology of the disorder. ADHD is more commonly diagnosed in males than females, with a ratio of 3:1 or even higher (Willcutt, 2012, Davies, 2014). Male ADHD patients predominantly exhibit symptoms of hyperactivity and impulsivity, whilst female ADHD patients tend to be more inattentive (Davies, 2014, Lahey et al., 1994). Furthermore, males generally have more severe ADHD symptom scores (Arnett et al., 2015) which could be

attributed to the more severely impaired cerebellar-prefrontal-striatal networks in male ADHD patients (Valera et al., 2010). Clinical studies have hinted that sex could have an effect on stimulant medication in ADHD patients, although this remains inconclusive due to the limited number of studies (Cornforth et al., 2010). These sex differences are also mimicked in animal models of ADHD, specifically; male SHRs have increased hyperactivity levels relative to female SHRs, whilst female SHRs are more inattentive compared to male SHRs (Berger and Sagvolden, 1998). Hence, better understanding of the biological factors underlying the male-bias in ADHD will be integral to understanding the cause(s) of ADHD and developing novel therapies with optimal effectiveness in each sex.

One biological factor that has been proposed to underlie the male bias in ADHD is the male sex-hormone testosterone. Despite the important role that prenatal testosterone plays in masculinising the developing male brain (Morris et al., 2004), overexposure to prenatal testosterone could increase the susceptibility of males to neurodevelopmental disorders such as ADHD and autism (Hines, 2008, Baron-Cohen et al., 2011). Multiple studies reported that males diagnosed with ADHD possessed lower finger-length ratios (a surrogate measure of prenatal testosterone exposure) (Martel et al., 2008, de Bruin et al., 2006), despite this measure of testosterone being somewhat controversial. In support, children with hyperandrogenism have increased risk of developing ADHD (Mueller et al., 2010). Aside from sex hormones, emerging evidence suggests that sex chromosome genes could also underlie the male bias in ADHD. Neuroprotective effects by X-inactivation gene escapees in females (ie. females will have twice the gene expression compared to males) have been proposed as a potential genetic mechanism involved. Individuals with Turner syndrome, whereby they possess a

45, XO karyotype (i.e. only one X-chromosome), have been linked to increased vulnerability to disorders associated with impaired memory, attention, and social interaction such as ADHD (Russell et al., 2006). A neuroimaging study showed that both 45, XO females and 46, XY males have larger amygdala (involved in emotion and social learning) volumes than 46, XX females, suggesting that having a single X-chromosome could be a risk factor. On the other hand, over dosage of Y-chromosomes has proven to be deleterious, whereby several cases of 47, XYY and 48, XXYY boys were associated with ADHD (Ross et al., 2009, Ruud et al., 2005, Tartaglia et al., 2012). Whilst it is unclear which sex-specific genes are crucial for driving the sex differences in ADHD, emerging studies indicate that the Y-chromosome gene, *SRY,* represents an excellent candidate for the sex-related endophenotype in ADHD (Dewing et al., 2006, Czech et al., 2012).

Whilst *SRY* is a key male sex-determining gene in the male gonads (Koopman et al., 1990, Sekido et al., 2004, Sinclair et al., 1990), there is a clear temporal segregation in the functionality of *SRY* in the male brain (Hacker et al., 1995, Jeske et al., 1995, Capel et al., 1993, Mayer et al., 2000). Brain mapping studies in rodent and human demonstrated that SRY is expressed in midbrain regions such as the substantia nigra pars compacta (SNc), substantia nigra pars reticulate (SNr), ventral tegmental area (VTA), and hypothalamus (Mayer et al., 1998, Lahr et al., 1995, Dewing et al., 2006, Milsted et al., 2004). In the male SNc and VTA, SRY protein co-localises with TH-positive neurons (Czech et al., 2012, Dewing et al., 2006). In keeping with the localization of SRY in DA neurons, work from Chapter 2 and others demonstrate SRY transcriptionally regulates DA machinery genes such as *tyrosine hydroxylase* (*TH*), *monoamine oxidase-a* (*MAO-A*) and dopamine D2 receptor (*DRD2*) *in vivo* and *in vitro* (Czech et al.,

2012, Milsted et al., 2004, Wu et al., 2009). In addition to midbrain DA neurons, SRY also co-localises with GABAergic neurons in both human (Czech et al., 2012) and rat SNr (Dewing et al., 2006) in males. Moreover, results in Chapter 2 demonstrate that SRY positively regulates the GABA synthesis enzymes, *Gad1* and *Gad2* in the rat SN, indicating a role for SRY in modulating DAergic and GABAergic functions in the male brain.

Considering the presence and function of *Sry* in male DA and GABA systems, dysregulation of *Sry* could affect DA and GABA transmission in the male brain, and consequently impact cognition and behaviour. Hence, dysregulated *Sry* expression could underlie male susceptibility to ADHD. I hypothesise that:

i) **Brain SRY expression is dysregulated in male ADHD patients**

ii) **Normalizing brain SRY expression in male ADHD will reduce brain chemical abnormalities and consequently symptoms of ADHD.**

To test these hypotheses, the main aims of this chapter are:

1) To assess the regulation of brain Sry expression in the SHR model of ADHD.
2) To determine the function of brain Sry in the SHR model of ADHD.

4.2 MATERIALS AND METHODS

SHR model of ADHD

The spontaneously hypertensive rat (SHR) model is by far the most widely used and accepted animal model of ADHD (Russell, 2011). SHRs mimic the key symptoms (i.e. inattention, hyperactivity and impulsivity) and pathophysiology (dysfunctional catecholamine and GABAergic transmission) of the human condition (Sagvolden et al., 2005, Sterley et al., 2013). SHRs also exhibit sex differences in attention and motor behavior (Berger and Sagvolden, 1998).

Study design

Aim 1. Assess the regulation of SRY expression in the SHR model of ADHD

To determine whether brain *Sry* expression is dysregulated in the SHR model of ADHD, *Sry* mRNA expression from various brain regions were compared between male SHRs and wild-type Wistar Kyoto (WKY) rats (see Figure 4.3). SHR and WKY rats aged 4 and 12 weeks were used to determine any age-dependent effects. Thus, the animals were divided into the following groups:

Group 1: Male WKY (4-week old)
Group 2: Male SHR (4-week old)
Group 3: Male WKY (12-week old)
Group 4: Male SHR (12-week old)

Locomotor activity of the SHRs and WKYs was assessed using the open field test. At the end of the open field test, the animals were culled and various brain regions were processed for RNA extraction for qRT-PCR measurements.

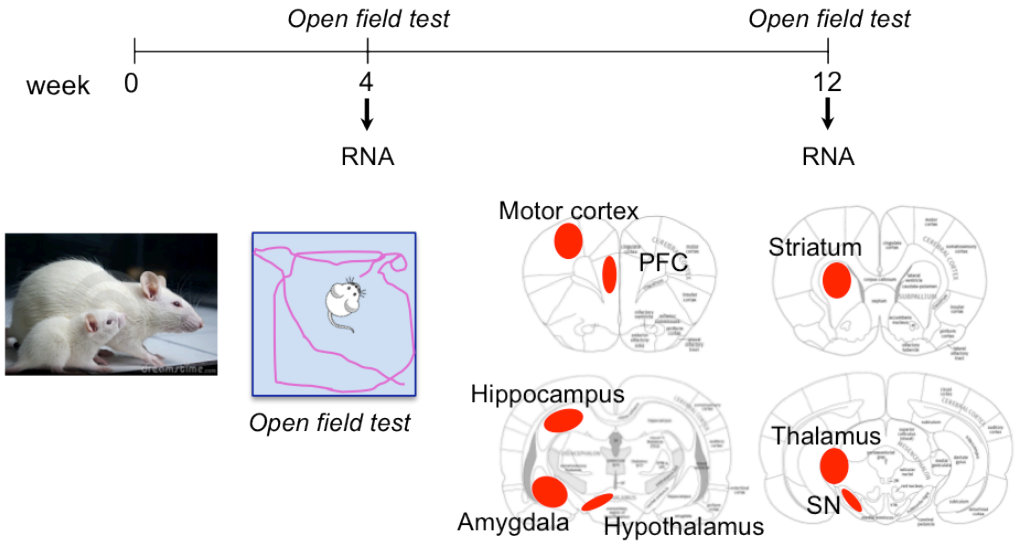


Figure 4.3 Schematic outline of Aim 1. Locomotor activity was assessed in 4 and 12-week old male SHRs and WKYs by open field test. Following the open field test, rats were culled and RNA was extracted from various brain regions for Sry mRNA measurements.

Aim 2. Determine the function of brain Sry in the SHR model of ADHD.

To determine the function of Sry in the SHR model of ADHD, the effect of reducing brain *Sry* expression on locomotor and cognitive behaviours were assessed in male SHRs and WKYs (see Figure 4.4). Brain Sry expression was reduced in male rats by repeated intracerebroventricular (ICV) Sry ASO infusion. At 7 days following the implantation of ICV cannula, male SHRs and WKYs were infused daily with ICV *Sry* ASO (or SO) for 7 days. A group of female WKYs were also used to account for any off-target effects of the Sry ASO. Thus, the animals were divided into the following groups:

Group 1: Male WKY (12-week old) / SO
Group 2: Male WKY (12-week old) / ASO
Group 3: Male SHR (12-week old) / SO
Group 4: Male SHR (12-week old) / ASO
Group 5: Female WKY (12-week old) / SO
Group 6: Female WKY (12-week old) / ASO

Motor and cognitive behaviours were assessed by the open field, novel object recognition (NOR) and spontaneous alternation tests at day 0 (pre-infusion) and 7 days post ASO infusion. At the end of the last behavioural test on day 7, the rats were culled, and RNA was isolated from the hippocampus and PFC for *Sry* mRNA measurements via qRT-PCR.

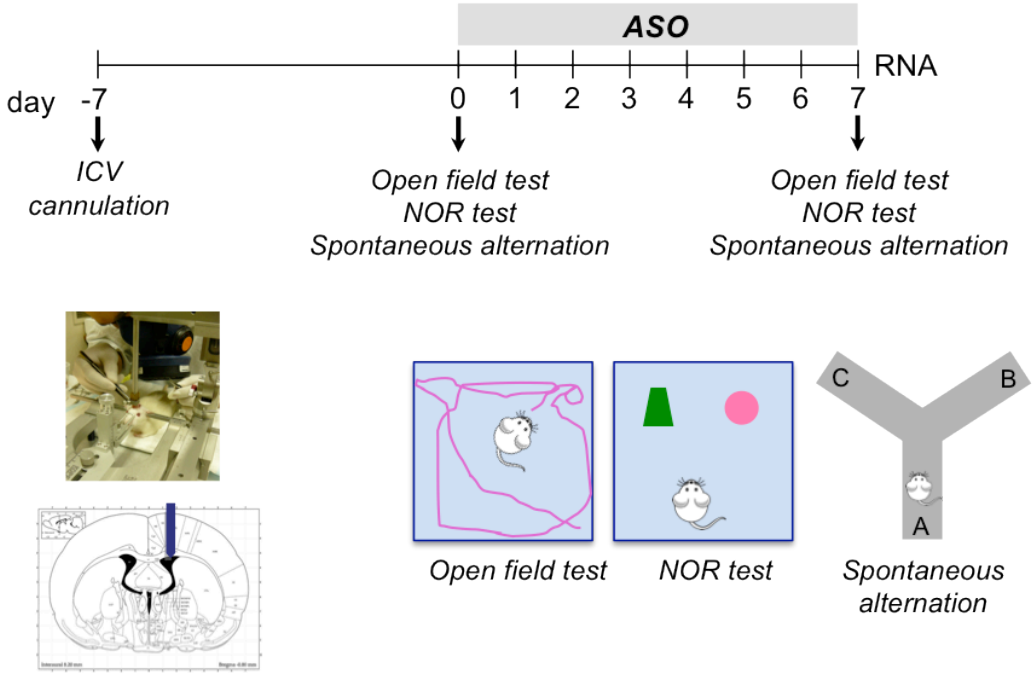


Figure 4.4 Schematic outline of experimental design for Aim 2. Stereotaxic surgery was performed to implant a intracerebroventricular (ICV) cannula above the right ventricle. At 7 days post surgery, *Sry* ASO (or SO) was infused daily for 7 days via the ICV cannula. Motor and cognitive behaviour were tested by the open field, novel object recognition (NOR) and spontaneous alternation tests at days 0 and 7 of *Sry* ASO (or SO) infusion. Following the last behavioural test, rats were culled, and RNA was extracted from various brain regions for mRNA measurements by qRT-PCR.

Intracerebroventricular cannulation in WKYs and SHRs

Unilateral guide cannula (22 gauge, Plastics One) directed at the right lateral ventricle was implanted at 0.8mm posterior, 1.5mm lateral from bregma, and

1.4mm 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 SRY antisense oligonucleotide (ASO) infusions

Brain *Sry* expression in male rats was reduced by repeated infusions of *Sry* ASO via the intracerebroventricular (ICV) cannula (Supplementary table 2). The *Sry* ASO used was a cocktail of three distinct ASOs directed against rat *Sry* mRNA added in equal proportions, as described previously (Dewing et al., 2006). ASOs were HPLC-purified (Invitrogen, Australia) and dissolved in artificial cerebrospinal fluid (aCSF) vehicle to a final concentration of 2μg/μL. 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 7 consecutive days.

Behavioural testing

Acclimatisation

Several measures were taken to minimize the level of stress of the rats before and during the behavioural tests. Three days prior to any behavioural testing, rats are handled for a minute daily. One day prior to behavioural testing, rats were allowed to acclimatise in the lab. On the day of behavioural testing, rats are placed in the lab at least one hour before the first behaviour test to acclimatise.

Open field test

The open field test measures general locomotor activity levels and anxiety in rodents. This test was conducted in a 60cm x 60cm x 50cm open field area. The rat was placed individually into the arenas for a 5 or 10 minutes testing period. A

video camera above the arena recorded the locomotion of the rats, and various parameters of movement (e.g. velocity, distance travelled) were analysed by the TopScan programme (CleverSys).

Novel object recognition test

The novel object recognition test was evaluated in a 60cm x 60cm x 50cm open field area. The objects used during the test were 330ml glass bottles for the familiar objects (FO) and T75 tissue culture flasks for the novel object (NO), which were stuck to the floor of the open field box. Rats were acclimatised in the open field box during the open field test performed prior to the NOR test. The NOR test consisted of three trials – habituation, familiarization and test trials. During the habituation trial, the rat was placed in the open field and allowed to freely explore for 10 minutes. 1 hour after the habituation trial, the familiarization trial was performed for 10 minutes (Data shown in Supplementary figure 2). Each rat was placed in the open field, containing two identical 330ml glass bottles placed at adjacent corners, located 15 cm from the open field walls and 30 cm between the bottles. The test trial was performed 1 hour later where one of the familiar objects (glass bottles) was replaced with a T75 tissue culture flask and each animal was allowed to explore for 5 minutes. The discrimination index was calculated by the difference in exploration time between the novel and familiar objects divided by the total object exploration time ((NO-FO)/(NO+FO)).

Spontaneous alternation test

Spontaneous alternation was determined by the Y-maze test that assesses spatial memory and attention. The Y-maze was performed one hour after the novel object recognition test. The apparatus consisted of three arms made of grey plastic that

are 50cm long, 15cm wide and 30cm high extending from a central platform. Each rat was placed in one of the arms and allowed to move freely between the three arms for 8 minutes. Arm entry was defined as the entry of all four paws into one arm. The Topscan software (CleverSys) tracked the movement of rats via a camera mounted directly above the maze. Alternation behavior was defined as consecutive entry into three different arms, with stepwise combinations in the sequence. Maximum number of possible alternations was calculated by subtracting two from the total of arm entries. Percentage of alternation was calculated by (actual alternations/possible alternations) x 100.

Histology and biochemical Assays

Rat brains were isolated fresh and processed for qRT-PCR. Coronal sections were cut serially through the prefrontal cortex, striatum, hippocampus, hypothalamus, SN, thalamus, motor cortex and amygdala (Figure 4.3), and stored at -80°C. Between each series, a 200μm slab was collected and brain regions were isolated for RNA processing using TRI-Reagent (Sigma Aldrich) according to the manufacturer's recommendations.

Quantitative RT-PCR

Total RNA (100-300ng) isolated from selected brain regions associated with ADHD using TRI-Reagent (Sigma Aldrich) was reverse-transcribed into cDNA (QuantiTect Reverse Transcription kit, Qiagen) and equal amount of cDNA template was added to QuantiNova SYBR Green master mix (Qiagen) using primers listed in Supplementary Table 1. The relative level of mRNA was interpolated from a standard curve prepared by serially diluting the cDNA reaction. Specificity of PCR product formation was confirmed by monitoring melting peaks.

All quantitative PCR reactions were conducted in triplicates. Final values represent fold change of gene expression relative to the housekeeping genes *TATA Box Binding Protein* (*TBP*).

Statistical analysis

All values are expressed as the mean ± S.E.M. All data was analysed using tools within Graphpad Prism 5. Comparisons of two experimental groups were performed using two-tailed unpaired Student t-test. Multiple comparisons of more than two experimental groups were performed using two-way ANOVA and Tukey's *post hoc* tests. Motor behaviour studies of the treatment groups across the days of testing were also analysed by two-way ANOVA and Tukey's *post hoc* test. 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.

4.3 RESULTS

Male SHRs are significantly more hyperactive than male WKYs

Previous studies have demonstrated that SHRs exhibit the key features of ADHD particularly inattention, hyperactivity and impulsivity (Sagvolden et al., 2005). To validate these findings, the hyperactivity of SHRs was characterized using the open field test. As illustrated by the trace images obtained from the open field test (Figure 4.5A), male SHRs exhibit significantly higher locomotor activity in the arenas compared to the male WKYs for both age groups. Two-way ANOVA revealed an overall significant difference in total distance ($F_{(1,28)}$=111.4, p<0.0001, Figure 4.5B) and velocity ($F_{(1,28)}$=103.6, p<0.0001, Figure 4.5B) travelled between the SHRs and WKYs. However, no significant age X strain interaction was observed in total distance ($F_{(1,28)}$=0.3261, p=0.57, Figure 4.5B) and velocity travelled ($F_{(1,28)}$=0.5471, p=0.47, Figure 4.5B). Post-hoc Tukey analysis revealed that male SHRs at both 4 and 12 weeks travelled far greater total distance (15.8 ± 1.0m and 13.4 ± 0.8m, 4w and 12w SHR, respectively, Figure 4.5B) compared to the male WKYs (4.4 ± 1.4m and 3.6 ± 0.5m, 4w and 12w WKY, respectively, p<0.0001 vs SHR, Figure 4.5B). Similarly, male SHRs travelled at a significantly higher velocity (0.056 ± 0.004m/s and 0.046 ± 0.002m/s, 4w and 12w SHR, respectively, Figure 4.5B) compared to the male WKYs (0.017 ± 0.005 and 0.013 ± 0.002m/s, 4w and 12 WKY, respectively, p<0.0001 vs SHR, Figure 3.5B). However, no age-dependent differences in locomotor activity were observed in the WKYs and SHRs (Figure 3.5B). Overall, the data from the open field test revealed that the SHRs are significantly more hyperactive than the WKYs, which supports previous studies (Sagvolden et al., 2005).

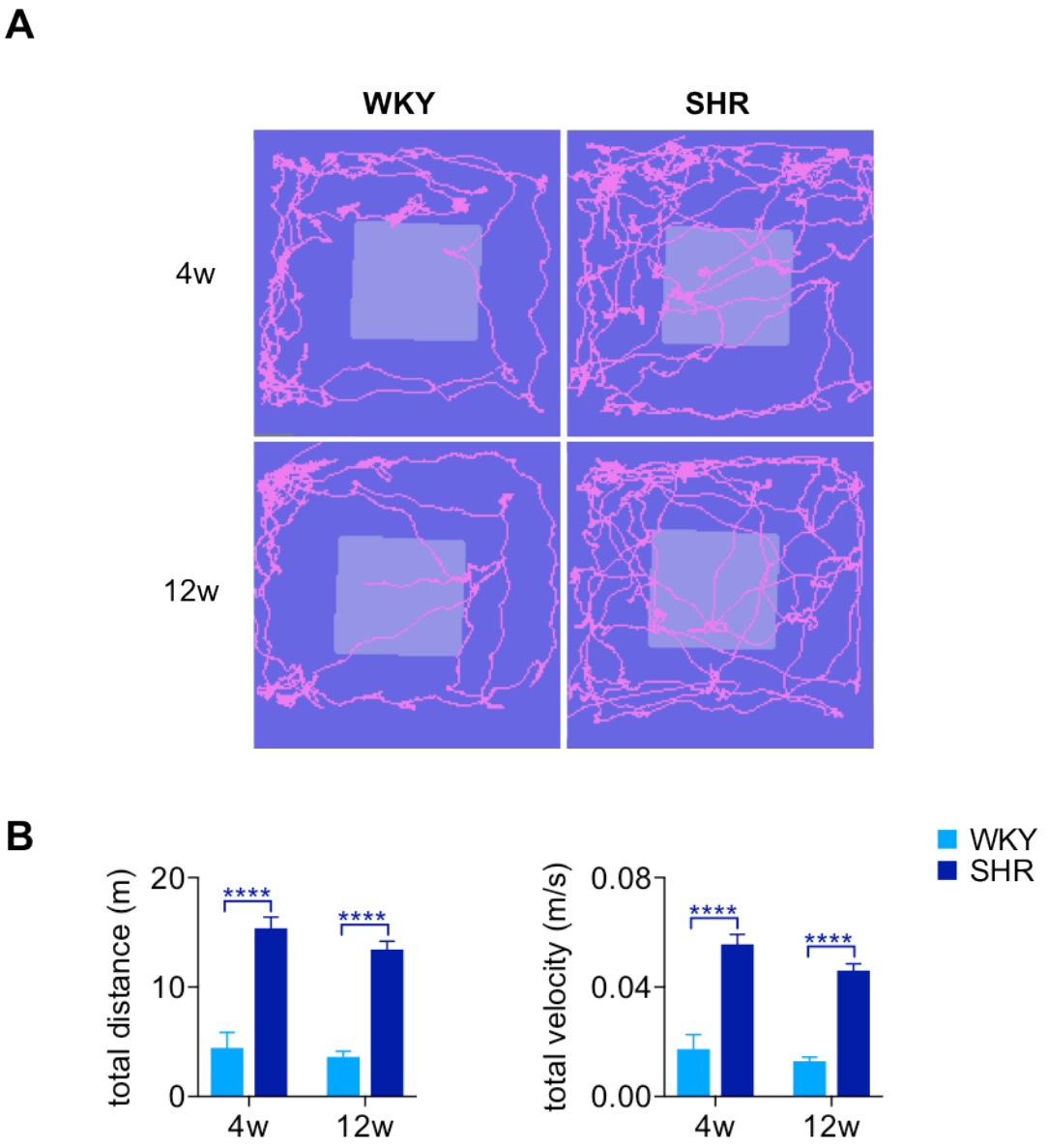


Figure 4.5 Total locomotor activity of 4 and 12 week old male WKYs and SHRs in the open field test. A) Trace image taken from WKYs and SHRs following 5 minutes in the open field test **B)** Total distance travelled and velocity were analysed at the end of the test (n=8/group, mean ± S.E.M. two-way ANOVA ****p<0.0001, compared to WKY in the same age group).

Sry mRNA expression is reduced in the male SHR brain

To determine whether *Sry* expression is dysregulated in an animal model of ADHD, *Sry* mRNA was measured in brain regions i) associated with ADHD symptoms or ii) known to express *Sry* in male SHRs and WKYs. Two-ANOVA analysis demonstrated significant strain differences in all the brain regions measured; (Figure 4.6A to F). Significant age differences were also found in the PFC (Figure 4.6A), striatum (Figure 4.6B), hippocampus (Figure 4.6C), SN (Figure 4.6D), hypothalamus (Figure 4.6E) and thalamus (Figure 4.6F). Moreover, age x strain interaction was present in the striatum (Figure 4.6B), hypothalamus (Figure 4.6E), SN (Figure 4.6D), thalamus (Figure 4.6F) and amygdala (Figure 4.6H).

Post-hoc analysis of strain revealed that *Sry* mRNA expression in 4 and 12-week male SHRs were markedly reduced in the PFC (11 and 21% of WKY, $p<0.05$ and $p<0.001$, respectively, Figure 4.6A), striatum (16 and 17% of WKY, $p<0.05$ and $p<0.001$, respectively, Figure 4.6B), SN (18 and 25% of WKY, $p<0.01$ and $p<0.001$, respectively, Figure 4.6D), hypothalamus (10 and 12% of WKY, $p<0.05$ and $p<0.001$, respectively, Figure 4.6E) and thalamus (27 and 50% of WKY, $p<0.0001$ and $p<0.05$, respectively, Figure 4.6F) compared to the 4 and 12-week WKYs. Furthermore, *Sry* mRNA expression was reduced in 4-week male SHRs for the motor cortex (11% of WKY, $p<0.05$, Figure 4.6G) and amygdala (15% of WKY, $p<0.05$, Figure 4.6H), and in 12-week male SHRs for the hippocampus (41% of WKY, $p<0.05$, Figure 4.6C) with respect to the WKYs.

Post-hoc comparisons between age groups revealed a developmental increase in brain *Sry* mRNA expression in the male WKYs, with increases observed in the 12-week WKY PFC (223% of 4w, $p<0.01$, Figure 4.6A), striatum (288% of 4w, $p<0.001$, Figure 4.6B), hippocampus (213% of 4w, $p<0.05$, Figure

4.6C), SN (200% of 4w, $p<0.01$, Figure 4.6D), and hypothalamus (245% of 4w, $p<0.01$, Figure 4.6E) compared to the 4 week WKYs. However, the *Sry* expression in the thalamus showed a reduction with increase in age at 12 weeks (22% of 4w, $p<0.0001$, Figure 4.6F). In contrast to the male WKYs, the age-dependent increase in *Sry* expression was absent in the male SHR brain, although a significant reduction in the thalamus was observed for the 12-week SHRs compared to 4 week SHRs (33% of 4w, $p<0.05$, Figure 4.6F).

Together, these findings reveal that i) *Sry* mRNA expression is significantly reduced in the male SHRs compare to wild type WKYs, particularly in brain regions associated with ADHD symptoms at both age groups and ii) the developmental increase of *Sry* expression in the male WKYs is absent in the SHRs. These findings suggest that stunted *Sry* expression in the male brain could contribute to male ADHD.

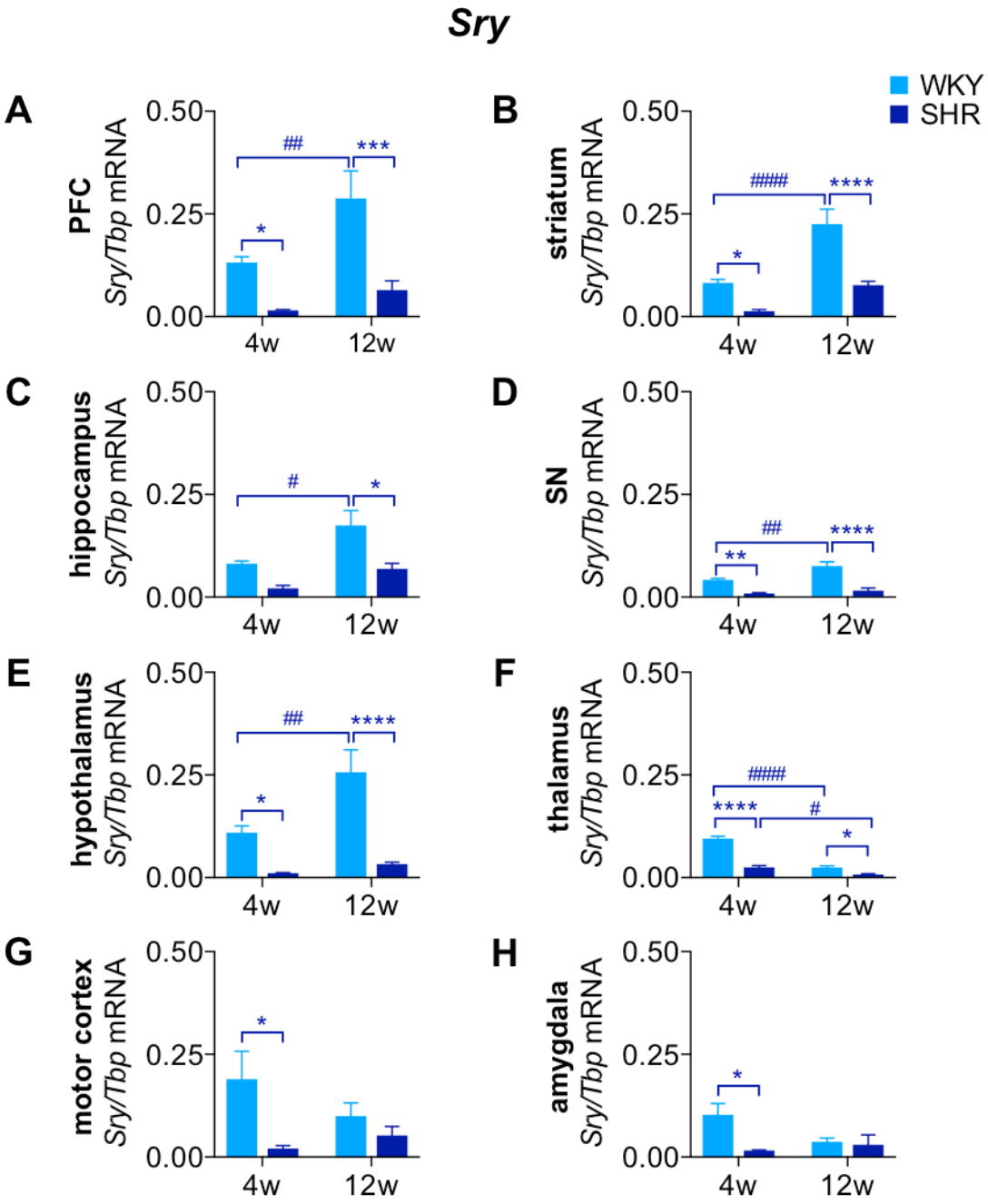


Figure 4.6 *Sry* mRNA expression in 4 and 12 week old male WKY and SHR A) PFC, **B)** striatum, **C)** hippocampus, **D)** SN, **E)** hypothalamus, **F)** thalamus, **G)** motor cortex and **H)** amygdala (n=4-5/group, mean ± S.E.M. two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to WKY in the same age group; #p<0.05, ##p<0.01, ####p<0.001, compared to 4w of the same strain).

Gadd45γ mRNA expression is not altered in the SHR brain

Previous studies have shown that induction of SRY expression is mediated by the *MAPK-GADD45γ* signaling pathway in embryonic gonads (Gierl et al., 2012, Warr et al., 2012). Thus, *Gadd45γ* was measured in 4 and 12 week-old SHRs and WKYs to determine whether *Sry* is regulated in the same manner. Two-way ANOVA analysis revealed that there were no significant differences in *Gadd45γ* expression between the SHRs and WKYs (Figure 4.7A to D). Significant age difference in *Gadd45γ* mRNA expression was observed in the striatum (Figure 4.7B) and hippocampus (Figure 4.7C), although post-hoc Tukey analysis did not show any significant age differences between any groups. Therefore, these results suggest that the reduction of brain *Sry* mRNA expression in male SHRs is not associated with changes in *Gadd45γ* expression, suggesting a Gadd45γ-independent mechanism of regulation in male SHRs.

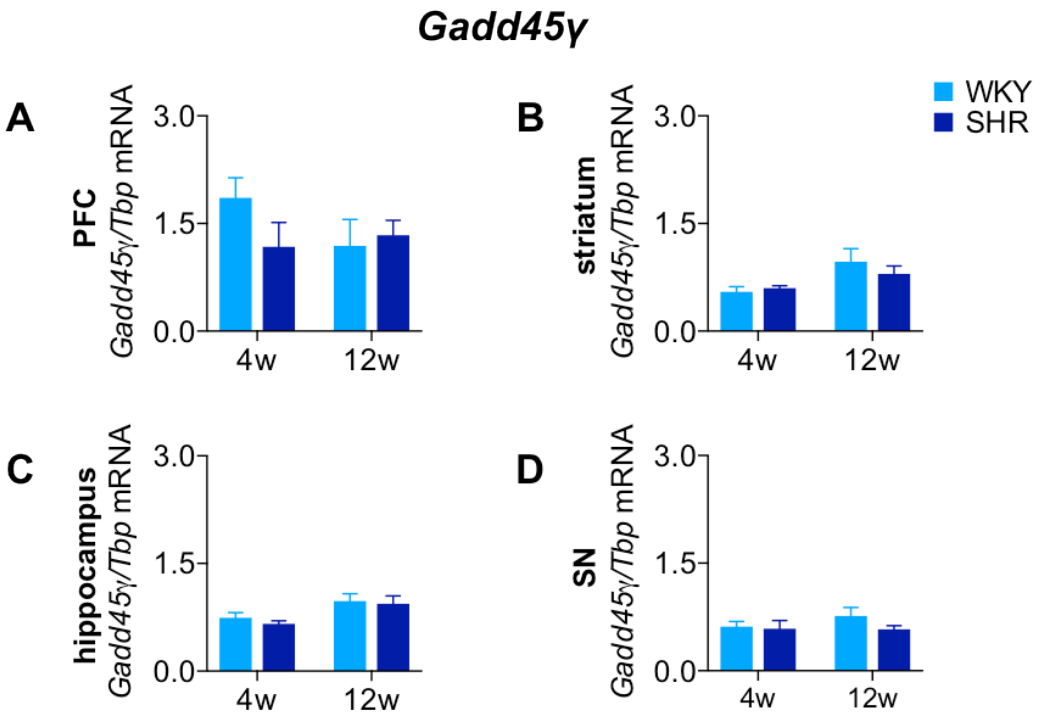


Figure 4.7 ***Growth arrest and DNA damage inducible γ (Gadd45γ)*** **mRNA expression in 4 and 12 week old male WKY and SHR A)** PFC, **B)** striatum, **C)** hippocampus and **D)** SN (n=4-5/group, mean ± S.E.M. two-way ANOVA).

Neurotransmitter synthesis enzymes are altered in the male SHR brain

Preclinical and clinical studies have demonstrated that ADHD symptoms could arise from deficits in neurotransmitter systems, particularly, the hypofunction of catecholamines and GABA as well as hyperfunction of glutamate (Arnsten and Pliszka, 2011, Edden et al., 2012, MacMaster et al., 2003).

To determine whether the reduced Sry mRNA expression in male SHRs is associated with hypofunction of the catecholamine system, the synthesis enzymes, *tyrosine hydroxylase* (*Th*) and *Dbh* mRNA expressions were measured. Two-way ANOVA revealed significant age differences in *Th* mRNA expression in the SN and hypothalamus (Figure 4.8A and C) as well as significant age X strain differences were present in the SN (Figure 4.8A). *Post-hoc* analysis revealed that *Th* mRNA expression was significantly lower in the SN of 12-week SHRs compared to 12-week WKYs (54% of WKY, $p<0.05$, Figure 4.8A). *Post-hoc* test revealed a significant increase in *Th* mRNA expression in the SN (297% of 4w, $p<0.001$, Figure 4.8A) and hypothalamus (483% of 4w, $p<0.001$, Figure 4.8C) of 12-week WKYs relative to 4-week WKYs, indicating a developmental increase in *Th* mRNA expression. In parallel, *Dbh* mRNA expression showed strain differences in the PFC and SN (Figure 4.8B and D) as well as age differences in the SN (Figure 4.8B). *Dbh* mRNA expression was significantly higher in the SN of 4-week SHRs compared to WKYs (200% of WKY, $p<0.01$, Figure 4.8B). Furthermore, there was a significant reduction in *Dbh* mRNA expression in the SN of 12-week old relative to the 4-week SHRs (7.5% of 4w, $p<0.001$, Figure 4.8B), suggesting an age-dependent effect. In summary, these results show that the developmental increase in midlbrain *Th* mRNA levels is absent in the SHRs.

To determine whether the reduced *Sry* mRNA expression in male SHRs could be associated with altered GABA and/or or glutamate levels, *Gad1* (synthesises cytosolic GABA) and *Gad2* (synthesises synaptosomal GABA) and *Gls* (synthesises glutamate) were measured (Soghomonian and Martin, 1998). The two-way ANOVA analysis showed significant strain differences of *Gad1* mRNA expression in the striatum (Figure 4.9B), hippocampus (Figure 4.9C), SN (Figure 4.9D) and thalamus (Figure 4.9F). Post-hoc Tukey's analysis showed that *Gad1* mRNA expression is significantly higher in the 4-week SHR hippocampus (307% of WKY, $p<0.01$, Figure 4.9C) and thalamus (268% of WKY, $p<0.05$, Figure 4.9F) compared to the 4-week WKYs. Similarly, Gad1 mRNA expression was elevated in the striatum (171% of WKY, $p<0.05$, Figure 4.9B) and SN (232% of WKY, $p<0.01$, Figure 4.9D) of 12-week SHRs compared to WKYs. Post-hoc Tukey test revealed that *Gad1* mRNA expression in 12-week SHRs was significantly higher in the SN (224% of 4w, $p<0.05$, Figure 4.9D) whilst lower in the thalamus (16% of 4w, $p<0.01$, Figure 4.9F) compared to 4-week SHRs. Unlike *Gad1*, two-way ANOVA analysis of *Gad2* mRNA expression did not reveal any strain differences between the SHR and WKYs. However, an age-dependent increase was observed across all the brain regions except the thalamus (Figure 4.10A to F). Therefore, these results suggest that *Gad1* expression is increased in SHRs compared to WKYs whilst no strain differences in *Gad2* expression were observed. Two-way ANOVA analysis of *Gls* mRNA expression did not show any strain differences between the SHR and WKYs. However, an age-dependent increase was observed across all the brain regions excluding the SN and thalamus (Figure 4.11A to F). These findings demonstrate that *Gls* mRNA expression does not differ between WKYs and SHRs.

In summary, these findings indicate that the reduced brain Sry expression in male SHRs is associated with dysregulation of brain *Th* and *Gad1* mRNA expression.

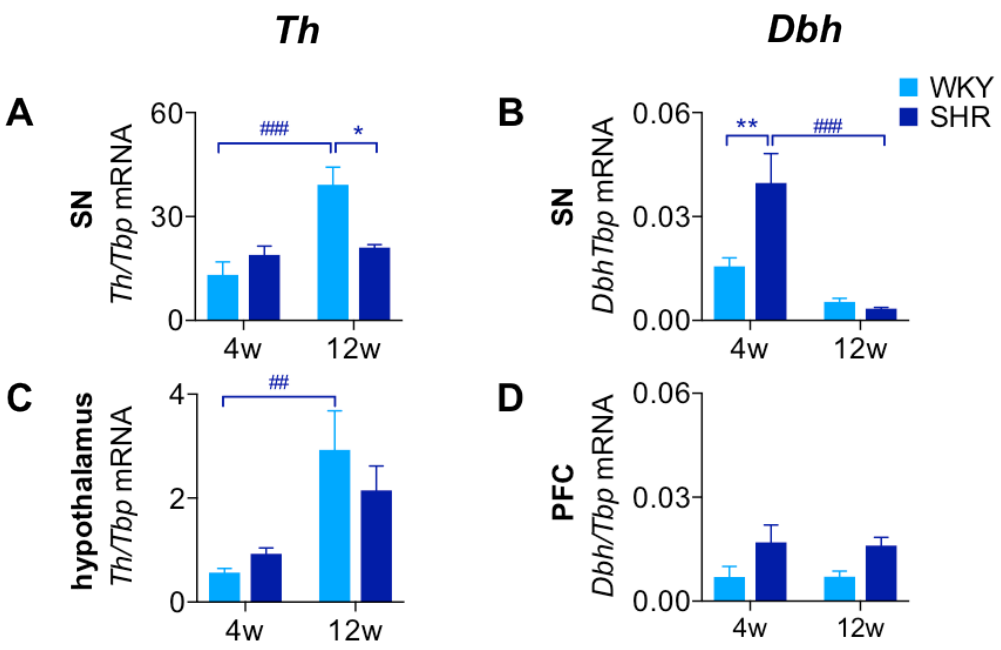


Figure 4.8 ***Tyrosine hydroxylase (Th) and dopamine beta hydroxylase (Dbh)*** **expression in 4 and 12 week old male WKY and SHR. A)** *Th* and **B)** *Dbh* mRNA in the SN, **C)** *Th* mRNA in the hypothalamus and **D)** *Dbh* mRNA in the PFC (n=5/group, mean ± S.E.M. two-way ANOVA, *p<0.05, **p<0.01, compared to WKY in the same age group; ##p<0.01, ###p<0.001, compared to 4w of the same strain).

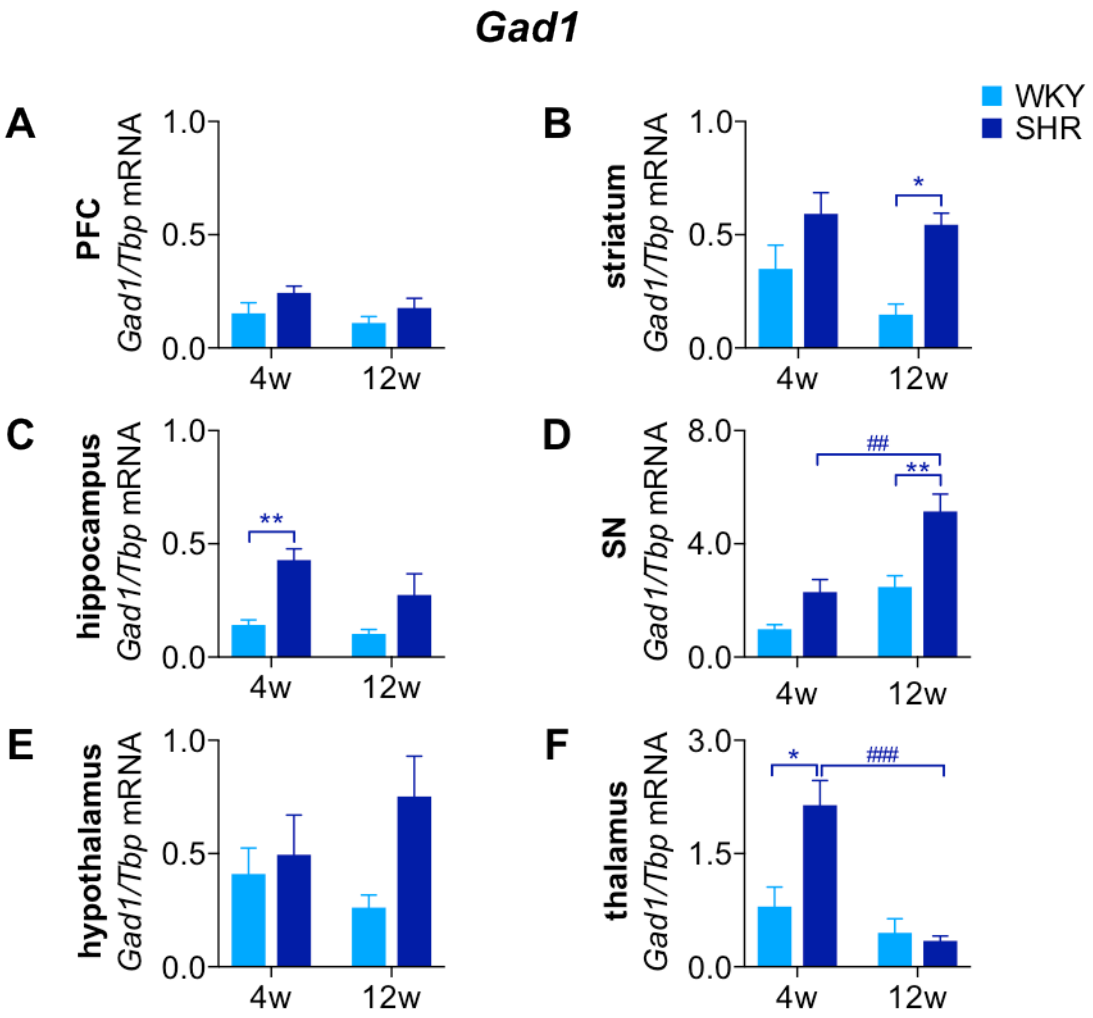


Figure 4.9 *Glutamate decarboxylase 1 (Gad1)* mRNA expression in 4 and 12 week old male WKY and SHR A) PFC, **B)** striatum, **C)** hippocampus, **D)** SN, **E)** hypothalamus and **F)** thalamus (n=4-5/group, mean ± S.E.M. two-way ANOVA, *p<0.05, **p<0.01, compared to WKY in the same age group; ##p<0.01, ###p<0.001, compared to 4w of the same strain).

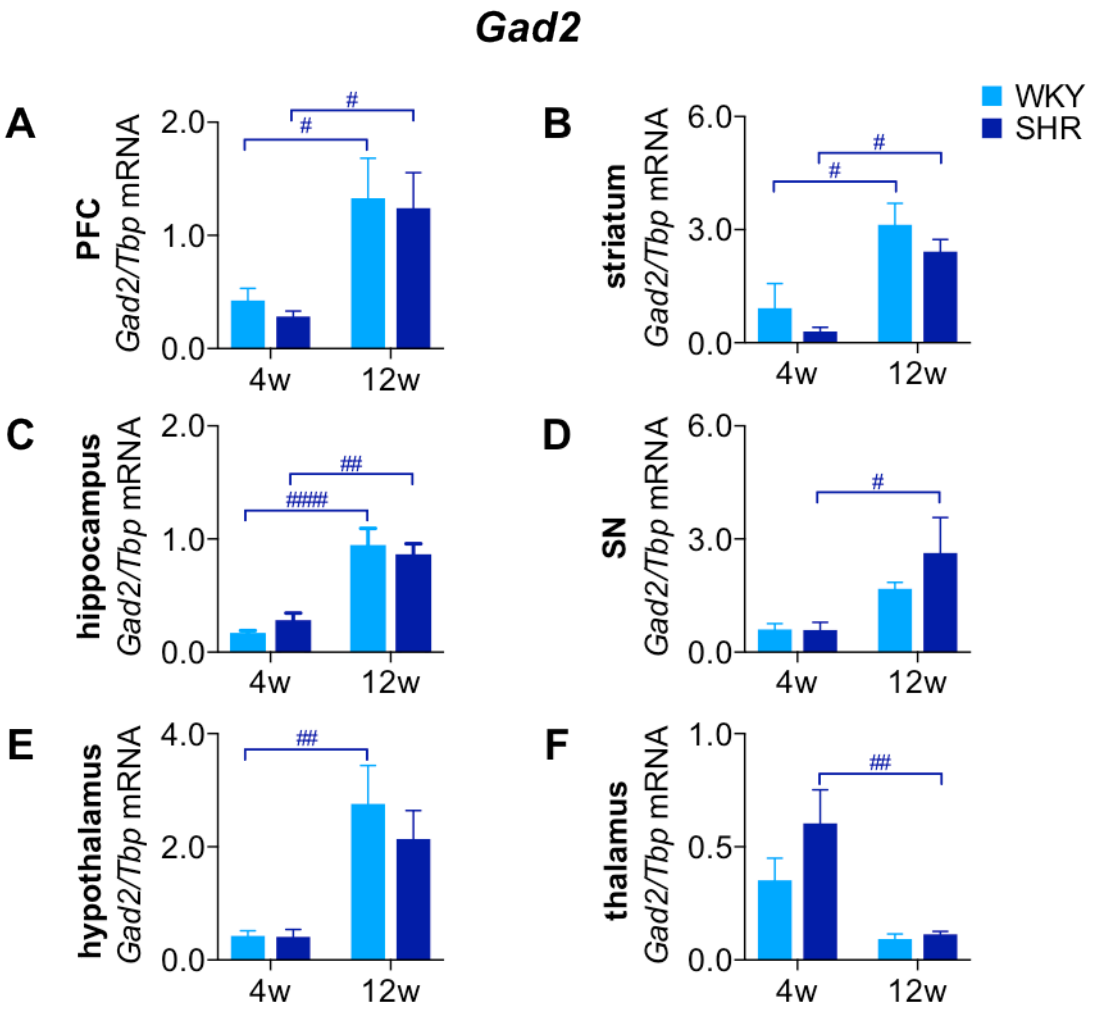


Figure 4.10 *Glutamate decarboxylase 2 (Gad2)* mRNA expression in 4 and 12 week old male WKY and SHR A) PFC, **B)** striatum, **C)** hippocampus, **D)** SN, **E)** hypothalamus and **F)** thalamus (n=4-5/group, mean ± S.E.M. two-way ANOVA, #p<0.05, ##p<0.01, ####p<0.0001, compared to 4w of the same strain).

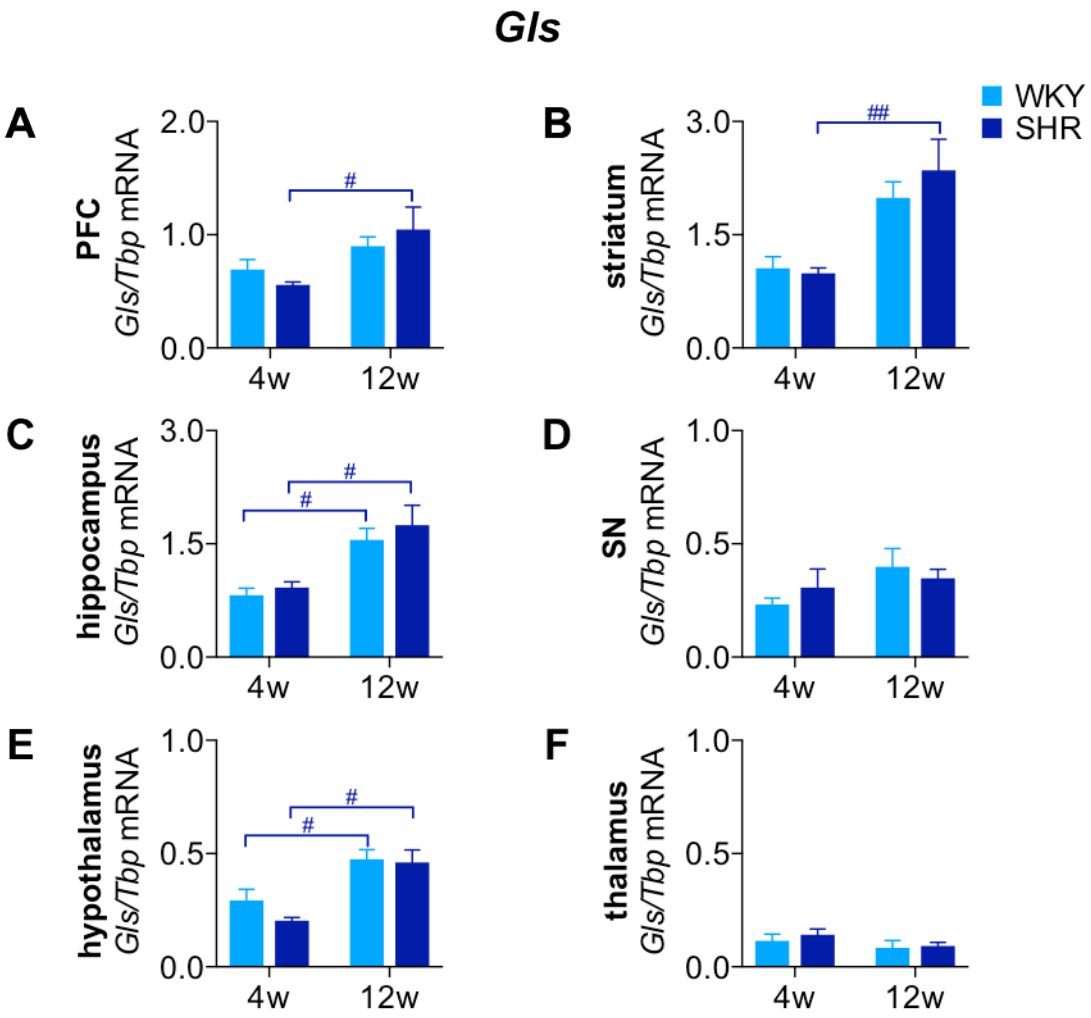


Figure 4.11 *Glutaminase (Gls)* mRNA expression in 4 and 12 week old male WKY and SHR A) PFC, **B)** striatum, **C)** hippocampus, **D)** SN, **E)** hypothalamus and **F)** thalamus (n=4-5/group, mean ± S.E.M. two-way ANOVA, #p<0.05, ##p<0.01, compared to 4w of the same strain).

Effect of Sry ASO infusion on cognitive and motor behaviour in male WKYs and SHRs

To determine whether the reduced *Sry* levels in the SHRs are responsible for the generation of symptoms, the effect of reducing *Sry* in the male WKYs was assessed by various motor and cognitive behavioural tasks. Brain *Sry* expression was reduced via repeated intracerebroventricular (ICV) Sry ASO-infusion in male WKYs and male SHRs. Motor and cognitive behaviours were assessed by open field, novel object recognition (NOR) and spontaneous alternation tests prior to and seven days post daily ASO-infusion.

Open field testing of male WKYs revealed that there were no significant differences in the total distance travelled, velocity and entries into the periphery and centre between the ASO and SO-infused groups at either pre or at post 7-day infusion (Figure 4.12A to D). However, 7 days of ASO-infusion in male WKYs significantly reduced discrimination index compared to the SO-infused group (-0.10 ± 0.12, ASO vs 0.32 ± 0.07, SO, $p<0.05$, Figure 4.13A) and pre-infusion (-0.10 ± 0.12, day 7 vs 0.33 ± 0.08, day 0, $p<0.05$, Figure 4.13A). Similarly, the ASO-infused male WKY rats showed preference for the novel object when measured by percentage of exploration time relative to day 0 (44.86 ± 5.68%, day 7 vs 66.58 ± 3.80%, day 0, $p<0.05$, Figure 4.13B) and a decreasing trend compared to the SO-infused group, although not statistically significant (Figure 4.13B). Furthermore, the ASO-infused male WKYs showed a significantly lower percentage of correct spontaneous alternation compared to the SO-infused WKYs (60.95 ± 5.49%, ASO vs 80.16 ± 2.12%, SO $p<0.01$, Figure 4.14A). The deficit in cognitive performance in the NOR and spontaneous alternation tests were associated with reduced *Sry* mRNA expression in the hippocampus (50% of SO,

$p<0.05$, Figure 4.15A). However, no significant change in Sry expression was observed in the PFC (Figure 4.15B) of ASO-infused WKYs. Together, these data indicate that reducing hippocampal expression, via *Sry* ASO-infusion, led to cognitive impairment in male WKYs.

At pre-infusion, open field testing of male SHRs showed a higher total distance travelled and velocity relative to male WKYs (Figure 4.12), validating that the male SHRs are more hyperactive. However, 7 days of ASO-infusion did not significantly affect the total distance travelled, velocity and entries into the periphery and centre when compared to the SO-infused SHRs or pre-infusion SHRs (Figure 4.12A to D). Unexpectedly, a significant decrease was observed in total distance (18.4 ± 1.1m, day 7 vs 26.1 ± 1.8m, day 0 $p<0.01$, Figure 4.12A) and velocity (0.03 ± 0.002m/s, day 7 vs 0.04 ± 0.003m/s, day 0, $p<0.01$, Figure 4.12B) in the SO-infused group post 7-day infusion compared to pre-infusion. At pre-infusion, the NOR test demonstrated that male SHRs had a lower discrimination index and percentage of novel object exploration time compared to the male SHRs, indicating that male SHRs are cognitively impaired at baseline (Figure 4.13). Unlike the male WKYs, 7 days of ASO-infusion did not worsen the discrimination index and percentage of novel object exploration time in male SHRs when compared to the SO-infused groups or pre-infusion of ASO (Figure 4.13A to B). At pre-infusion, no differences were observed in the percentage of correct spontaneous alternations between male SHRs and WKYs, however the male SHRs had higher total arm entries, suggesting increased locomotor activity (Figure 4.14). Furthermore, the test showed no significant differences in the percentage of correct spontaneous alternations between the ASO and SO-infused groups at either pre or at post 7-day infusion (Figure 4.14A). However, an unexpected

significant reduction in total arm entries in the SO-infused group post 7-day infusion compared to pre-infusion (25.4 ± 2.73, day 7 vs 15.8 ± 1.58, day 0, $p<0.01$, Figure 4.14B). As shown previously in Figure 4.6, *Sry* mRNA expression was reduced in the hippocampus and PFC of SO-infused male SHRs compared to SO-infused male WKYs (Figure 4.15). However, 7 days of ASO infusion did not further reduce *Sry* mRNA expression in male SHRs when compared to SO-infused SHRs or pre-infusion SHRs (Figure 4.15 A and B). The lack of ASO effect on Sry mRNA expression may be due to low endogenous *Sry* levels in male SHRs, which is in keeping with the lack of ASO effect on cognitive function in male SHRs.

Behavioural testing of female WKYs revealed no significant differences in the open field, NOR or spontaneous alternation tests compared to the male WKYs at day 0 (Figure 4.12, 4.13 and 4.14), indicating that there are no significant sex differences in locomotive and cognitive function for the WKY strain. Furthermore, female WKYs showed no significant differences between the ASO and SO-infused groups in any of the behavioural tests (Figure 4.12, 4.13 and 4.14), confirming that the effect of *Sry* ASO-infusion in male WKYs was male (and Sry) specific.

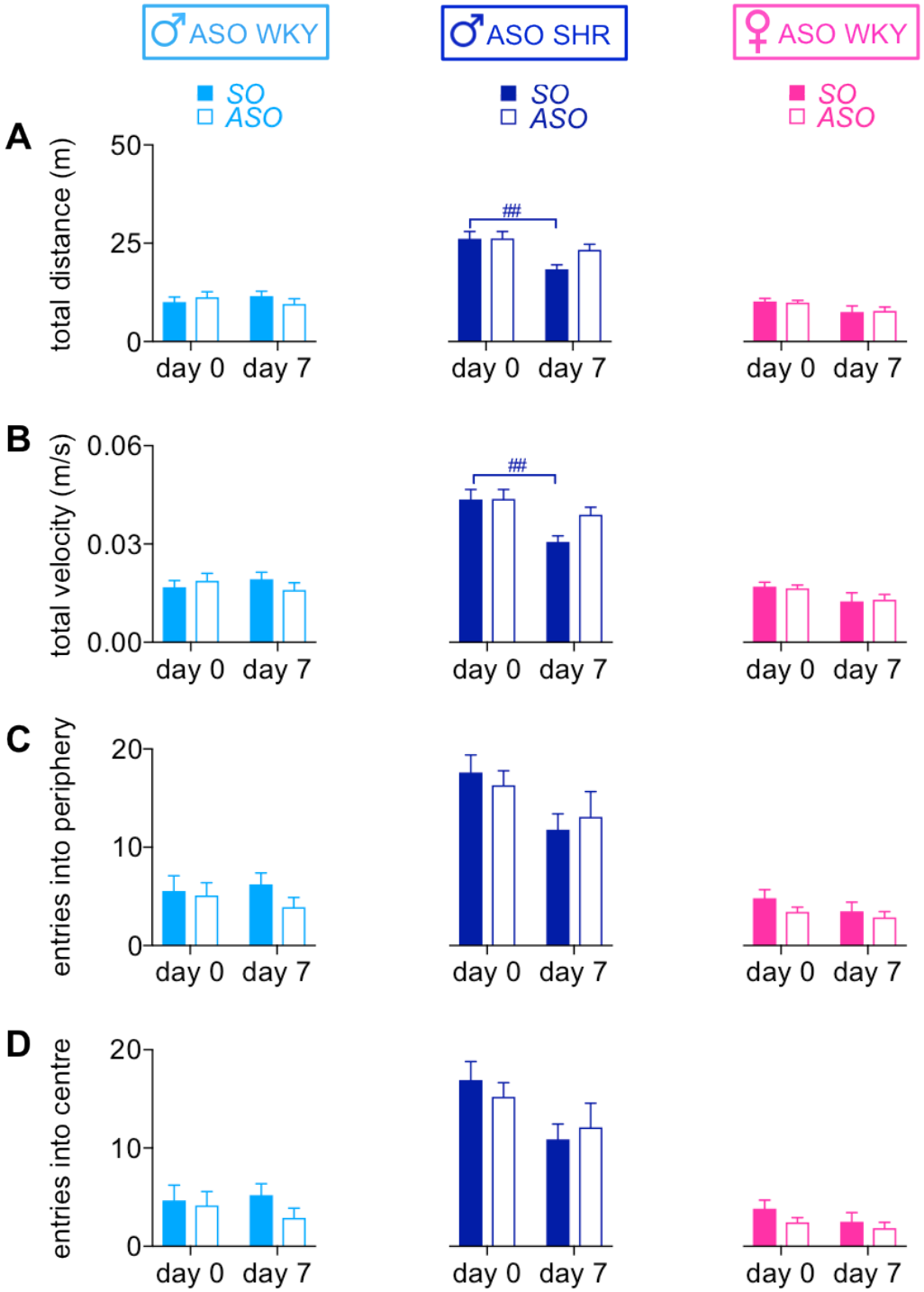


Figure 4.12 Effect of ICV *Sry* ASO infusion on locomotor activity. Male WKY, male SHR and female WKY rats were assessed at day 0 and 7 of ICV *Sry* ASO (or SO) infusion by the open field test. **A)** Total distance travelled, **B)** velocity, **C)** entries into the periphery and **D)** entries into the centre in the open field test (n≥6/group, mean ± S.E.M. two-way ANOVA, ##p<0.01, compared to day 0).

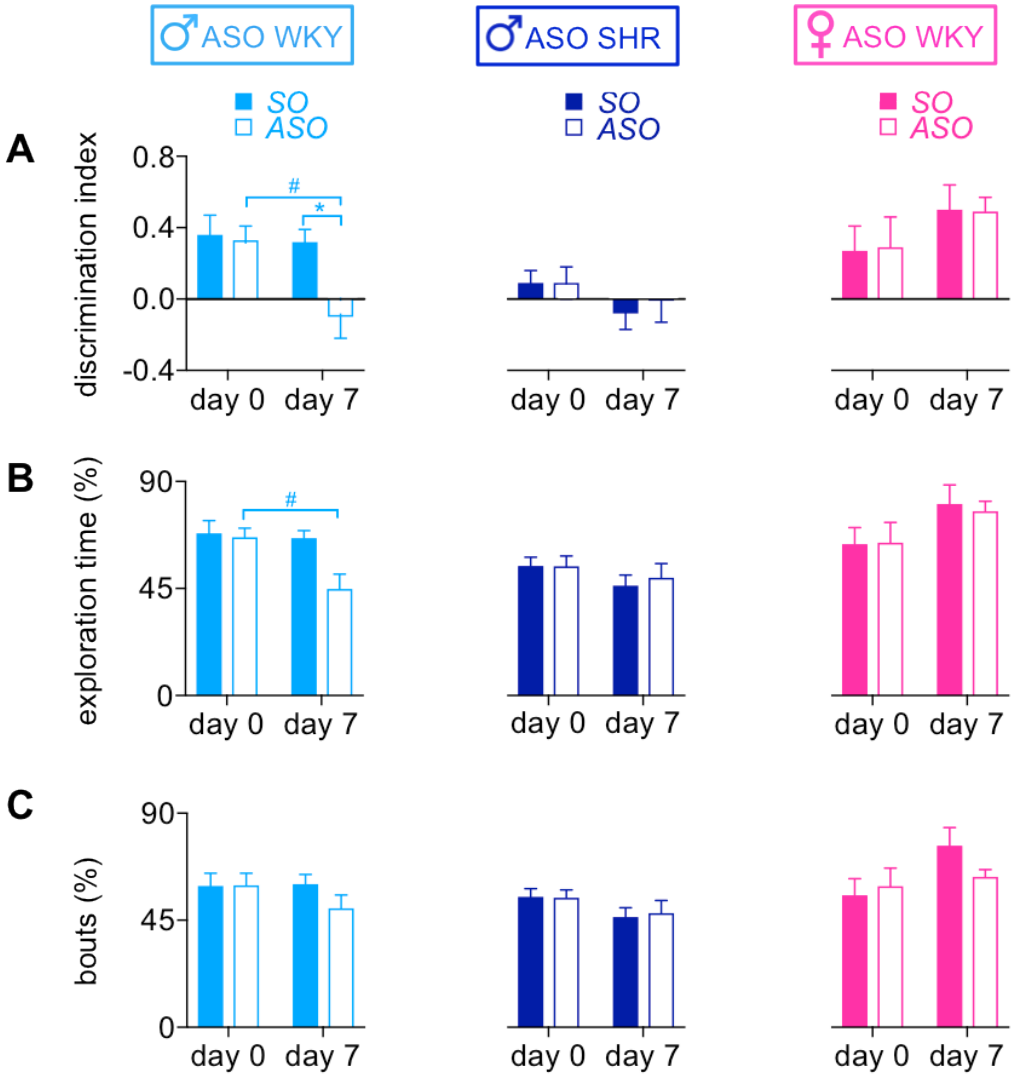


Figure 4.13 Effect of ICV *Sry* ASO infusion on recognition memory. Male WKY, male SHR and female WKY rats were assessed at day 0 and 7 of ICV *Sry* ASO (or SO) infusion by the novel object recognition test. **A)** Discrimination index, **B)** percentage of time spent exploring the novel object (NO) and **C)** percentage of bouts made to explore the NO during the test trial were calculated (n≥6/group, mean ± S.E.M. two-way ANOVA, *p<0.05 compared to SO; $^{\#}$p<0.05 compared to day 0).

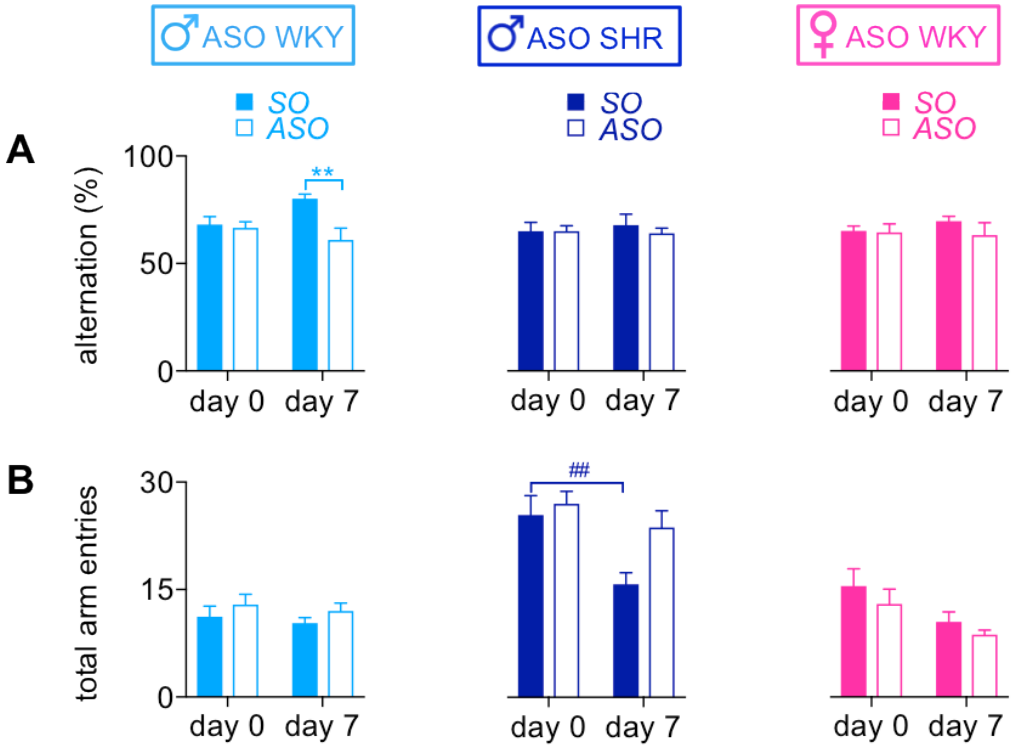


Figure 4.14 Effect of ICV *Sry* ASO infusion on spatial memory. Male WKY, male SHR and female WKY rats were assessed at day 0 and 7 of ICV *Sry* ASO (or SO) infusion by the spontaneous alternation test. **A)** Percentage of correct alternations and **B)** total arm entries were analysed (n≥6/group, mean ± S.E.M. two-way ANOVA, **p<0.01 compared to SO; $^{\#\#}$p<0.01 compared to day 0).

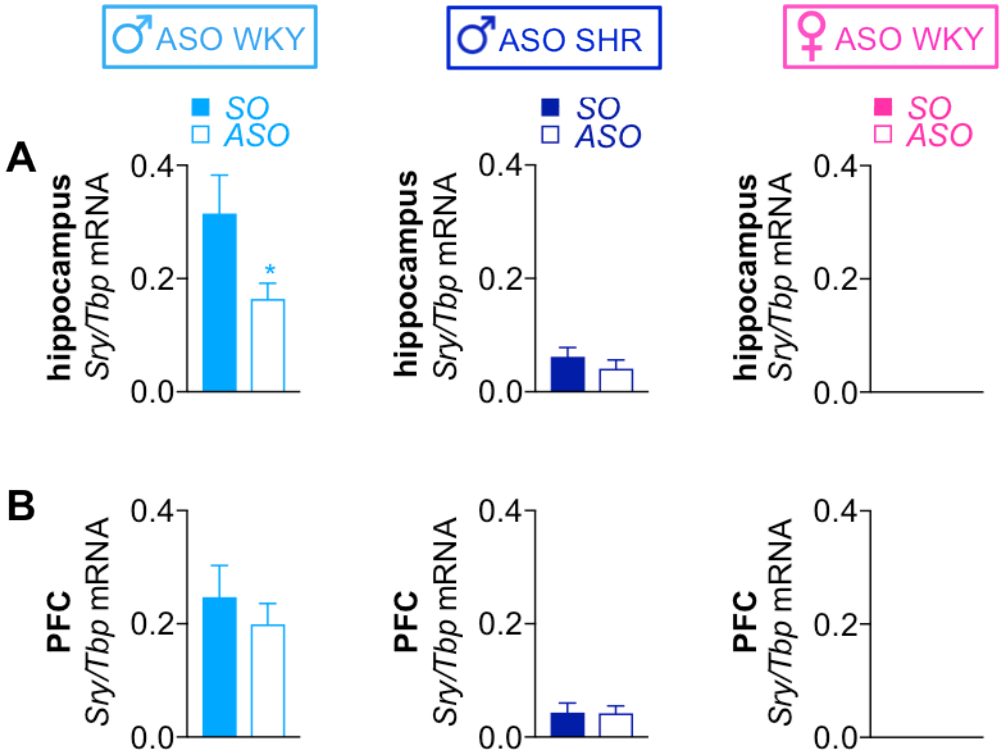


Figure 4.15 Effect of ICV *Sry* ASO infusion on *Sry* mRNA expression. *Sry* mRNA expression was measured following 7 days of ASO-infusion in the male WKY, male SHR and female WKY **A)** hippocampus and **B)** PFC (n≥6/group, mean ± S.E.M. student t-test, *p<0.05, compared to SO).

4.4 DISCUSSION

In this chapter, the findings provide evidence that, SRY, a male-specific Y-chromosome gene, underlies the male bias in ADHD. To summarise, *Sry* mRNA expression is reduced and developmentally stunted in the male SHR model of ADHD. In parallel, catecholamine and GABA synthesis enzymes were altered in the SHRs. Finally, suppressing Sry synthesis, by Sry antisense oligonucleotide (ASO)-infusion via ICV in male WKYs resulted in cognitive impairments that were similar to male SHRs. Importantly, the effect of Sry ASO-infusion on cognition was absent in female WKYs, demonstrating for the first time a direct interaction between cognition and a sex-specific gene.

Sry mRNA expression is reduced in brain regions associated with ADHD symptoms in male SHRs

The results from the present chapter provide compelling evidence that brain *Sry* mRNA expression is reduced in multiple brain regions of male SHRs (Figure 4.6). In particular, *Sry* mRNA expression was reduced in the PFC, hippocampus, striatum, hypothalamus and thalamus, brain regions that work independently or interdependently with each other to generate pathophysiology of ADHD. Indeed, reduced Sry expression in PFC, the pivotal brain region implicated in ADHD, could contribute to impaired working memory resulting in inappropriate movement and responses, and inattention in ADHD patients (Brennan and Arnsten, 2008). Together with the PFC, suppressed *Sry* expression in the basal ganglia (includes the striatum and SN) function could underlie the poor motor performance due to the inability to inhibit unintentional movement in ADHD (Prince, 2008, Gaddis et al., 2015). Furthermore, reduced *Sry* expression could disrupt thalamic function resulting in inattention and impulsivity symptoms (Bailey and Joyce, 2015). On the

other hand, reduced *Sry* expression in the striatum and the hippocampus could lead to poor performance in cognitive tasks and learning disabilities (de Wit et al., 2012, Plessen et al., 2006). In parallel, lower hypothalamic *Sry* expression could underlie the negative stress response in ADHD patients (Ma et al., 2011). Whilst the link between Sry and these ADHD symptoms need to be elucidated, a clinical study showed that a boy with two copies of *SRY* displayed ADHD-like symptoms, in particular, hyperactivity and impulsivity (Mulligan et al., 2008), indicating that dysfunctional *SRY* expression is associated with clinical ADHD.

Results in this chapter also revealed that developmental increase in brain *Sry* mRNA expression was absent in male SHRs (Figure 4.6). These results indicate that stunted Sry transcription during development could contribute to the susceptibility of males to ADHD, and identification of pathways involved in the regulation of *SRY* expression in the ADHD brain may reveal male-specific causes of ADHD. Previous studies demonstrate an essential role for the *Gadd45γ*-p38-MAPK pathway in the regulation of *Sry* expression in mouse embryonic gonads (Warr et al., 2012, Gierl et al., 2012) and in a human cell culture model of PD (Czech et al 2014). However, there were no significant differences in *Gadd45γ* mRNA expression between WKYs and SHRs (Figure 4.7) in any of the brain regions measured. This suggest that the *Sry* expression in male SHRs is likely to be mediated by a pathway independent of *Gadd45γ*-MAPK signalling, such as the D2 receptors. Given that SRY expression is positively regulated by the D2 receptor (Chapter 2), reduced DA levels in male SHRs may lead to reduced *Sry* expression. In turn, the reciprocal regulation of the DA pathway by Sry could be disrupted and exacerbate the symptoms in male ADHD. In support, a subtype of the D2 receptor (DRD4) has been implicated in male ADHD (Das Bhowmik et al.,

2013), and decreased D2 receptor availability is associated with increased impulsivity in male rats (Dalley et al., 2007). Thus, assessing the effects of pharmacological or genetic manipulation of the D2 receptor in male SHRs could provide some important insights on the regulation of SRY expression in male ADHD.

The interaction between DA and the glucocorticoid system could potentially be involved in the regulation of SRY expression in male ADHD. Low levels of cortisol, a glucocorticoid stress hormone, have been linked to the hyperactivity, inattentive and impulsive symptoms in children with ADHD (Ma et al., 2011). Whilst the impact of stress on male and females with ADHD remains elusive, human and animal studies have suggested that stress is more closely linked with cognition processes in males compared to females (Cornelisse et al., 2011, Espin et al., 2013, Zorawski et al., 2005). Furthermore, stress regulation of cognition in men, is associated with cortisol levels (Zorawski et al., 2005, Wolf et al., 2001), whilst stress and cognition is modulated by ovarian hormones in females (Schoofs and Wolf, 2009, Maki et al., 2015, Zeidan et al., 2011). Various studies focusing on the male VTA systems have demonstrated that glucocorticoid stress hormones impact the mesolimbic and mesocortical DA systems, which are associated with the symptoms of ADHD (Piazza and Le Moal, 1996, Lindley et al., 1999, Krishnan et al., 2007, Trainor, 2011, Lemos et al., 2012, Barik et al., 2013). In this thesis, the results revealed that reduced levels of *Sry* could be associated with the cognitive deficits in the SHRs. Whilst the mechanism underlying the reduced *Sry* expression remain elusive, hormonal regulation of SRY via the glucocorticoid system could be a potential mechanism underlying the male bias in ADHD.

Reduction in hippocampal Sry levels is associated with impaired cognitive function

Direct *in vivo* actions of *Sry* in the brain were first demonstrated by assessing the anatomical and behavioural consequences of down-regulating *Sry* expression in the rat SN (Dewing et al., 2006). Knockdown of nigral *Sry* in the male rats, via repeated *Sry* ASO injections, resulted in reduced motor performance, indicating that *Sry* exerts direct actions on voluntary movement in males (Dewing et al., 2006). Various rodent models have also demonstrated that *Sry* (solely or in combination with other sex-linked genes) could be involved in influencing impulsivity, feeding behavior, anxiety phenotypes and blood pressure (Davies, 2014, Kopsida et al., 2013, Ely et al., 2007, Ely et al., 2009).

To determine the function of Sry in the male ADHD brain, the effect of reducing brain Sry expression, via repeated ICV ASO infusion, was assessed in male WKYs and SHRs. Behavioural testing of male SHRs revealed hyperactive behaviour and impaired cognitive function at baseline (i.e. pre-infusion), as previously described (Russell, 2011, Sagvolden, 2000, Sagvolden et al., 2005). However, Sry ASO infusion did not exacerbate the cognitive deficit or further reduce brain Sry expression in male SHRs, indicating a ceiling effect of Sry expression on cognitive function in the male brain. On the other hand, reducing *Sry* levels in male WKYs led to a cognitive decline in the NOR and spontaneous alternation tests, similar to those observed in the male SHRs (Figure 4.14 and 4.15). The ASO-induced decline in cognitive function was associated with reduced hippocampal Sry expression, demonstrating that reduced brain Sry expression is directly responsible for impaired cognitive function in male rats. In parallel, Sry ASO-infusion did not affect any behaviour in the female WKYs, confirming that the Sry ASO-infusion effects in the male WKYs are male-specific and off target effects

are absent. Unexpectedly, the SO-infused male SHRs exhibited decreased locomotor activity in the open field and spontaneous alternation tests following the repeated SO infusions. Heightened levels of stress by an unknown cause could have contributed to this unexpected finding.

Whilst ICV ASO infusion reduced hippocampal Sry expression in male WKYs, it did not significantly affect Sry expression in the PFC. This suggests that ICV ASO infusion may be effective in reducing Sry expression in brain regions proximal to the lateral ventricles, such as the hippocampus (Figure 4.15), but to a lesser extent in distal regions such as the PFC. Indeed, the lack of ASO effect on locomotor activity in male WKYs may be related to inefficient knockdown of Sry expression in brain regions that mediate motor function such as striatum and SN. Thus, site-specific *Sry* ASO infusion in other Sry-positive brain regions are necessary to better understand the physiological and pathophysiological brain functions modulated by *Sry*.

Although the current chapter suggest that reduced brain SRY expression underlies cognitive deficits in SHRs, downstream targets of Sry in the SHR brain is unclear. Considering that Sry regulates DA and GABA biosynthesis in the male brain, reduced brain Sry expression may lead to dysregulation of these neurotransmitter systems. In support, the lack of developmental increase in nigral and hypothalamic *Th* mRNA expression in male SHRs is associated with reduced Sry expression in the same brain regions. Furthermore, *Gad1* mRNA expression is dysregulated in multiple brain regions in the male SHRs, which may be potentially driven by SRY. Whilst these findings have provided a hint on the targets of Sry in male ADHD, further work is required to better understand the direct effect of *Sry* ASO infusion on the neurotransmitter systems in the normal and ADHD brain.

In summary, these novel findings have revealed that SRY directly mediates cognitive function in the healthy male brain; hence reduced SRY expression could be underlying the cognitive deficits in male ADHD.

Contribution of sex-specific genes and sex-hormones in the ADHD brain

The findings in this chapter revealed that the reduced brain Sry expression in male SHRs could underlie the susceptibility of male to ADHD. This is somewhat intriguing, as this implies that the lack of SRY in females should increase the vulnerability of females to ADHD. Considering that ADHD is clearly a male-biased disorder (Arnett et al., 2015), this paradox may be explained by an alternate mechanism for SRY in the female brain. Whilst oestrogen is an obvious candidate, the oestrogen surge in females only occurs at puberty – after when ADHD has already manifested. Another mechanism could be the protective effects of X-inactivation escapee genes in females – i.e. females will possess two alleles whilst males will only possess one allele. One such mechanism is the X-inactivation escapee genes in females, which may be protective in ADHD. Indeed, an X-linked escapee gene, *steroid sulfatase* (*STS*), which acts to modulate neurotransmitter receptors, is potentially protective in females (Davies, 2012) as the lack of functional *Sts* has been linked to inattention and hyperactivity in mice (Davies et al., 2009, Trent et al., 2012). In support, STS activity in the brain is higher in females compared to males in both humans and non-human primates, and the sex difference might be of a greater magnitude during pre-pubertal period (Cuevas-Covarrubias et al., 1993, Kriz et al., 2005, Steckelbroeck et al., 2004). Hence, X-inactivation gene escapees could be a compensatory mechanism in females that provide neuroprotection from ADHD.

Several human and animal studies have revealed that overexposure of testosterone underlies the male bias in ADHD (Hines, 2008, Baron-Cohen et al., 2011, Mueller et al., 2010, Bucci et al., 2008, Li and Huang, 2006, Toot et al., 2004). These findings highlight the importance of considering the effect of testosterone underpinning the male bias in ADHD, and whether SRY might be interacting with testosterone. During arousing conditions in rodents, circulating testosterone levels is associated with testis size, which can be influenced by *Sry* (Preston et al., 2012, Satou and Suto, 2015). A similar interaction could be occurring in the brain, whereby elevated testosterone levels could be a consequence of dysregulated SRY expression. In support, the stunted brain *Sry* mRNA expression could be linked to the higher serum testosterone levels in male SHRs compared to WKYs (Toot et al., 2004). Considering that SRY negatively regulates transcriptional activity of androgen receptors (Yuan et al., 2001), the reduced levels of *Sry* in male SHRs could result in increased AR activation and testosterone signaling. This suggests that SRY could potentially directly target some genes such as TH and indirectly regulate other genes via its interaction with testosterone.

Future studies

Together, the current findings have provided evidence for a novel role of *Sry* as a risk factor for male ADHD. To better understand the function of *Sry* in male SHRs, an unbiased approach, such as RNA-sequencing and Sry chromatin immunoprecipitation (ChIP)-sequencing are essential. These genome-wide studies will provide a greater understanding of novel downstream targets and signaling pathways of *Sry* in the male SHRs. In this chapter, *Sry* levels were reduced in male SHRs compared to WKYs. In addition, reducing *Sry* levels via ICV resulted in

impaired cognition in male WKYs that were similar to male SHRs. Therefore, it is important to determine whether increasing *Sry* expression using a viral vector can rescue the symptoms in male SHRs. If successful, this could potentially lead to male-specific modifying therapy for males with ADHD.

To confirm and validate my findings that *Sry* is a male susceptibility factor for ADHD, it is important to determine whether *Sry* expression is dysregulated in other animal models of ADHD such as the Naples High Excitability (NHE) rat and the DAT-KO murine models. Furthermore, these animal models should be used to determine whether overexpressing or normalising *Sry* expression could attenuate the ADHD-like symptoms.

Considering that overexposure of testosterone plays a role in underlying the male bias in ADHD, further studies on understanding the link between SRY and testosterone in the brain should be performed. Firstly, measuring various markers of testosterone including the androgen receptors as well as synthesis and metabolic enzymes in the WKY rats that received *Sry* ASO-infusion would reveal any modulation of Sry on androgen biosynthesis, regulation and function. Secondly, presuming that testosterone levels are elevated in male SHRs, gonadectomised male SHRs will be useful to study the targets of testosterone.

Human studies examining SRY expression in ADHD post mortem sections are crucial to help us better understand the role of SRY in ADHD. Furthermore, clinical studies investigating SRY polymorphisms in male ADHD patients would be beneficial. If SRY mutations are identified, this could potentially lead to earlier diagnosis and intervention of ADHD in males.

Conclusion

The current findings in this chapter indicate that the aberrant developmental regulation of SRY may provide a mechanistic basis for the pronounced male bias in ADHD. Another significant finding is that SRY could be involved in modulating learning and attention. Hence, in addition to the deleterious effects of elevated testosterone levels, reduced *Sry* mRNA expression proved to be detrimental and may contribute to some of the ADHD symptoms. SRY is definitely not solely responsible for underlying the pathophysiology and symptoms in ADHD considering that females are also diagnosed with ADHD. However, this study suggests that *SRY* could be an additional susceptibility factor contributing to the male bias in ADHD, by interacting with other genes and/or hormones.

CHAPTER 5:

General discussion and conclusion

5. General discussion and conclusion

Sex differences in the brain are more pervasive than many appreciate. Emerging studies have revealed that the vast majority of brain sex differences are present in the neuroanatomy and neurochemistry, as well as neuronal structure and connectivity of the brain. These brain sex differences are thought to underlie the startling sex differences observed in several neurological disorders. For example, females are more likely to develop depression, anxiety (Weissman et al., 1996, Nolen-Hoeksema, 1987) and Alzheimer's disease (Hebert et al., 2013), whilst males are more susceptible to Parkinson's disease (PD) (Wooten et al., 2004), attention deficit hyperactivity disorder (ADHD) (Balint et al., 2009), and autism spectrum disorders (ASD) (Gillberg et al., 2006) and early-onset schizophrenia (Hafner et al., 1993). This highlights the importance of exploring sexual dimorphism in the brain for their impact and therapeutic implications for neurological disorders.

The biological basis underlying the sexual dimorphism in the brain has predominantly been attributed to the influences of gonadal sex steroids, in particular oestrogen. Several clinical and pre-clinical studies have demonstrated that oestrogen positively regulates components of multiple neurotransmitter systems. In the DA pathway, oestrogen modulates DA synthesis, release, metabolism and receptor binding in females (Pasqualini et al., 1995, McDermott et al., 1994, Di Paolo et al., 1985, Demotes-Mainard et al., 1990). In parallel, oestrogen suppresses GABA synthesis and neurotransmission whilst promoting receptor binding (Saad, 1970, Murphy et al., 1998, McGinnis et al., 1980, Perez et al., 1988). Furthermore, oestrogen regulates acetylcholine levels and receptor binding activity (Avissar et al., 1981, Hortnagl et al., 1993, Egozi et al., 1982). It is

well established that oestrogen exerts neuroprotective actions in females, as worsening of symptoms in PD and schizophrenia are associated with the declining levels of oestrogen at the onset of menopause (Quinn and Marsden, 1986, Riecher Rossler, 1994), whilst oestrogen replacement therapies attenuated the symptoms (Shulman and Bhat, 2006, Riecher Rossler, 1994). Whilst prenatal testosterone is important for the masculinization of the developing male brain (Morris et al., 2004), overexposure to prenatal testosterone increases the susceptibility of males to neurodevelopmental disorders such as ADHD and ASD (Hines, 2008, Baron-Cohen et al., 2011).

In addition to the influence of sex hormones on the healthy and diseased brain, emerging evidence reveals that sex chromosomes also contribute (Arnold, 2004, Carruth et al., 2002, Dewing et al., 2003, Beyer et al., 1992, Ngun et al., 2011, McCarthy and Arnold, 2011, McCarthy et al., 2012). Animal studies revealed that sexually dimorphic gene expression in the brain occurs prior to hormonal influence (Dewing et al., 2003, Beyer et al., 1992), thus suggesting that sex chromosome gene might also underlie brain sex differences. Furthermore, the "four core genotype" mouse model, which partitions the effect of sex chromosome genes from the actions of gonadal sex hormones, have demonstrated significant differences between XY and XX mice such as DA cell numbers (Carruth et al., 2002), social behaviour (McPhie-Lalmansingh et al., 2008) and susceptibility to autoimmune disease (Smith-Bouvier et al., 2008). In addition, X-inactivation gene escapees in females (ie. females will have twice the gene expression compared to males) are neuroprotective in females, particularly in neurodevelopmental disorders such as ADHD. Individuals with a 45, XO karyotype (i.e. only one X-chromosome), have been linked to increased vulnerability to disorders associated

with impaired memory, attention, and social interaction such as ADHD (Russell et al., 2006). Hence, possessing both X-inactivation gene escapees is important for neuroprotection in females, suggesting that having only one X-chromosome in males could be a risk factor. However, another potential susceptibility factor in males are the genes on the Y-chromosome, such as, *SRY*.

The discovery of the sex determining gene *SRY* led to the identification and characterization of genes involved in the sex determination pathway and gonadal development (Ono and Harley, 2013, Svingen and Koopman, 2013). The finding that *SRY* is expressed in tissues outside the gonad, such as the brain, heart and adrenal glands, sparked speculation about the non-gonadal function of SRY (Clepet et al., 1993). In the male brain, *SRY* is expressed in the midbrain such as the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) and the hypothalamus, which are abundant with DA neurons (Dewing et al., 2006, Czech et al., 2012, Mayer et al., 1998). In the male SNc, SRY positively regulates transcription of DA machinery genes such as *tyrosine hydroxylase* (*TH*) and *monoamine oxidase-a* (*MAO-A*) (Dewing et al., 2006, Czech et al., 2012, Wu et al., 2009) and consequently, voluntary movement (Dewing et al., 2006). For the first time this suggested a direct genetic link between a sex-specific gene and behaviour. Therefore, the general aim of this thesis was to extend these findings to ultimately better understand the contribution of SRY in the healthy male brain as well as in male-biased neurological disorders such as PD and ADHD.

Regulation of SRY in the healthy and diseased male brain

In the developing male gonad, the activation of Sry is dependent on the Gadd45γ pathway (Gierl et al., 2012, Warr et al., 2012). We have also shown that the upregulation of SRY following 6-OHDA treatment (Czech et al., 2014) is mediated

by the GADD45γ-MAPK signalling pathway, demonstrating that SRY levels is altered in pathophysiological conditions. The work from chapters 2 and 3 add further support to these findings, as SRY and *GADD45γ* expression were aberrantly upregulated in response to cellular injury resulting from supra-physiological levels of DA *in vitro* or 6-OHDA or rotenone treatment in *in vitro* cell culture and *in vivo* rat models of PD. This SRY upregulation occurred prior to DA cell loss, suggesting a compensatory increase in response to cellular injury.

In addition to the pathophysiological upregulation of SRY expression, chapter 2 revealed a novel physiological form of SRY regulation that was *GADD45γ*-independent and responsive to D2 receptor stimulation. The potential regulation of SRY expression by the D2 receptor pathway, and the reciprocal regulation of the DA system by SRY indicate a feedback loop to maintain optimal levels of SRY and DA in the male brain. Given the well-established sex differences in the expression and function of D2 receptors (Pohjalainen et al., 1998, Andersen and Teicher, 2000), D2 receptor-dependent regulation of *SRY* may underlie these sex differences. Moreover, dysregulation of this D2 receptor feedback mechanism may be responsible for sex differences observed in male-biased neurological disorders such as ADHD and early-onset schizophrenia.

In contrary to experimental models of PD, the results from chapter 4 revealed reduction in brain *Sry* expression in the SHR model of ADHD, particularly in brain regions associated with ADHD symptoms. Moreover, developmental increase in brain *Sry* expression observed in the control WKY strain was stunted in the male SHRs, which may underlie the developmental deficits in cognitive function in ADHD. Indeed, reducing brain *Sry* levels in male WKYs, resulted in cognitive deficits to levels that were similar to male SHRs, revealing for the first

time an interaction between a sex-specific gene and cognitive function. Unlike the experimental models of PD, regulation of Sry in the SHRs was independent of the Gadd45γ-pathway, indicating *Sry* is regulated by a different mechanism in ADHD. Given that SRY regulates DA biosynthesis genes, and DA regulates SRY via the D2 receptor, dysregulation of this feedback loop may underlie the reduced SRY expression and hypodopaminergic transmission in ADHD. Pharmacological or genetic approaches to reduce D2 receptor and/or Sry expression in animal models will be necessary to validate this hypothesis. Together, these findings demonstrate that SRY expression is i) regulated by D2 receptor under physiological conditions, ii) upregulated in conditions of cellular injury via a GADD45γ-dependent mechanism and iii) downregulated during development in an animal model of ADHD. Therefore, better understanding of this regulation may reveal sex-specific causes and consequently novel therapies for male biased neurological disorders.

Function of SRY in the healthy and diseased male brain

The contrasting effect of reducing Sry expression in healthy and toxin-induced rat models of PD suggests an opposing role for Sry in the healthy and diseased male brain. In line with previous *in vitro* studies (Czech et al., 2012, Milsted et al., 2004, Wu et al., 2009), this thesis demonstrates that Sry regulates a wide range of DA biosynthesis and catabolic genes *in vivo*, notably, *Th, Mao-a, Ddc, Dbh, Dat, Drd1* and *Drd2*. In parallel, Sry also regulates GABA synthesis genes, *Gad1* and *Gad2*, which are predominantly expressed in the SNr. Considering the presence of Sry in both the SNc and SNr, where it regulates DA and GABA biosynthesis genes, Sry is likely to mediate input and output activity of the basal ganglia to potentially fine tune basal ganglia functions, such as voluntary movement and goal-directed actions in males. However, in 6-OHDA or rotenone-induced models of PD, the

aberrant upregulation of nigral *Sry* mRNA expression was concomitant with activation of several PD pathogenesis pathways. Conversely, reducing *Sry* expression attenuated the motor deficits and nigral degeneration, indicating that *Sry* upregulation is detrimental in male PD.

In contrast to experimental models of PD, *Sry* expression was reduced and developmentally stunted in the SHR model of ADHD. Moreover, reducing hippocampal *Sry* levels in male WKYs resulted in cognitive deficits to levels that were similar to male SHRs. Whilst the target genes of Sry in ADHD remain elusive, dysregulated *Th* and *Gad* genes are likely to occur given that they are modulated by Sry. The paradox of reduced *Sry* expression in male ADHD and the lower incidence of ADHD in females (which do not have SRY) suggest an alternate mechanism for SRY in females. One such mechanism is the X-inactivation escapee genes in females, which may be protective in ADHD. S*teroid sulfatase* (*STS*) is an X-linked escapee gene, which acts to modulate neurotransmitter receptors, is potentially protective in females (Davies, 2012). The lack of functional *STS* has been linked to inattention and hyperactivity in mice (Davies et al., 2009, Trent et al., 2012). In support, STS activity in the brain is higher in females compared to males in both humans and non-human primates, and the sex difference might be of a greater magnitude during pre-pubertal period (Cuevas-Covarrubias et al., 1993, Kriz et al., 2005, Steckelbroeck et al., 2004).

Thus, work from my thesis demonstrates multiple roles for SRY in the normal, PD, and ADHD male brain. In particular, aberrant up-regulation of Sry was shown to detrimental in experimental PD models, whilst diminished brain Sry expression was responsible for cognitive deficits in ADHD. These results suggest that SRY exerts its action via different transcriptional targets during physiological

and pathophysiological conditions, which will need to be considered when designing therapeutics targeting SRY in the male brain.

Homeostatic regulation of SRY expression is essential for the healthy male brain

Chapter 2 has demonstrated that a D2 receptor mediated pathway regulates SRY and in turn, Sry regulates DA biosynthesis and metabolism. On the other hand, Chapters 3 and 4 revealed that the aberrant upregulation or downregulation of *Sry* could be predisposing males to PD or ADHD, respectively. As illustrated in Figure 5.1, it is tempting to speculate that the homeostatic regulation of SRY expression is important to maintain the healthy functioning of the male brain. However, a DNA-damage event could trigger an aberrant upregulation of SRY expression and consequently exacerbate the symptoms and pathophysiology in male neurodegenerative disorders such as PD. This upregulation of SRY could be a compensatory mechanism in response to injury and cellular stress. On the contrary, the disruption of SRY expression during developmental stages, which persist through adulthood, could predispose males to neurodevelopmental disorders that have a significant male bias such as ADHD and ASD. Similarly in females, oestrogen levels are important for healthy functioning of the female brain as a decline in oestrogen predisposes females to neurological disorders, such as PD and schizophrenia (Quinn and Marsden, 1986, Riecher Rossler, 1994). Given that oestrogen replacement therapies have been effective in treating PD and schizophrenic female patients (Shulman and Bhat, 2006, Riecher Rossler, 1994), designing therapies to target SRY expression in males with neurological disorders might be beneficial. In particular, novel therapies that reduce the levels SRY in males with neurodegenerative disorders such as PD or increase the expression of SRY in males with neurodevelopmental disorders such as ADHD and ASD.

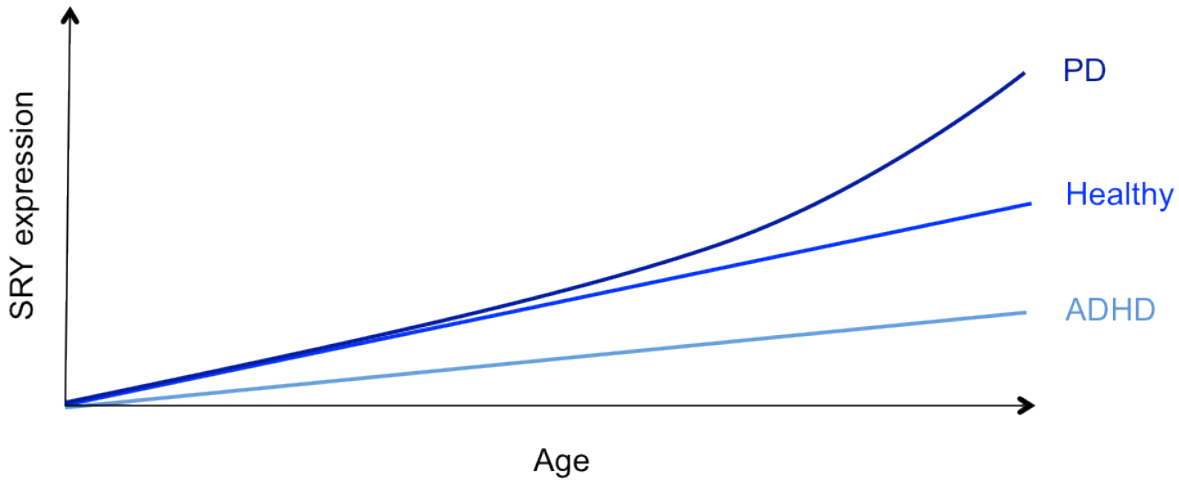


Figure 5.1 Proposed model for SRY regulation in healthy and diseased male brain. *SRY* is developmentally regulated from birth through to adulthood in the healthy male brain. However, stunted development of SRY expression during the developmental stage could predispose males to ADHD. In contrast, aberrant increase in SRY expression during adulthood could underlie male susceptibility to PD.

Potential role of SRY in other male biased neuropsychiatric and neurodegenerative disorders

The results from my thesis have revealed for the first time a sex-specific genetic basis underlying the male-bias in neurological disorders such as PD and ADHD. Considering the wide distribution and functions of SRY in the male brain, dysregulation of *SRY* expression could underlie other male biased neuropsychiatric and neurodegenerative disorders.

ASD is a neuropsychiatric and neurodevelopmental disorder with one of the strongest male bias whereby four males to every female diagnosed with ASD (Fombonne, 2003) and this ratio is further increased to eleven to every one female in severe autism (Gillberg et al., 2006). Both ASD and ADHD are neurodevelopmental disorders that share similar clinical features and affects children in the same three central areas – communication, social interaction and

behavior. Given the presence of Sry expression in the brain regions affected in ASD such as the PFC, hippocampus and the amygdala (Bachevalier and Loveland, 2006, Baron-Cohen et al., 2000, Santangelo and Tsatsanis, 2005), it is tempting to speculate that dysregulated Sry expression underlies the vulnerability of males to ASD.

Another male-biased neuropsychiatric disorder is early-onset schizophrenia, which is diagnosed in males twice as more often compared to females (Hafner et al., 1993). Notably, the positive symptoms of schizophrenia (e.g. hallucinations and delusions) are associated with increased presynaptic dopaminergic activity, leading to excessive DA release (Laruelle, 1996, Miyake et al., 2011) in the PFC originating from the ventral tegmental area (VTA) (Laruelle, 1996, Joyce and Meador-Woodruff, 1997). Furthermore, the hippocampus, which is the central region involved in psychosis, experiences an overly responsive DA system in schizophrenic patients. Considering that SRY is expressed in both the human male VTA (Czech et al., 2012) and hippocampus, dysregulation of SRY expression in these regions might contribute to the increased DA activity in the mesolimbic system and predisposing males to early-onset schizophrenia.

Aside from PD, amyotrophic lateral sclerosis (ALS) is another neurodegenerative disorder that displays a significant male bias. ALS is characterized by the progressive degeneration of motor nerve cells in the brain (McCombe and Henderson, 2010). Apart from being four times more prevalent among males during early onset of the disease (Haverkamp et al., 1995), ALS is usually manifested at a younger age in males relative to females (Norris et al., 1993). As neuroinflammation has been implicated as one of the main

pathogenesis pathways activated in ALS (Evans et al., 2013), the presence of SRY in the microglia could underlie the male susceptibility in ALS.

Whilst a significant male bias is observed in many neurological disorders, the biological basis underlying the sex differences have yet to be fully revealed. Hence, further examining the expression and function of SRY in animal models and human tissues samples in these male-biased disorders might reveal SRY as novel sex-specific target for the diagnosis and/or therapy.

Future studies

My thesis has provided important insights into the role of SRY in the healthy and diseased male brain. However, findings from my thesis have generated some critical questions that need to be addressed.

Given the different regulatory mechanisms of SRY in the healthy and diseased male brain, identifying the downstream targets that are activated by Sry in the normal, PD and ADHD brain by genome-wide approaches, such as RNA and chromatin-immunoprecipitation sequencing is essential to advance our understanding brain sex differences. These approaches have the potential to reveal the precise mechanism(s) underlying the aberrant up-regulation of SRY expression in male PD and down-regulation of SRY in ADHD and identify pathways that can be modulated as a sex-specific neuroprotective strategy for PD.

Although work from the current chapter and our group (Czech et al., 2012) have provided a link between Sry and DA or GABAergic neurons in the male brain, the precise role of Sry in these neurons remain unclear. To better understand the role of Sry in specific cell types, generating mice using the Cre/lox system with Sry floxed and a cre specifically driven by genes specific to the cell - for example, TH in DA neurons, would reveal whether SRY is crucial for the development and

survival of DA neurons. Given that SRY is expressed in brain regions that have not been previously shown such as the hippocampus and cerebellum, overexpressing or reducing Sry levels in these brain regions in rodents using a viral vector or ASO technology, respectively, would uncover new behavioural roles for Sry in males.

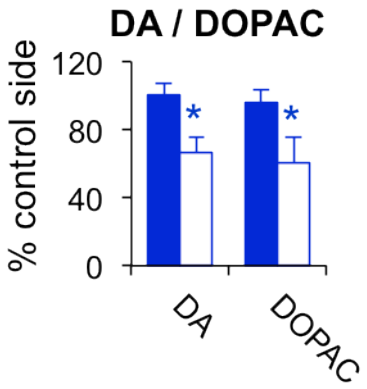
Results from the current study indicate the importance of considering the role of SRY when investigating the mechanisms underlying brain sex differences. However, further work is needed to better understand the interactions between the hormonal and genetic influences in the healthy and diseased male brain. 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 brain sex differences. Overall, the characterisation of molecular mechanisms responsible for differences between the male and female brain will give the basic biological tools necessary to understand the occurrence of sex differences observed in neurological disorders.

Conclusion

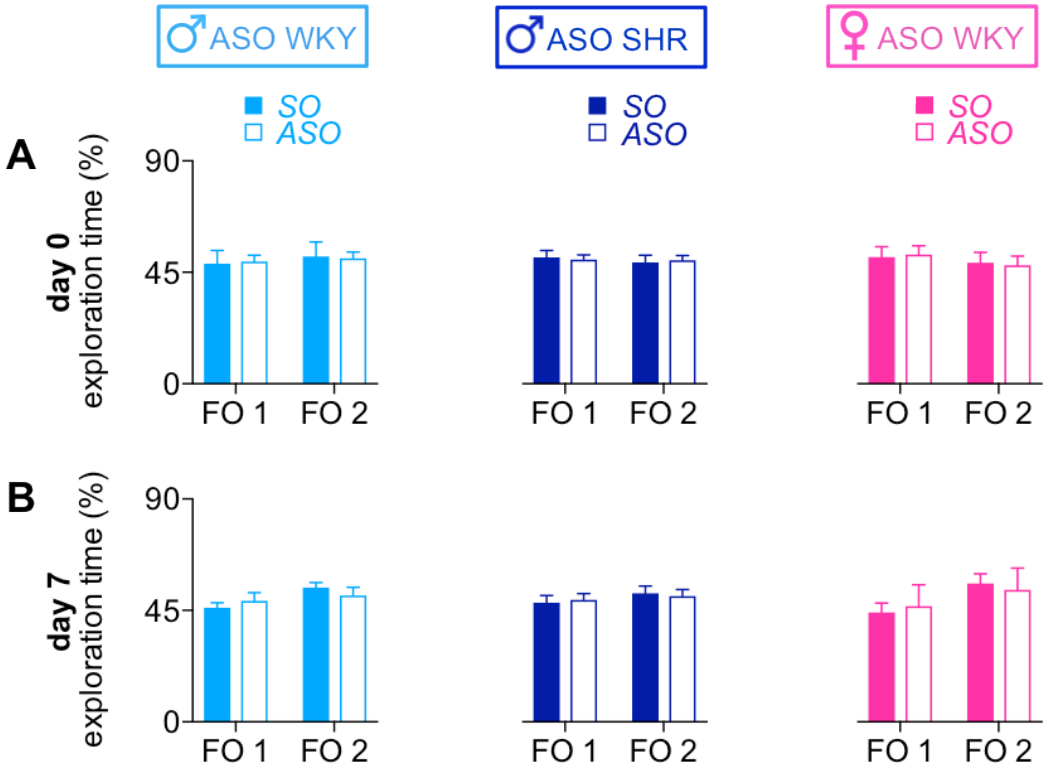
The work presented in this thesis confirms and expands upon the knowledge of the SRY in the healthy male brain as well as in male-biased neurological disorders, PD and ADHD. These findings challenge the current dogma that gonadal sex hormones are the sole regulators of sexual dimorphism in the brain. Furthermore, it presents a new framework that includes sex chromosome genes, particularly the Y-chromosome gene, *SRY*, for sex differences in the brain. *SRY* appears to play a wider role than previously thought, whereby it is involved in the healthy regulation of DA and GABA neurotransmission in the male brain. Thus, the dysregulation *of SRY* expression could predispose males to neurological disorders such as PD and ADHD and normalization of SRY may be protective in males. Future research aimed at better understanding the role of SRY in the male brain is necessary for the development of disease modifying therapies for male-biased neurodegenerative and neuropsychiatric disorders.

APPENDIX 1:

Supplementary figures and tables



Supplementary figure 1 Effect of nigral Sry ASO infusion on striatal DA and DOPAC levels in male rats. Following repeated nigral *Sry* antisense or sense ODN injections (2µg/daily for 10 days) brains were processed for striatal DA and DOPAC levels measurements (n≥8/group, mean ± S.E.M. student t-test, *p<0.05, compared to sense).



Supplementary figure 2 Percentage of time spent exploring the familiar objects (FO) during the familiarisation phase at A) day 0 and B) day 7. All groups of animals equal exploration time for each object. (n≥6/group, mean ± S.E.M. two-way ANOVA).

Species	Gene identifier	Gene Name	Forward sequence (5' – 3')	Reverse sequence (5' – 3')
Rat	Sry	Sex determining region Y	ttccaggaggcgcagagactga	tgttgaggcaacttcacgctgca
Rat	Th	Tyrosine hydroxylase	actgtggaattcgggctatg	cattgaagctctcggacaca
Rat	Ddc	Dopa decarboxylase	tgactatctggacggcattgagg	ggaagtaagcgaagaagtaggg
Rat	Mao-a	Monoamine oxidase- a	acgctcaggaatgggacaagatg	cccacactgcctcacataccaca
Rat	Dbh	Dopamine beta hydroxylase	agccccttcccttaccaca	tgcgttctccatctcacctc
Rat	Dat	Dopamine reuptake transporter	tgctggtcattgttctgctc	gctccaggaagggtaactcc
Rat	Drd1	Dopamine D1 receptor	gccatagagacggtgagcat	ggcatgagggatcaggtaaa
Rat	Drd2	Dopamine D2 receptor	gtcctctacagcgccttcac	atgaggtctggcctgcatag
Rat	Gad1	Glutamate decarboxylase 1	tccaagaacctgctttcctgt	ggatatggctcccccaggag
Rat	Gad2	Glutamate decarboxylase 1	gcccgctataagatgtttccag	gctgaaagaggtaggaagcatg
Rat	Gls	Glutaminase	ttgatcctcggagagaaggag	gacgtgtcgtcacttgactcag
Rat	Sox3	SRY-related HMG-box 3	aacgctttcatggtgtggtcc	gtccgggtactccttcatgtg
Rat	Sox6	SRY-related HMG-box 6	ctgcctctgcaccccataatg	ttgctgagatgacagaacgct
Rat	Bax	Bcl-2-associated X protein	ctcaaggccctgtgcactaaa	cccggaggaagtccagtgt
Rat	Bcl-2	B-cell lymphoma 2	ggaggctgggatgcctttg	ctgagcagcgtcttcagagaca
Rat	Sod1	Superoxide dismutase 1	cactgcaggacctcattttaatcc	gtctccaacatgcctctcttcat
Rat	Sod2	Superoxide dismutase 2	cgctggccaagggagat	ccccgccattgaacttca
Rat	Gpx1	Glutathione peroxidase 1	ctcggtttcccgtgcaat	tcggacatacttgagggaattca
Rat	iNos	Inducible nitric oxide synthase	cggaagagacgcacaggcagaggtt	aaggcagcaggcacacgcaatgatg
Rat	Pgc1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	cagtcaagctgtttttgacgac	cggagagttaaaggaagagcaa
Rat	Cyt C	Cytochrome c oxidase	actacccttgcctgatgtg	actcattggtgcccttgttc
Rat	Nd1	NADH-ubiquinone oxidoreductase chain 1	ttaattgccatggccttcctcacc	tggttagagggcgtatgggttctt
Rat	Txnrd1	Thioredoxin reductase 1	tcaaggtgaccgctaagtcc	tcttcccggtcttttcattg
Rat	Gadd45γ	Growth arrest and DNA-damage-inducible protein GADD45 gamma	gctgcgagaacgacattgaca	cggctctcctcgcagaacaa
Rat	Puma	p53 upregulated modulator of apoptosis	agtgcgccttcactttgg	caggaggctagtggtcaggt
Rat	Tbp1	TATA-box-binding	gggagctgtgatgtgaagt	gtggtcttcctgaatcccttta

		protein 1		
Rat	*Tnfα*	*Tumour necrosis factor alpha*	gccctaaggacacccctgaggg agc	tccaaagtagacctgcccggact cc
Rat	*Il-1β*	*Interleukin 1 beta*	aaaaatgcctcgtgctgtct	tcgttgcttgtctctccttg
Rat	*Il-10*	*Interleukin 10*	taagggttacttgggttgcc	tatccagagggtcttcagc
Rat	*Tgf-β1*	*Transforming growth factor beta 1*	tggaaatcaatgggatcagtc	ggagctgtgcaggtgttgag
Human	*SRY*	*Sex determining Region Y*	ttcccgcagatcccgcttcggtac tctg	ttttttttttttttgaaatgaataag
Human	*GADD45γ*	*Growth arrest and DNA-damage-inducible protein GADD45 gamma*	actagctgctggttgatcgc	caactcatgcagcgctttc
Human	*β2M*	*Beta-2-Microglobulin*	tgaattgctatgtgtctgggt	cctccatgatgctgcttacat

Supplementary table 1 Primer sequences

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

Supplementary table 2 Base sequences and positions of rat antisense (ASO) and sense (SO) oligonucleotides. Blue indicates that the residue is phosphorothioated.

APPENDIX 2:

Other publications during enrolment

Journal of Neurochemistry

JNC

JOURNAL OF NEUROCHEMISTRY | 2017 | 142 | 790–811

doi: 10.1111/jnc.14107

REVIEW

The malleable brain: plasticity of neural circuits and behavior – a review from students to students

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Received December 29, 2016; revised manuscript received April 30, 2017; accepted June 6, 2017.

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Abbreviations used: AMPAR, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor; ASD, autism spectrum disorders; CaMK-II, calcium/calmodulin-dependent protein kinase 2 alpha; CaN, PP2B/calcineurin; CREB2, also known as ATF4, cAMP-responsive element binding protein-2; CRTC-1, CREB-regulated transcription coactivator 1; DLPFC, dorsolateral prefrontal cortex; ERK, extracellular signal-regulated kinase; FMRP, fragile X mental retardation protein; LTD, long-term depression; LTP, long-term potentiation; M1, primary motor cortex; MAPK, mitogen-activated protein kinase; MB, mushroom body; MD, monocular deprivation; MMP, matrix metalloproteinase; NMJ, neuromuscular junction; PFC, prefrontal cortex; PKA, cAMP-dependent protein kinase; PV cell, parvalbumin-expressing basket cells; RBP, RNA-binding protein; REM, rapid eye movement; RIM, Rab3-interacting molecule; SMA, supplementary motor area; SPW-R, sharp wave ripple complex; SWS, slow-wave sleep; VS, ventral striatum; VTA, ventral tegmental area.

REVIEW

forms, offers new insights into the underlying mechanisms and behavioral relevance, and provides directions for future research in the field of synaptic plasticity.

Keywords: associative learning, critical period, Hebbian plasticity, homeostatic plasticity, motorskill learning, synaptic plasticity.
J. Neurochem. (2017) **142**, 790–811.

Read the Editorial Highlight for this article on page 788.
***Cover Image for this issue: doi: 10.1111/jnc.13815*.**

The mammalian brain has the fascinating ability of processing and storing information in highly organized neuronal networks (Hofman 2014). Synaptic plasticity can be defined as the potential of neural activity patterns generated by experiences to induce alterations in synaptic connectivity (Bliss & Lomo 1973; Citri & Malenka 2008), thereby playing key roles in brain function. This concept was first proposed by Donald Hebb in 1949, who stated that concurrent activation of pre-synaptic and post-synaptic neurons increases the strength of their synaptic connections (reviewed in Cooper 2005).

The nervous system modulates connectivity of its networks depending on activity of neurons that receive inputs from their surroundings (Mayford *et al.* 2012) which is, for instance, widely discussed as a mechanism of memory formation (Fauth & Tetzlaff 2016). Such modulations could appear at the functional level, for example, through changes in transmitter release probability that result in altered strength of synaptic transmission at pre-existing synapses (Castellucci *et al.* 1970), and by changes in neuronal excitability (McKay *et al.* 2013). On the other hand, structural modifications are also possible through the emergence or disappearance of dendritic spines (Engert & Bonhoeffer 1999) or synapses (Martin & Kandel 1996).

Animal models have allowed a more complete understanding of multiple features of the nervous system at molecular, cellular, physiological, anatomical, and behavioral levels. Simpler animal models are characterized by a number of technical advantages, such as a short lifespan, large numbers of offspring, mapped neural networks, and a wide range of options for genetic modification (Jennings 2011).

In this context, *Drosophila melanogaster* (Frank *et al.* 2013), the transparent nematode *Caenorhabditis elegans* (Corsi 2006; Lau *et al.* 2013), as well as the snail *Aplysia californica* (Bailey & Chen 1983, 1988) have been widely used in neuroscience research during the past decades to study, for example, neurotransmitter release in synaptic transmission (Schwarz 2006), axon guidance (Dickson & Zou 2010), as well as potential neuroprotective strategies in disease (Cutler *et al.* 2015; Sandin *et al.* 2016). Fundamental mechanisms in fish locomotor control were uncovered by application of functional imaging approaches on larval zebrafish (Portugues & Engert 2009; Valente *et al.* 2012), which share basic brain organization with their vertebrate counterparts (Friedrich *et al.* 2010; Engert & Wilson 2012; Leung *et al.* 2013).

Nonetheless, the most commonly used models in neuroscience are rodents. Mice (*Mus musculus*) and rats (*Rattus norvegicus*) are particularly used in transgenic approaches, since they offer a great variety of opportunities for studying complex behaviors along with powerful genetic tools (Van Meer & Raber 2005; Ward *et al.* 2011; Kim *et al.* 2013; Rincon-Cortes & Sullivan 2016). Rodent hippocampal slice preparations are only one example of broadly used tools to study mammalian synaptic function and plasticity from genetic and epigenetic to protein and structural levels (Hofer *et al.* 2009; Ch'ng *et al.* 2012). Despite their advantages, mouse models alone cannot reflect the diversity of all organisms. Variations in motor systems and behavior explain why larger animal models are often more suitable (Courtin *et al*. 2014). For example, early studies on the effects of the visual cortex (Wiesel & Hubel 1963; Shatz & Stryker 1978; Shatz & Luskin 1986) and description of the mechanisms underlying plasticity on the proteomic level (Cnops *et al*. 2008) were carried out in kittens (*Felis silvestris* catus).

Although improvements in modeling have enabled considerable progress in understanding synaptic plasticity, many mechanistic, structural, and regulatory factors remain elusive. To understand fully the computation of the brain in health and disease will therefore be a vital task in present and future neuroscience. By covering topics from developmental, molecular, and systematic neuroscience, this review aims to provide an update on current knowledge in synaptic plasticity, as well as offer directions for future research in the field. The authors participated in the first Flagship School hosted by the International Society for Neurochemistry (ISN) and the *Journal of Neurochemistry* (JNC) on 'The Malleable Brain' in September 2016 in Alpbach, Austria.

Synaptic plasticity during the critical period

During certain periods of development, brain circuits are highly receptive to specific experiences and undergo maximum plasticity rates. These sensitive temporal windows of heightened experience-dependent neural plasticity are known as critical periods (Hartley & Lee 2015), which can be defined as the timeframe in which development leads to permanent and irreversible changes in the neuronal networks

of different brain regions (Ismail et al. 2016). Critical periods are initiated when the cortical circuitry begins to receive input from the sensory epithelium (Trachtenberg 2015) and are crucial for refining brain circuits based on environmental inputs (Hensch 2005), leaving major impacts on behavior (Knudsen 2004). Timing, duration, and strength of critical periods can be affected by neurological diseases that are associated with abnormal plasticity mechanisms, such as the autism spectrum disorders (ASD), Rett, Fragile X, and Angelman syndromes (Ismail et al. 2016).

Knowledge of neuroplasticity within critical periods emerged primarily from research on sensory systems such as the visual system. The development and plasticity of the primary visual cortex have been studied using various animal models (Wiesel & Hubel 1963; Horton & Hocking 1997; Crowley & Katz 2000; Smith & Trachtenberg 2007; Faguet *et al.* 2009). Following eye opening, the primary visual cortex has a preference for inputs from one eye, known as ocular dominance, but this is reduced during the critical period (Hubel & Wiesel 1962; Espinosa & Stryker 2012). Monocular deprivation during early postnatal life causes connections to be eliminated in the closed eye and strengthens connections in the open eye in both humans and rodents (Wiesel 1982; Antonini & Stryker 1993). This leads to loss of visual acuity and a clinical condition called amblyopia (Wiesel 1982). Studies have also reported that children who develop discordant vision or a cataract in one eye during a critical period suffer from permanent defects in vision through that eye (Parsons-Smith 1953). As later-life monocular deprivation effects are not severe, it is apparent that visual experience during the critical period in early life is crucial for optimal development of the visual cortex (Nabel & Morishita 2013).

The initiation and closure of the visual critical period is driven by the maturation of the inhibitory circuitry in the cortex (Hensch *et al.* 1998; Katagiri *et al.* 2007). The shift to a predominantly inhibitory activity culminates in consolidation of neuronal circuits through curtailing development of new synapses and pruning of existing ones. Although the role of inhibition in the initiation of the critical period is still elusive, it probably mediates plasticity by altering the composition of NMDARs (Kanold *et al.* 2009) and/or facilitating long-term depression (LTD) (Choi *et al.* 2002). Moreover, its inhibition directly plays a role in ocular dominance shift. This was exemplified when intravenous injections of the GABAR antagonist bicuculline reinstated binocular responses in a significant proportion of neurons in the visual cortex of cats with vision loss (Duffy *et al.* 1976). Further studies have shown that transplantation of GABAergic precursor cells in mice after the closure of the critical period induces ocular dominance plasticity (Southwell *et al.* 2010; Tang *et al.* 2014).

The extracellular environment is also important for visual cortical plasticity, particularly perineuronal nets (PNN), which consist of chondroitin-sulfate proteoglycans. With age, GABAergic parvalbumin-expressing interneurons (PV cells) will eventually be encased by PNNs, thus marking the end of the critical period. Visual experience is essential for maturation of PV cells, and accurate critical period timing as dark rearing from birth results in a prolonged critical period because of stunted maturation of the PNNs (Pizzorusso *et al.* 2002). PNNs mediate the transfer and facilitate internalization of orthodenticle homeobox 2 homeoprotein in PV cells, in which specific orthodenticle homeobox 2-binding sites are present to maintain critical period closure (Beurdeley *et al.* 2012).

During the development of the primary auditory system, synapse elimination occurs in the brainstem in the first postnatal week of rodents, prior to hearing onset (Kim & Kandler 2003). Following the onset of hearing at P11-P12, the critical period is initiated in the auditory cortex (Blatchley *et al.* 1987; de Villers-Sidani *et al.* 2007) where synchronized and coherent environmental acoustic inputs are crucial for maturation of the primary auditory system (Zhang *et al.* 2001; Chang & Merzenich 2003). Exposure to degraded noise conditions (i.e., incoherent continuous noise and absence of tones that stand out) causes a delay in the critical period leading to poor development of the auditory cortex (Zhang et al. 2001; Chang & Merzenich 2003; Hensch 2004). Thus, these studies indicate that, besides sensory deprivation, poor environmental noise can potentially halt or delay development of hearing in children (Zhang et al. 2001; Chang & Merzenich 2003). Learning occurring in a rapid, period-sensitive manner independently of the behavioral consequences, and accompanied by synaptic elimination, comprises the concept of imprinting. Many species, including birds and mammals, exhibit imprinting, in which they display an innate tendency to follow the first suitable moving stimulus. The first extensive systematic study involving critical periods required for imprinting was carried out by Konrad Lorenz in the first half of the 20th century (Lorenz 1937). He demonstrated that incubator-hatched geese imprint on the first moving visual object during the critical period between the first 13–16 h. Female offspring of monkeys (Harlow & Zimmermann 1959) or rats (Liu *et al.* 2000) that experience stress and anxiety during early postnatal days tend to display poor maternal care behavior later on in life. Parental care mediates the effects of environmental adversity on the development of the nervous system and, therefore, early postnatal experiences may subdue the genetic predispositions (Meaney 2001).

Similarly, slow-wave sleep, which makes up most of adult sleep as compared to rapid eye movement-like sleep during early life, augments critical period plasticity in the visual system (Jouvet-Mounier *et al.* 1970). It has also been suggested that sleep is configured and structured by experience (Miyamoto *et al.* 2003). Sound localization is another example of an intricate exercise that displays several aspects of critical period development (Knudsen *et al.* 2000). Here,

the auditory and visual experiences during early life cause adaptive changes in the auditory localization behavior and, correspondingly, in the functional and anatomical properties of the midbrain localization pathway. Extensive studies by Allison Doupe explained the need of young songbirds to hear adult sound (songs) during a sensitive period to learn their songs, which appears to be similar to speech acquisition in humans (Brainard & Doupe 2002, 2013). We now have sufficient evidence to recognize critical periods as the windows that provide insights into the neural mechanisms of learning and synaptic plasticity.

Cellular and molecular mechanisms of synaptic plasticity

Synaptic plasticity requires functional and/or structural modifications at synapses upon persistent stimulation (Lepeta *et al.* 2016). Functional plasticity is essential to allow adaptation to different contexts and learning (Kandel *et al.* 2014; Bailey *et al.* 2015), which rely on precise, local and dynamic control of synapse function (Petzoldt *et al.* 2016). Pre-synaptic plasticity involves alteration of neurotransmitter release tonus or dynamics, while post-synaptic plasticity usually encompasses alterations in receptor number, availability or properties (Yang & Calakos 2013; Lepeta et al. 2016).

While several forms of structural and functional alterations in different neurotransmitter systems are speculated to exist, plasticity at glutamatergic synapses has been the most extensively characterized. Canonical mechanisms of functional plasticity at glutamatergic synapses involve changes in neurotransmitter receptor presence at the cleft with consequent modifications of post-synaptic ionic balance and kinase/phosphatase activation. For example, long-term plasticity mechanisms that relate to memory [namely, long-term potentiation (LTP) and LTD] appear to derive from persistent stimulation of synapses at high or low frequency, respectively (Bliss & Lomo 1973; Nabavi *et al.* 2014). Persistent stimulation triggers ion influx through NMDARs that, in turn, determines the rate of exposure of α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPARs) through post-translational modifications at the post-synaptic density. Fine-tuning of such complex cascades will therefore establish differential synaptic responses (Luscher & Malenka 2012; Kandel et al. 2014).

Despite some controversies (MacDougall & Fine 2014; Padamsey & Emptage 2014), pre-synaptic mechanisms, including increased neurotransmitter release, appear to contribute to LTP (Bliss & Collingridge 2013). Although mechanistic details are still not fully known, recent evidence has highlighted the role of extracellular proteases, including matrix metalloproteinases 7 and 9, ADAM10, and tissue plasminogen activator (Szklarczyk *et al.* 2007; Peixoto *et al.* 2012; Wiera *et al.* 2013; Borcel *et al.* 2016; Jeanneret *et al.* 2016), and pre-synaptic cannabinoid receptor 1 as potential drivers of pre-synaptic contributions (Madronal *et al.* 2012). For instance, endocannabinoids could work as liaisons that bridge post- and pre-synaptic signals toward plasticity (Wang *et al.* 2016), in addition to well-known factors such as neurexins and ephrins (Sudhof 2008; Klein 2009).

In the short term, synaptic plasticity is protein synthesis-independent and relies on modification of the already existing proteins. For longer lasting changes, local translation in response to stimulation that uses the local machinery is crucial, and can take place even in the dendritic compartment (Aakalu *et al.* 2001; Sutton & Schuman 2006). However, this can only last for several hours, suggesting that transcriptional changes are required for maintaining long-lasting plasticity, such as LTP and LTD (Frey *et al.* 1996; Alberini & Kandel 2014).

Transcriptional regulation and synaptic plasticity

Nuclear transcription sets the cell at a ready state, after which the locally regulated translation at the synapse permits quick and space-restricted responses. From a canonical perspective, the set of persistent activity-driven molecular modifications at the synapse (e.g., ion influx, post-translational modifications) will drive the activation of a subset of transcription factors ultimately resulting in transcriptional reprogramming to maintain plasticity (Alberini & Kandel 2014). This may be further regulated by epigenetic status and by several classes of non-coding transcripts, including long non-coding RNAs (lncRNAs) and piwi-related RNAs (piRNAs) (Rajasethupathy *et al.* 2012; Maag *et al.* 2015). Nonetheless, two additional types of signals appear to shape the final response of a single synapse: the signal for translation of specific mRNAs already at the synapse and retrograde signals from the stimulated synapse to the nucleus, allowing for *de novo* transcription.

Several mechanisms that mediate retrograde signals from synapse to nucleus have been identified, including rapid electrochemical signaling, regenerative calcium waves orchestrated by the endoplasmic reticulum, and physical transport of signaling molecules (Ch'ng & Martin 2011). Synapse-localized importins that bind the nuclear localization signal-containing cargoes play a crucial role in mediating retrograde communication by translocating rapidly to the nucleus in response to a synaptic stimulus (Thompson *et al.* 2004; Jeffrey *et al.* 2009). This could offer a mechanism through which specific synapses could drive nuclear transcriptional changes. The list of proteins that translocate from synapses to the nucleus upon stimulation has been ever growing and includes cAMP-responsive element binding protein-2, CAM associated protein, ErbB4, Jacob, nuclear factor kappa B (NF–κB), ErbB4, proline-rich protein 7, ring finger protein 10, and the amyloid precursor protein intracellular domain (Murata *et al.* 2005; Lee *et al.* 2007; Dieterich *et al.* 2008; Lai *et al.* 2008; Ch'ng & Martin

2011; Karpova *et al.* 2013; Dinamarca *et al.* 2016; Kravchick *et al.* 2016; Melgarejo da Rosa *et al.* 2016).

Recently, cAMP-response element binding protein (CREB)-regulated transcription coactivator 1 has emerged as an important coincidence detector that undergoes rapid nuclear import upon stimulation and subsequent CREB binding, coupling specific synaptic stimulation with activity-dependent transcription because of its nuclear import and localization (Ch'ng *et al.* 2015). CREB-regulated transcription coactivator 1 appears to undergo a complex phosphorylation pattern that is controlled by both calcium influx/calcineurin activation and cAMP-dependent signaling. (Ch'ng et al. 2012).

Additional factors promoting synapse-nucleus communication remain to be identified in order to establish the full repertoire of mechanisms controlling activity-dependent transcription. For instance, determining the molecules that modulate the strength and target specificity of activity-induced transcription, as well as establishing how activity of a specific group of synapses could drive transcription to sustain their own plasticity, are current gaps in the field.

Local protein synthesis as an emerging mechanism of plasticity

Local regulation of protein synthesis is a key process allowing for focal physiological responses in many cell types. This is especially relevant for larger and polarized cells, conferring on them the ability to quickly respond to the local cues at a single site in a nucleus-independent manner (Sutton & Schuman 2006). Given the diversity of synapses that can be present in the dendritic arborization of a single neuron, engagement of local mechanisms for regulating synapse strength and function coordinates multiple signals and facilitates proper responses (Rangaraju *et al.* 2017).

To date, multiple mRNAs required for neuronal function have been shown to undergo synaptic translation in response to local stimulation, including Arc, calcium/calmodulin-dependent protein kinase 2 alpha, and PSD95 (Aakalu et al. 2001; Bramham & Wells 2007). Furthermore, components of the translational machinery are present in dendritic spines (Steward & Levy 1982), together with Dicer and other components of the RNA-induced silencing complex (RISC) as well as pre-miRNAs that are further cleaved to mature miRNA (Wang *et al.* 2012; Bicker *et al.* 2013). Importantly, some studies have determined that some miRNAs are synaptically enriched, and may play potential synapse-specific roles in translation (Lugli *et al.* 2008; Siegel *et al.* 2009; Sambandan *et al.* 2017). However, given that the translational machinery is not evenly distributed in dendrites, a single spine may not host the whole translational apparatus, and short stretches of the dendritic shafts presumably harbor larger subcellular structures, including stretches of endoplasmic reticulum, Golgi outposts, and ER-Golgi intermediate compartments (Horton & Ehlers 2003; Horton *et al.* 2005; Cui-Wang *et al.* 2012; Hanus *et al.* 2014; Mikhaylova *et al.* 2016).

The dendritic pool of synaptically translated mRNAs considerably differs from the cell soma, as recently shown by deep RNA sequencing of microdissected regions from hippocampal CA1 (Cajigas *et al.* 2012). Interestingly, sequencing of 3′UTRs allowing the identification of multiple polyadenylation sites in mRNAs revealed that longer 3′UTR isoforms are mainly localized to distal parts of the cell, whereas the shorter ones mainly localize to the soma, suggesting a differential enrichment of mRNA variants of the same gene at synaptic sites (An *et al.* 2008; Will *et al.* 2013; Epstein *et al.* 2014; Vicario *et al.* 2015). A longer 3′UTR provides more flexibility for regulation, because of the presence of more miRNA and RNA-binding protein (RBP) recognition motifs.

Synapse-targeted mRNAs are usually transported in RBP-containing granules by microtubule-dependent transport (Doyle & Kiebler 2011). Although the precise cues that trigger local translation of specific mRNAs are yet to be determined, recent studies suggested that calcium-dependent retrograde netrin-1/DCC receptor signaling could be one such mechanism (Kim & Martin 2015). In addition, activity-dependent regulation of the fragile X mental retardation protein (FMRP) has been shown to promote a number of synaptic mRNAs by direct interaction (Darnell *et al.* 2001). FMRP is an RBP that physiologically represses mRNA translation. Upon synapse stimulation, however, FMRP becomes inhibited, allowing several mRNAs to incorporate into the translational machinery (Liu-Yesucevitz *et al.* 2011; Sidorov *et al.* 2013). In accordance, deregulation of FMRP levels has been implicated in multiple neurological disorders with a synaptic background, including bipolar disorder, schizophrenia, and affective disorders (Bryant & Yazdani 2016). Lack of FMRP expression leads to Fragile X Syndrome, an ASD with severe cognitive impairments, further confirming the importance of precise regulation of local translation at the synapse (Bagni & Greenough 2005).

Controlling synaptic proteostasis to shape plasticity

The requirement of coordinated mRNA transport and local translation for synaptic plasticity adds support to the notion that maintaining protein homeostasis, or proteostasis, is critical for synapse stability and function (Hanus & Schuman 2013). In addition to local protein synthesis, protein degradation by the proteasome also emerges as a critical regulator of plasticity. In fact, neuronal activity recruits the proteasome to synaptic sites (Bingol & Schuman 2006) through CamKIIα anchoring (Bingol *et al.* 2010). Synaptic localization of the proteasome, in turn, promotes PSD protein degradation (Bingol & Schuman 2004), regulates neurotransmitter receptor trafficking (Ferreira *et al.* 2015), and plays important roles in shaping structural and functional

plasticity (Djakovic *et al.* 2012; Hamilton *et al.* 2012; Santos *et al.* 2015; Li *et al.* 2016).

Schuman and colleagues have recently employed a variation in metabolic labeling termed 'bio-orthogonal non-canonical amino acid tagging' to determine proteome fluctuations during homeostatic plasticity and found the proteome size to be similar regardless of the increased cell activity in response to stimulation (Schanzenbacher *et al.* 2016). Their findings suggest that, while several proteins are up-regulated during synaptic scaling, others are down-regulated, maintaining the number of unique proteins being synthesized at a stable level (Schanzenbacher et al. 2016). These findings are in agreement with the revised 'synaptic tagging' hypothesis, which states that expression of the so-called plasticity-related products, and not only early LTP formation, is required for long-lasting plasticity and, for example, memory formation (see Redondo & Morris 2011 for a review). Thus, maintaining synaptic proteostasis through tight control of protein synthesis and degradation allows plastic stimuli to express, thereby remodeling synaptic function (Fig. 1).

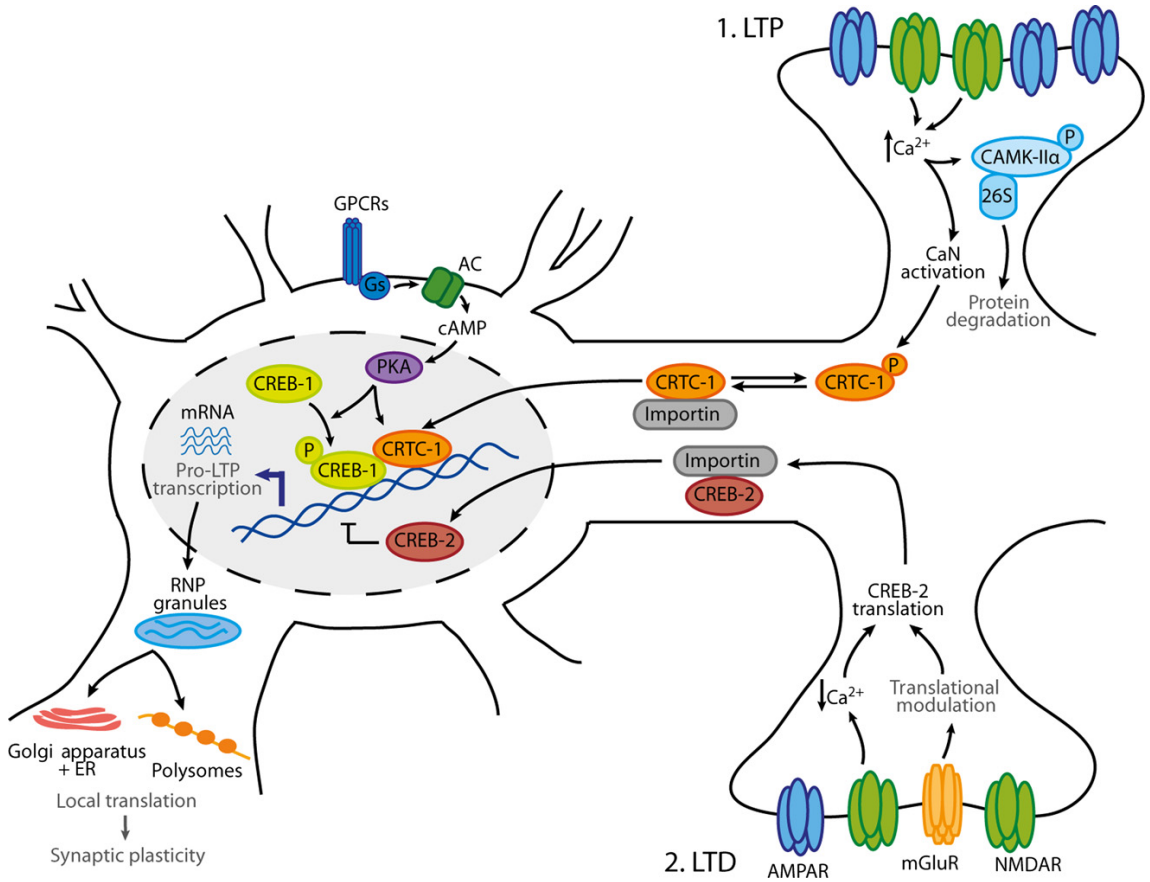

Fig. 1 Synapse-to-nucleus communication and local proteostasis in synaptic plasticity. (1.) LTP induces post-synaptic AMPAR (green) insertion, enabling elevated calcium (Ca^{2+}) influx through NMDAR (blue) activity. Increased Ca^{2+} levels lead to autophosphorylation and activation of CaMK-IIα, which acts as a scaffold translocating the 26S-proteasome to the synapse, thereby inducing degradation of polyubiquitinated proteins and facilitating protein turnover and synaptic plasticity. Increased Ca^{2+} influx further activates PP2B/calcineurin (CaN), which dephosphorylates CREB-regulated transcription coactivator 1 (CRTC-1), a transcriptional coactivator that mediates cAMP-response element binding protein (CREB)-dependent transcription. CRTC-1 thus binds to importin and translocates to the nucleus. Activation of GPCRs coupled to Gs stimulate adenylyl cyclase (AC) to produce cAMP, thereby leading to activation of cAMP-dependent protein kinase (PKA). PKA phosphorylates CREB-1, which together with nuclear CRTC-1 induces transcription of pro-LTP factors. These newly produced mRNAs may be stored in ribonucleoprotein granules (RNPs) and transported to Golgi and endoplasmic reticulum (ER) outposts or to free polysomes at synapses, where they will be locally translated. (2.) On the other hand, LTD-dependent reduction in post-synaptic AMPARs decreases Ca^{2+} influx and facilitates CREB-2 translation. Furthermore, mGluRs are activated, leading to modulation of translation factors with additional induction of CREB-2 translation. CREB-2 translocates to the nucleus via binding to importin, drives LTD-dependent transcription, and antagonizes the actions of CREB-1, thus reducing pro-LTP gene expression.

In addition to maintaining levels of synaptic proteins, it is noteworthy that post-translational modifications comprise an important step toward ultimate functional modulation of synaptic proteins. For instance, most neurotransmitter receptors, ion channels, and scaffold proteins present at synaptic sites are post-translationally modified. Membrane insertion of synaptic AMPARs, for example, has been shown to depend on ubiquitination cycles (Mabb & Ehlers 2010; Goo *et al.* 2015).

Furthermore, tight control of protein maturation and stability is required for plasticity to develop. Several lines of evidence have indicated that local ER compartments regulate the availability of synaptic proteins, including glutamate receptors, in an activity-driven manner (Xia *et al.* 2001; Mu *et al.* 2003; Cui-Wang et al. 2012; Hanus et al. 2014; Pick *et al.* 2017). However, the precise mechanisms of subsequent Golgi sorting and additional post-translational modification have still been a matter of debate. Even though there is not a consensus on whether most dendrites harbor Golgi outposts (Gardiol *et al.* 1999; Horton & Ehlers 2003), data have suggested that some proteins might undergo Golgi-independent maturation, while others might be matured in tiny specialized ER compartments (Jeyifous *et al.* 2009) or in Golgi satellites, which diverge from traditional outposts (Mikhaylova et al. 2016). It is conceivable, therefore, that multiple modes of protein maturation could contribute to proteostasis and synaptic plasticity. Future studies are warranted to dissect the relative contribution of each of these mechanisms in the expression of plasticity.

Structural and homeostatic plasticity

Dendritic spines are primary sites for structural modifications during memory formation (Bailey et al. 2015). Spines are highly dynamic, as they grow, shrink, disappear, and change forms throughout lifetime (Parnass *et al.* 2000; Hering & Sheng 2001; Chen *et al.* 2014). There is an increase in spine turnover (growth and elimination) during adolescence, compared to adulthood (Holtmaat *et al.* 2005). Experiences such as exercise, learning, and environmental enrichment can influence spine turnover (Chklovskii *et al.* 2004; Lamprecht & LeDoux 2004; Leuner & Gould 2010; Lovden *et al.* 2013; Attardo *et al.* 2015).

For instance, monocular deprivation doubles the firing rate of spines in apical dendrites of pyramidal neurons in the mouse visual cortex, thereby resulting in an overall increase in spine density in layer 5 apical dendrites (Hofer et al. 2009). Thus, the shape and proportion of spines is constantly regulated by synaptic activity (Meyer *et al.* 2014) which, in turn, influences different aspects of spine function including abundance of receptors, diffusion of small molecules between spine and shaft, along with spine mobility and stability (Nimchinsky et al. 2002; Lai et al. 2008).

Reorganization of the spine cytoskeleton leads to modifications in spine morphology. The spine cytoskeleton is composed of actin filaments (F-actin) and microtubule components (Landis & Reese 1983; Cingolani & Goda 2008a). Although there is no apparent direct interaction between cytoskeletal elements (Geraldo & Gordon-Weeks 2009; Dent *et al.* 2011), proteins like drebrin (Dun & Chilton 2010), p140Cap (Jaworski *et al.* 2009), and Q motif-containing GTPase-activating protein 1 (Jausoro *et al.* 2012) are involved in mediating such interactions. There is an increase in actin stability and subsequent enlargement of spines upon induction of LTP, whereas LTD reduces F-actin stability, leading to decreased spine volume.

Association between structural and functional plasticity

Over the past 20 years, structural modifications that occur at the synaptic level during LTP stimulation have been extensively investigated. It is now well understood that LTP in hippocampal slices results in filopodia outgrowth and/or enlargement or bifurcation of pre-existing dendritic spines (Engert & Bonhoeffer 1999; Maletic-Savatic *et al.* 1999; Toni *et al.* 1999). Whether LTP-induced spine growth leads to formation of functional synapses remained unanswered for several years. By reconstructing dendritic spines with serial section electron microscopy from the barrel cortex of mice undergoing sensory experience by whisker trimming (removal of vibrissae), spine growth was found to precede synapse formation (Knott *et al.* 2006). In particular, nascent spines preferentially formed synapses with multi-synapse boutons 4 days after their formation. In a second study, Nägerl and colleagues performed time-lapse two-photon microscopy and serial section electron microscopy analysis in organotypic hippocampal slices (Nagerl *et al*. 2004). This revealed that LTP induces formation of new spines, which make close contacts with pre-synaptic boutons and form functional synapses within 15–19 h. A follow-up study in hippocampal slices showed a delay of just 1 h between spontaneous spine growth and synapse formation (Zito *et al.* 2009). On the contrary, LTD-like low-frequency stimulation of dendritic spines in CA1 pyramidal neurons induces NMDAR-dependent spine retractions and causes loss of functional synapses (Nagerl *et al.* 2004; Okamoto *et al.* 2004; Zhou *et al.* 2004). Taken together, these observations show that there is bidirectional activity-dependent regulation of structural plasticity.

Homeostatic plasticity and synaptic scaling

Hebbian plasticity is a form of synaptic plasticity which creates positive feedback loops of activity-dependent changes in synaptic strength, that, in turn, cause perturbations in the stability of neuronal networks. Hebbian plasticity triggers long-lasting activity-dependent changes in synaptic strength resulting from both LTP and LTD (Fig. 2). These durable forms of plasticity require correlated precise and strong firing of the pre- and post-synaptic neurons specific to active input, and therefore are thought to facilitate the empowerment of particular synaptic connections (Vitureira & Goda 2013).

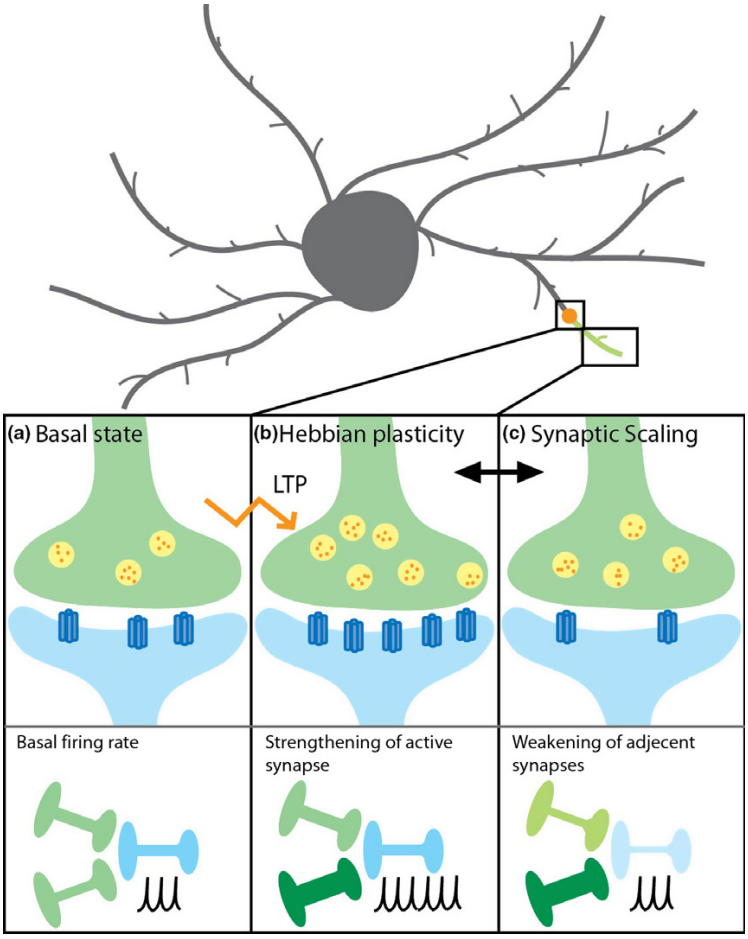

Fig. 2 Hebbian and non-Hebbian forms of synaptic plasticity. During basal state, synapses process stimuli via AMPARs (a, upper panel), leading to a defined firing rate (a, lower panel). LTP induces Hebbian plasticity, during which synapses increase the number of AMPARs in an input-specific fashion (b, upper panel), thereby strengthening synapses. Increased stimuli lead to an increased firing rate (b, lower panel). Synaptic scaling, the most studied form of non-Hebbian plasticity, acts via post-synaptic reduction in AMPARs (c, upper panel) through weakening synapses adjacent to the potentiated synapse to restore homeostasis and optimal global firing rate (c, lower panel).

To counterbalance unsustained activity arising from LTP or LTD processes, neurons have developed negative-feedback homeostatic mechanisms. The proper functioning of mammalian brain relies on joint interplay of homeostatic and Hebbian plasticity (Fernandes & Carvalho 2016). As such, homeostatic forms of synaptic plasticity not only reduce synaptic strength during elevated excitability conditions but also play a crucial role in preventing unnecessary synapse loss by increasing synaptic strength during chronic activity suppression conditions (Vitureira & Goda 2013) (Fig. 2). It is interesting that these two opposing phenomena likely cooperate at the molecular level by regulating effectors at the synapses (Yger & Gilson 2015). Recently, disruptions in homeostatic plasticity have been associated with brain disorders such as ASD, schizophrenia, epilepsy, Alzheimer, and Huntington diseases (Fernandes & Carvalho 2016).

Synaptic scaling of excitatory synapses is the most studied form of homeostatic plasticity in the central nervous system. It is involved in the stabilization of neuronal networks and synaptic strength in the brain. Synaptic scaling keeps the firing rates within a set-point range by increasing or decreasing the accumulation of glutamate receptors (in particular, the AMPARs) at synaptic sites (Turrigiano *et al.* 1998; Turrigiano 2008). Compensatory changes in the accumulation of glutamate receptors are achieved through sustained increase in activity using bicuculline or through prolonged activity deprivation using pharmacological agents such as tetrodotoxin (Turrigiano et al. 1998; Maffei & Turrigiano 2008; Hengen *et al.* 2013; Gainey *et al.* 2015). These changes maintain synaptic strength and overall network dynamics.

Scaling AMPARs up or down through post-synaptic accumulation and/or changes in their subunit composition, requires a wide range of proteins. These include transcriptional and translational regulators, such as MeCP2 (Blackman *et al.* 2012; Qiu *et al.* 2012), FMRP (Soden & Chen 2010): the scaffolding proteins Stargazin (Louros *et al.* 2014), PICK1 (Anggono *et al.* 2011), GRIP1 (Gainey et al. 2015), and Arc/Arg3.1 (Shepherd *et al.* 2006; Gao *et al.* 2010), cell-adhesion/trans-synaptic signaling molecules like

β3-integrins (Cingolani & Goda 2008b; Cingolani *et al.* 2008), β-catenin (Okuda *et al.* 2007; Vitureira *et al.* 2011), and the released soluble factors Brain-derived neurotrophic factor (BDNF) (Rutherford *et al.* 1998) and tumor necrosis factor alpha (TNFα) (Stellwagen & Malenka 2006).

Synaptic scaling can globally adjust responses of all synapses in a given network (Turrigiano 2008). For instance, in a certain neuron all the synaptic populations activate simultaneously to increase/decrease AMPARs upon synaptic scaling (Turrigiano 2008). Thus, synaptic scaling helps to maintain synaptic homeostasis without interfering with the individual differences in stronger versus weaker synapses.

Pre-synaptic homeostatic plasticity

The homeostatic modulation of pre-synaptic plasticity was first described at the *Drosophila* neuromuscular junction (NMJ) (Davis & Goodman 1998; Davis *et al.* 1998). Subsequent studies have revealed that homeostatic modulation of the NMJ is evolutionarily conserved from flies to humans (Cull-Candy *et al.* 1980; Plomp *et al.* 1992).

Pre-synaptic homeostasis induces a fast, persistent, and accurate modulation of pre-synaptic vesicle fusion in response to the levels of ongoing neuronal activity (Davis & Muller 2015). Pre-synaptic homeostasis principally requires increases in pre-synaptic Ca^{2+} influx through CaV2.1 channels (Muller & Davis 2012) and in the readily releasable vesicle pool (Weyhersmuller *et al.* 2011; Muller *et al.* 2012).

Several proteins are required for homeostatic signaling at the pre-synaptic NMJ site. Proteins such as dysbindin and snapin are involved in Ca^{2+} dependent synaptic vesicle endocytosis (Dickman & Davis 2009; Dickman *et al.* 2012) and influence the pre-synaptic strength of the NMJ. In addition, both ab3 and Rab3-interacting molecules are essential regulators of pre-synaptic homeostasis. Rab3-interacting molecule interacts with the pre-synaptic protein ELKS/CAST, pre-synaptic voltage gated channels, and munc13 (Sudhof 2012), and is required for the homeostatic plasticity-induced increase in the readily releasable pool of vesicles (Muller et al. 2012). However, further studies are required to understand how pre-synaptic homeostasis plasticity can be integrated with activity-dependent plasticity.

Associative learning

Synaptic plasticity is now widely accepted to be a neural correlate of at least some types of learning and memory, enabling us to adapt to the environment and shape our behavior accordingly. In order to guide adaptive behavior, incoming sensory information needs to be integrated into existing brain circuitry that drives behavioral output (Naumann *et al.* 2016). That is, the animal needs to encode the valence of a stimulus, form an association between the stimulus and previously executed behavior, use that information to make an accurate prediction about future outcomes, and alter its behavior accordingly.

Associative learning of involuntary behavior, known as classical conditioning, involves the experience of a neutral stimulus and a subsequently occurring appetitive or aversive stimulus eliciting an automatic response. Following learning, presentation of the previously neutral stimulus alone is sufficient to elicit the associated response (Fanselow & Wassum 2015).

In *Drosophila*, olfactory conditioning is linked to the convergence of signals from Kenyon cells (encoding olfactory cues) and dopaminergic neurons (encoding stimulus valence) onto the mushroom body (MB), which is a key learning center in flies (Davis 1993; de Belle & Heisenberg 1994; Aso *et al.* 2014). For example, optogenetic activation of dopamine cells during odor presentation induces LTD of MB synapses, and promotes olfactory aversive learning (Hige *et al.* 2015). Yamagata *et al.* (2016) extended these findings and identified a specific subset of protocerebral anterior medial dopamine cluster neurons, PAM-γ3 neurons, which relay reward signals to the MB. They also demonstrated that the inhibitory neuropeptide allostatin A is necessary and sufficient to suppress PAM-γ3 basal activity via $G\alpha_o$ recruitment, and drive appetitive learning.

In the mammalian brain, neural substrates of classical learning have been studied extensively utilizing fear conditioning paradigms in rodents and is thought to involve synaptic plasticity in the amygdala (Romanski *et al.* 1993), auditory cortex (Letzkus *et al.* 2011), and prefrontal cortex (Courtin *et al.* 2014). On the molecular level, fear learning requires NMDAR activation (Fanselow & Kim 1994), while the MAPK/extracellular signal-regulated kinase signaling cascade is required for the expression and maintenance of fear memories (Atkins *et al.* 1998).

Because of its diversity and complexity, learning of voluntary behavior, also known as operant or instrumental conditioning, is less well understood. In the mammalian brain, changes in the phasic firing rate of dopaminergic neurons in the ventral tegmental area are thought to exert motivational control by encoding a value prediction error, that is, the discrepancy between the actual and predicted outcome (Schultz *et al.* 1997; Eshel *et al.* 2016; Matsumoto *et al.* 2016). These neurons project to the ventral striatum (VS) (Ikemoto 2007), which receives additional inputs from other memory-associated regions such as the hippocampus (Ito *et al.* 2006; Lansink *et al.* 2016), amygdala (Everitt *et al.* 1991; Ito et al. 2006), and prefrontal cortex (Gourley *et al.* 2016). Thus, the VS is ideally situated to integrate signals from different circuits involved in goal-directed behavior and to initiate the appropriate behavioral output. Supporting this view, the plasticity-related immediate early gene Zif268 has been shown to be up-regulated in the VS during instrumental learning (Maroteaux *et al.* 2014) whereas, blocking plasticity-related processes in the VS

impaired spatial learning (Ferretti *et al.* 2010). Reward associative learning requires the activation of NMDARs (Zellner *et al.* 2009) and dopamine D1 receptors (Kravitz *et al.* 2012; Higa *et al.* 2017), as well as its downstream targets cAMP and cAMP-dependent protein kinase (PKA) (Beninger & Gerdjikov 2004; Lorenzetti *et al.* 2008), and the MAPK signaling cascade (Michel *et al.* 2011). In keeping with this, increased extracellular signal-regulated kinase 2 activity facilitates striatal LTP and long-term memory formation (Mazzucchelli *et al.* 2002; Ferguson *et al.* 2006).

Another line of evidence has linked specific patterns of synchronized cell firing, in particular theta rhythmic activity, to plasticity and memory mechanisms (Buzsaki & Draguhn 2004). Cell firing at theta frequency in the VS appears to be phase-locked to hippocampal theta waves, and reward-expectancy enhances theta and beta band activity (Berke *et al.* 2004; Lansink et al. 2016). Moreover, connectivity between hippocampal, cortical, and striatal regions (driven by theta-alpha power) was correlated with memory retrieval in humans (Herweg *et al.* 2016), providing evidence that synchronization of cell firing across memory-related neural circuits may enable association between stimuli encoded in different aspects of the brain. Disrupting BDNF expression on the other hand alters theta power in the hippocampus and prefrontal cortex, decreases theta phase synchrony between these areas, reduces late LTP, and impairs fear extinction (Sakata *et al.* 2013; Hill *et al.* 2016).

For memories to guide future behavior, they need to be transformed into enduring representations (Dudai *et al.* 2015), which involve processes operating both at synapse (Frankland *et al.* 2004; Karunakaran *et al.* 2016) and system levels (Tse *et al.* 2007; Wei *et al.* 2016). Perhaps unsurprisingly, given their role in synaptic plasticity and learning, memory consolidation and reconsolidation require the activation of PKA (Kemenes *et al.* 2006), CREB-mediated transcription (Bourtchuladze *et al.* 1994; Limback-Stokin *et al.* 2004), and NFκB (Freudenthal *et al.* 2005). There is also growing interest in specific activity patterns, such as the sharp wave-ripple complex (SPW-R), as a potential memory consolidation mechanism (Buzsaki 2015). Changes in SPW-Rs have been correlated with operant learning (Ponomarenko *et al.* 2008) and learning-induced plasticity (Grosmark & Buzsaki 2016), while blocking SPW-Rs impaired memory consolidation (Nakashiba *et al.* 2009), spatial learning (Ego-Stengel & Wilson 2010), and goal-directed behavior (Jadhav *et al.* 2012). It is thought that SPW-Rs generate time-compressed replay loops of neural activity that occurred during an earlier state (Carr *et al.* 2011; Girardeau & Zugaro 2011). This allows for the repetition of new experiences and enables mechanisms supporting synaptic plasticity (Nadasdy *et al.* 1999; Buzsaki 2015; Jahnke *et al.* 2015; Sadowski *et al.* 2016). Several studies have highlighted the importance of interneurons in such system-wide modifications. In particular, plasticity of PV cells is crucial for maintaining gamma and SPW-R oscillations, synaptic plasticity, and long-term memory consolidation (Karunakaran et al. 2016; Zarnadze *et al.* 2016; Polepalli *et al.* 2017).

While the specific neural systems involved in associative learning might differ depending on the specific model or tasks, it is now well established that synaptic plasticity phenomena drive the formation of a link between separate neural systems. The mechanisms underlying some forms of memory formation, for example, fear conditioning and spatial learning have been well described, but less is known about the specific signaling cascades driving more complex behavioral change. A large body of evidence has suggested a role of a rhythmic firing pattern in the formation and maintenance of memory. However, molecular and network mechanisms are often studied separately and more research is needed to integrate our understanding of molecular events at the synapse level with system-wide modifications.

Synapse plasticity and motor skill learning

Motor skill learning plays a fundamental role in many aspects of our lives, without which it would be impossible to master a piano piece or to learn how to hit a tennis ball. Motor skill learning comprises the acquisition of movement sequences and is characterized by executing movements faster and more accurately with practice (Willingham 1998). When starting to learn a motor skill, the movement is often disjointed, poorly controlled, and executed with considerable variation and immense attention (Penhune & Steele 2012). Once learned, however, the skill is retained for a long period of time with minimal decay (Luft & Buitrago 2005).

It has been proposed that motor skill learning can be divided into separable acquisition stages with an early 'fast' phase, characterized by rapid and considerable learning improvements, and a later 'slow' stage, in which a nearly asymptotic level is reached and further improvements are gained only slowly (Dayan & Cohen 2011). Although different models exist regarding the brain areas and connections involved in processing motor skill learning, it is thought that interactions between cortico-thalamic-striatal and cortico-thalamic-cerebellar structures and the limbic system are essential to successfully build a motor memory trace (Hikosaka *et al.* 2002; Doyon & Benali 2005). These networks seem to be active during different time points of motor learning and, within each of them, specific associative-premotor and sensorimotor networks are activated (Lehericy *et al.* 2005; Coynel *et al.* 2010; Lohse *et al.* 2014). Furthermore, motor learning can modulate functional connectivity of the cortical motor network, and early skill learning has been shown to lead to enhanced inter- and intra-hemispheric coupling (Sun *et al.* 2007).

The primary motor cortex (M1) seems to play a crucial part in fast motor learning (Greenough *et al.* 1985; Kolb *et al.* 2008). Rodent studies have shown that motor learning can induce recruitment of neurons in the M1 and modulate synaptic efficacy through LTP and LTD (Rioult-Pedotti *et al.* 1998, 2000; Costa *et al.* 2004; Monfils & Teskey 2004). These results are supported by human studies, which also suggest that LTP-like plasticity in the M1 is involved in motor learning. While LTP-like effects are reversed after a period of motor learning, LTD-like effects were shown to be either enhanced or unchanged (Ziemann *et al.* 2004; Stefan *et al.* 2006; Rosenkranz *et al.* 2007).

Early motor learning increases dendritic spine number in M1 pyramidal cells (Harms *et al.* 2008; Xu *et al.* 2009; Fu *et al.* 2012). These learning-related spines cluster around or within a subset of dendritic branches (Fu et al. 2012; Yang & Lisberger 2014). Increased spine density is accompanied by expansion of the movement-related neuronal ensembles, as well as an induction of excitatory neuron activity (Peters *et al.* 2014). Furthermore, it is thought that stabilization of learning-induced nascent spines is essential for building durable memories (Xu et al. 2009; Yang *et al.* 2009). After the learning process, the overall spine density gradually returns to basal levels through selective elimination (Xu et al. 2009; Chen *et al.* 2015).

The cellular mechanisms underlying synaptic plasticity in the M1 still remain to be elucidated. *De novo* protein synthesis is essential for most of the plastic changes following motor learning (Bisby & Tetzlaff 1992; Alvarez *et al.* 2000) and inhibition of protein synthesis in the M1 impedes motor learning in rats (Luft *et al.* 2004). Furthermore, alterations in M1 gene expression were demonstrated in rats after learning a reach-and-grasp task (Cheung *et al.* 2013). Gene expression in the M1 after motor learning is thought to have distinct steps, with an initial suppression of genes influencing transcription, followed by genes that support mRNA translation and, finally, increased expression of genes that mediate plastic changes (Hertler *et al.* 2016).

Rodent studies suggest that BDNF is required for induction of neural plasticity related to motor learning (Kleim *et al.* 2003; Schabitz *et al.* 2004, 2007; Ploughman *et al.* 2009). Furthermore, it has been proposed that the Val66Met BDNF gene polymorphism reduces experience-dependent plasticity of human motor cortex and influences motor skill learning (Kleim *et al.* 2006; Cheeran *et al.* 2008; Gajewski *et al.* 2011), although the functional implications of this polymorphism regarding motor learning are still unclear (Li Voti *et al.* 2011; Nakamura *et al.* 2011; McHughen & Cramer 2013).

Consolidation, occurring after both fast and slow motor learning, is defined by offline behavioral skill improvements and is characterized by a reduction in fragility of a motor memory trace (Robertson *et al.* 2004a). However, the neuronal processes that support motor memory consolidation remain to a large extent unknown. Studies suggest that the M1 and the striatum play major roles during memory consolidation (Jenkins *et al.* 1994; Muellbacher *et al.* 2002; Ungerleider *et al.* 2002; Fischer *et al.* 2005; Yin *et al.* 2009), and that functional connectivity in frontoparietal networks support consolidation after motor learning (Albert *et al.* 2009; Ma *et al.* 2011; Taubert *et al.* 2011; Sampaio-Baptista *et al.* 2015).

It has further been suggested that sleep has an important function in memory consolidation after procedural learning (Maquet *et al.* 2000; Walker *et al.* 2002; Fischer et al. 2005; Morin *et al.* 2008; Ramanathan *et al.* 2015). Even short daytime naps can mediate these offline improvements (Walker *et al.* 2003; Korman *et al.* 2007; Nishida & Walker 2007). However, sleep does not seem to influence implicit learning (Robertson *et al.* 2004b; Song *et al.* 2007; Hotermans *et al.* 2008; Reis *et al.* 2015). It has been proposed that age-related changes may impact sleep-dependent memory processes (Spencer *et al.* 2007; Pace-Schott & Spencer 2011; Mander *et al.* 2014), and that changes in the gray matter of hippocampus and cerebellum are responsible for these deficits in sleep-related motor sequence memory consolidation (Fogel *et al.* 2016).

Acquisition, consolidation, and retention of motor skills require neural plasticity in different brain areas. However, molecular mechanisms driving and supporting motor learning, as well as the underlying synapse plasticity still remain to be fully elucidated. Local inhibitory circuits may act as key regulators of synaptic changes during motor learning, memory consolidation, and retrieval (Donato *et al.* 2013; Chen et al. 2015). Moreover, it has been proposed that the mesocortical dopaminergic pathway connecting the ventral tegmental area with the M1 is essential for successful motor skill learning (Hosp *et al.* 2011). Dopamine D1 receptors have been shown to be critically involved in LTP induction and D2 receptors (D2Rs) mediate spine addition in the M1 (Guo *et al.* 2015). Another study suggested that dopamine receptor activity influences motor skill acquisition and synaptic LTP via phospholipase C signaling (Rioult-Pedotti *et al.* 2015). Furthermore, the cAMP/PKA pathway has also been proposed to be critically involved in the acquisition of new motor skills (Qian *et al.* 2015). Nonetheless, more studies highlighting the molecular aspects of motor skill learning are needed.

Challenges and future directions

Neurodevelopmental research has made major contributions to our understanding of synaptic plasticity and learning. However, the mechanisms driving the development of synaptic plasticity, especially during critical periods, still remain to be clearly defined. Further studies are required to map the extent of learning-dependent changes that occur in developing brain,

including variations in dendritic spine number (Chen *et al.* 2016) and connectivity (Karim *et al.* 2016), as well as molecular details underlying such modifications.

Establishing the molecular mechanisms underlying synapse plasticity is a key step toward understanding the malleable properties of the brain. For instance, synapse-to-nucleus translocation and the exact signals that drive information back to synapses still remain elusive. Application of novel metabolic labeling techniques to visualize newly synthesized proteins at the synapse (Aakalu et al. 2001; Dieterich *et al.* 2010; tom Dieck *et al.* 2015; Bowling *et al.* 2016) could fill gaps regarding the coordination of local translation and the cues that trigger space-restricted responses. Such approaches are further expected to unveil novel mechanisms connecting local translation to synaptic plasticity and to provide clues into how synapse proteostasis becomes deregulated in several neurological disorders, including Alzheimer's disease and ASD (Buffington *et al.* 2014; Lourenco *et al.* 2015).

While general principles are thought to govern synaptic responses, a wide variety of mechanisms appear to be stimulus-, region-, and neuron type-specific, ultimately resulting in different behavioral outputs. For instance, although LTP has been regarded as a universal event driving plasticity, new LTP mechanisms have been continually uncovered. A recent work demonstrated that diffusible factors released by glia can travel considerable distance and affect the likelihood of LTP onset at given synapses (Kronschlager *et al.* 2016). More of such yet unconventional mechanisms are expected to emerge in the future.

Emerging evidence has further highlighted the role of non-Hebbian forms of synapse plasticity in brain function. Such mechanisms, including homeostatic plasticity, have the potential to help to explain the diversity and complexity of cognitive and non-cognitive behaviors arising from synaptic modulation.

Recent efforts have allowed significant advances in the understanding of neural substrates of complex behavior. Nevertheless, significant challenges comprise uncovering mechanisms that drive specific behavioral responses, as well as to track molecular responses in behaving animals. New techniques such as opto- or chemogenetics have fostered significant progress in our understanding of learning, memory, and the synaptic changes during memory consolidation by allowing precise spatiotemporal control of synapses and network.

The field of synaptic plasticity is extremely broad, gathering molecular and cellular phenomena to system and higher order properties of the brain. Nonetheless, integrating genomic, molecular, cellular, and system data still has been a caveat in the field, and should be a priority in current and future research. Identifying properties, mechanisms, and regulations of synaptic plasticity may provide new avenues for treatment of neurological disorders.

Acknowledgments and conflict of interest disclosure

This review was initiated at the 1st International Society for Neurochemistry (ISN) and *Journal of Neurochemistry* (JNC) Flagship School in Alpbach, Austria, in September 2016. We thank the International Society for Neurochemistry (ISN) Flagship School faculty and organizers, especially Drs. Alois Saria and Jörg Schulz. All authors wish to acknowledge International Society for Neurochemistry (ISN) for the financial and educational support, and declare no conflict of interest. Anthony J. Turner is the Chair of the Publication Committee of the ISN.

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