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DECIPHERING THE NEURAL MECHANISMS OF OXYTOCIN'S PROSOCIAL AND ANTIPSYCHOTIC-LIKE EFFECTS

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Abstract

Both clinical and preclinical research has indicated administration of the endogenous neuropeptide oxytocin (OXY) may help restore normal social functioning in patients with social deficits, such as those seen in schizophrenia. In addition, 'antipsychotic-like' effects of OXY have also been reported in rats, through the attenuation of phencyclidine (PCP)-induced hyperactivity. OXY has affinity at both the oxytocin (OXTR) and vasopressin 1a (V1aR) receptors, and evidence suggests both are involved in social behaviour. However, the neural mechanisms underpinning these behavioural effects of OXY remain poorly understood. The overall aims of this thesis are to gain an insight into the neural mechanisms that underlie the prosocial and antipsychotic-like effects of subcutaneous (s.c.) OXY in healthy rats, through the use of selective antagonists and intracranial micro-injections of the OXTR targeting toxin, OXY-saporin, into discrete brain nuclei.

First, we developed an *in vitro* calcium assay to determine whether a conjugation of OXY to a novel cell-penetrating peptide, GET, was biologically active and could be used to enhance intranasal OXY administration. This experiment demonstrated that conjugation of OXY to GET hinders biological activity at the OXTR, and therefore was unsuitable for intranasal administration. A low dose of s.c. OXY was subsequently selected for the behavioural experiments performed, due to the fact it can modulate brain OXY levels without having any profound concomitant peripheral effects, such as hypothermia or sedation.

Second, we demonstrated s.c. OXY can both enhance social interaction between two male rats and attenuate PCP-induced hyperactivity, indicative of an antipsychotic-like effect and consistent with previous work from this laboratory. Utilising selective V1aR and OXTR antagonists, SR 49059 and L-368,899 respectively, we demonstrated activity at the OXTR was responsible for OXY enhancement of social behaviour following systemic administration. In contrast, activity at both the OXTR and V1aR are involved in the ability of OXY to reverse PCP-induced hyperactivity, such that both antagonists reduced OXY-attenuation of PCP-hyperactivity.

Finally, using discrete micro-injections of the OXTR targeted toxin, OXY-saporin, we sought to determine the role of NAc and VTA OXTRs on OXYs prosocial and antipsychotic-like effects. OXY was no longer able to increase social interaction following microinjection of NAc OXY-saporin, however, removal of NAc OXTR expressing neurons had no effect on the ability of OXY to attenuate PCP-induced hyperactivity. In the same way, removal of OXTR expressing neurons in the VTA also prevented OXY enhancement of prosocial behaviours but had no effect on OXY's attenuation of PCP-induced hyperactivity.

Overall, our results suggest that OXYs prosocial and antipsychotic-like effects are modulated by different neural mechanisms. Our findings strongly implicate mesolimbic dopamine in the prosocial effect of s.c. OXY and are consistent with work suggesting mesolimbic dopamine can enhance social reward and social interaction. The antipsychotic-like effects of s.c. OXY are likely modulated by an alternative neural mechanism, such as modulation of the nigrostriatal pathway. Overall, this work has significantly contributed to our understanding of OXYs neural mechanisms, providing robust evidence peripheral OXY can modulate behaviour through central activity. Although further work is required, the use of OXY to treat social deficits in psychiatric disorders remains promising.

Acknowledgements

Firstly, thank you to my supervisors Kevin Fone and Maddy King for providing me with the opportunity to conduct this research and providing me with guidance throughout.

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Mum, Dad, and Sophie, thank you so much for your unwavering love and support, and for always being at the end of the phone. I can't wait to go to the pub and celebrate.

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Conference Proceedings

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List of Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
ADHD	attention-deficit-hyperactivity-disorder
AM	acetoxymethyl
ANOVA	analysis of variance
AON	anterior olfactory nucleus
ARRIVE	animal Research: Reporting of In Vivo Experiments
ASD	autism spectrum disorders
AWERB	Animal Welfare and Ethical Review Body
BBB	blood brain barrier
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CRH	corticotrophin-releasing hormone
CSF	cerebral spinal fluid
D1	Day 1
D2	Day 2
DABCO	1,4-diazabicyclo[2.2.2]octane
DAG	1,2-diacylglycerol
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	dimethyl sulfoxide
FCS	foetal calf serum
FM	frequency modulated
fMRI	functional magnetic resonance imaging
GABA	gamma-Aminobutyric acid
GAG	glycosaminoglycan
GDP	guanosine diphosphate
GET	glycosaminoglycan-binding enhanced transduction
GPCR	G-protein coupled receptor

GPe	external globus pallidus
GPi	internal globus pallidus
GRKs	G-protein receptor kinases
GTP	guanosine triphosphate
HBSS	Hanks Balanced Salt Solution
HEK293T	human embryonic kidney 293T cells
HEP	heparin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
ICj	islands of Calleja
IP_3	inositol trisphosphate
IU	international units
K_B	equilibrium dissociation constant
kHz	kilohertz
L-368,899	(2S)-2-Amino-N-[(1S,2S,4R)-7,7-dimethyl-1-[[[4-(2-methylphenyl)-1-piperazinyl]sulfonyl]methyl]bicyclo[2.2.1]hept-2-yl]-4-(methylsulfonyl)butanamide
LDCV	large dense core vesicles
LMA	locomotor activity
MAM	methylazoxymethanol acetate
MCF-7	Michigan Cancer Foundation-7
MDCV	medium dense core vesicles
MDD	major depressive disorder
MDMA	3,4 methylenedioxymethamphetamine
MS	maternal separation
MSN	medium spiny neurons
NAc	nucleus accumbens
nm	nanometres
NMDA	*N*-methyl-D-aspartate
NMDA-R	NMDA receptor

NTS	nucleus tractus solitarius
OB	olfactory bulb
OCT	optimal cutting temperature compound
OXTR	oxytocin receptor
OXY	oxytocin
OXY-GET	oxytocin glycosaminoglycan-binding enhanced transduction conjugation
PANSS	Positive and Negative Syndrome Scale
PBS	phosphate buffered saline
PCP	phencyclidine
PFA	paraformaldehyde
PFC	prefrontal cortex
PIP_2	phosphatidylinositol bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PVN	paraventricular nucleus of the hypothalamus
RAGE	receptor for advanced glycation end-products
RCT	randomised control trial
RFU	raw fluorescent units
RIP	ribosome inactivating protein
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
s.c.	subcutaneous
S1	Schedule 1
SBNN	social behaviour neural network
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SN	substantia nigra
SNc	substantia nigra pars compacta
SNP	single nucleotide polymorphism
SNr	substantia nigra pars reticulata

SON	supraoptic nuclei of the hypothalamus
SR 49059	(2S)-1-[[(2R,3S)-5-Chloro-3-(2-chlorophenyl)-1-[(3,4-dimethoxyphenyl)sulfonyl]-2,3-dihydro-3-hydroxy-1H-indol-2-yl]carbonyl]-2-pyrrolidinecarboxamide
STN	subthalamic nucleus
TMD	transmembrane domain
Tolvaptan	N-[4-[(7-Chloro-2,3,4,5-tetrahydro-5-hydroxy-1H-1-benzazepin-1-yl)carbonyl]-3-methylphenyl]-2-methylbenzamide
USV	ultrasonic vocalisations
V1aR	vasopressin 1a receptor
V1bR	vasopressin 1b receptor
V2R	vasopressin 2 receptor
VTA	ventral tegmental area

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COVID-19 Impact Statement

Due to the COVID-19 global pandemic and the U.K. 2020 Lockdown the University of Nottingham was shut for 5 months, with no access to research laboratories. Following re-opening we were subject to shortages of laboratory supplies and delays in getting risk assessments approved. Therefore, during the course of this PhD there was an 8 month period where there was no laboratory access (March 2020 until October 2020).

Statement of Contribution

Abigail Abrahams conceived and designed all the experiments presented in this thesis. Throughout this thesis all data analysis, interpretation, figure preparation, and writing was conducted independently by the author, Abigail Abrahams.

The data presented in Chapter 2 was collected independently, but in parallel, with Sara Wong. During the collection of the data presented in Chapters 3, 4, and 5, technical support was provided by Dr. David Watson and Clare Spicer, specifically during the stereotaxic surgery and social interaction task, under the instruction of Abigail Abrahams.

All immunohistochemical procedures reported in this thesis were independently performed by Abigail Abrahams.

Chapter 1: General Introduction

1.1. Thesis overview

Social withdrawal and social deficits are transdiagnostic, seen across a wide variety of neuropsychiatric disorders including Alzheimer's disease, autism spectrum disorders (ASD), schizophrenia, and major depressive disorder (MDD) (Porcelli *et al.*, 2019). Social dysfunction is often the first symptom seen in these disorders, and is an indication of cognitive deficits and a poorer prognosis (Javed & Charles, 2018). Both clinical and preclinical research has indicated administration of the endogenous neuropeptide, oxytocin (OXY), may help restore normal social functioning in patients with social deficits (Fernandez-Sotos *et al.*, 2018; Jarskog *et al.*, 2017; Keech *et al.*, 2018; Kohli *et al.*, 2019). Endogenous OXY, released both centrally (from the hypothalamus) and peripherally (from the posterior pituitary into the blood stream), plays a critical role in social bonding, reproduction, and parturition in both humans and animals (Caldwell, 2017). When acting within the central nervous system (CNS) OXY has very similar behavioural effects in males and females; enhancing social behaviour (Ross & Young, 2009; Striepens *et al.*, 2011). In contrast, peripheral effects of OXY differ significantly between the sexes, due to OXY receptor (OXTR) expression in the reproductive system (Garrison *et al.*, 2012; Jurek & Neumann, 2018). For example, in females, OXY triggers uterine contraction and milk let-down through contraction of myoepithelial cells in the mammary glands, while in males, peripheral OXY increases the contractility of the seminiferous tubules and epididymis, and enhances sperm motility (Fuchs *et al.*, 1989; Jurek & Neumann, 2018; Thackare *et al.*, 2006). In addition to the prosocial effects of OXY there is also preclinical evidence to suggest OXY may also have antipsychotic-like efficacy (Kohli *et al.*, 2019).

The evidence for both prosocial and antipsychotic-like effects of OXY suggests it has a strong therapeutic potential in the treatment of psychiatric disorders, such as schizophrenia. However, the neural mechanisms underpinning these behavioural effects of OXY remain poorly understood. The overall aims of this thesis are to gain

an insight into the neural mechanisms that underlie the prosocial and antipsychotic-like effects of subcutaneous (s.c.) OXY in healthy rats, through the use of selective antagonists and intracranial micro-injections of the OXTR targeting toxin, OXY-saporin, into discrete brain nuclei.

1.2. Oxytocin

1.2.1 Overview and history

OXY was first discovered by Sir Henry Dale in 1909, following the observation that a sample taken from the posterior pituitary gland caused the contraction of a pregnant cat's uterus (Dale, 1909). The structure of OXY was later discovered in 1983, while the protein sequence of the OXTR was deciphered in 1992 (Kimura *et al.*, 1992). OXY is a nonapeptide with a cyclic structure, due to the presence of a disulphide bridge between two cysteine residues at positions one and six; resulting in a six amino acid cyclic structure with a three amino acid C-terminal tail (Figure 1.1) (Gimpl & Fahrenholz, 2001). Structurally, OXY only varies by two amino acids from vasopressin, also a neuropeptide involved in the regulation of social behaviour (Caldwell, 2017). The similarities between OXY and vasopressin are described in more detail in section 1.2.5.

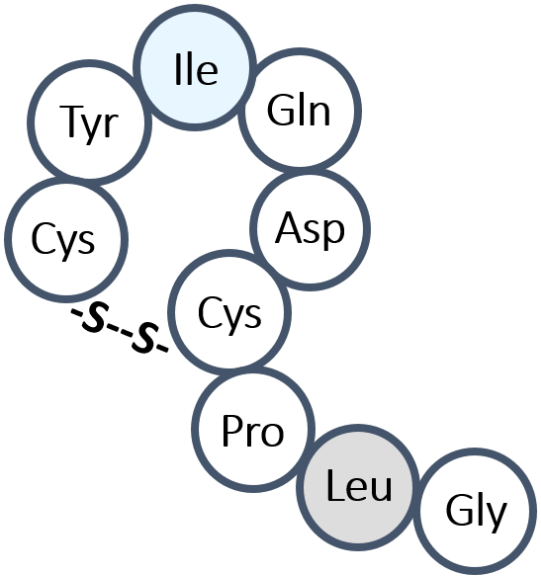

Figure 1.1: The cyclic structure of OXY, demonstrating the disulphide bridge between two cysteine residues at positions one and six; resulting in a six amino acid cyclic structure with a three amino acid C-terminal tail (Gimpl & Fahrenholz, 2001).

OXY is a neuropeptide that can act both centrally and peripherally to impact a huge variety of behaviours and physiological functions. When released into the synapse OXY acts as a neurotransmitter, influencing neuronal activity. In addition, OXY can also act as a neurohormone, acting at peripheral receptors distant from the site of release (Dölen, 2015; Ludwig & Leng, 2006). Dual action of OXY as a neurohormone and neurotransmitter gives OXY a great amount of flexibility in its actions (Donaldson & Young, 2008).

Early research indicated a role for OXY in parturition and lactation, through the contraction of smooth muscle cells in the myometrium and mammary gland alveoli (Augustine *et al.*, 2017; Gimpl & Fahrenholz, 2001; Kota *et al.*, 2013). More recently research has demonstrated a critical role of OXY in promoting social behaviours and reproduction (Donaldson & Young, 2008; Ross & Young, 2009). This discovery has promoted more recent research on whether OXY can be utilised in the treatment of disorders with socio-behavioural deficits (Dumais & Veenema, 2016; Kanat *et al.*, 2014; Kosfeld *et al.*, 2005; Love, 2014). The most significant advances in OXY research are shown in Figure 1.2, from its discovery through to the present day.

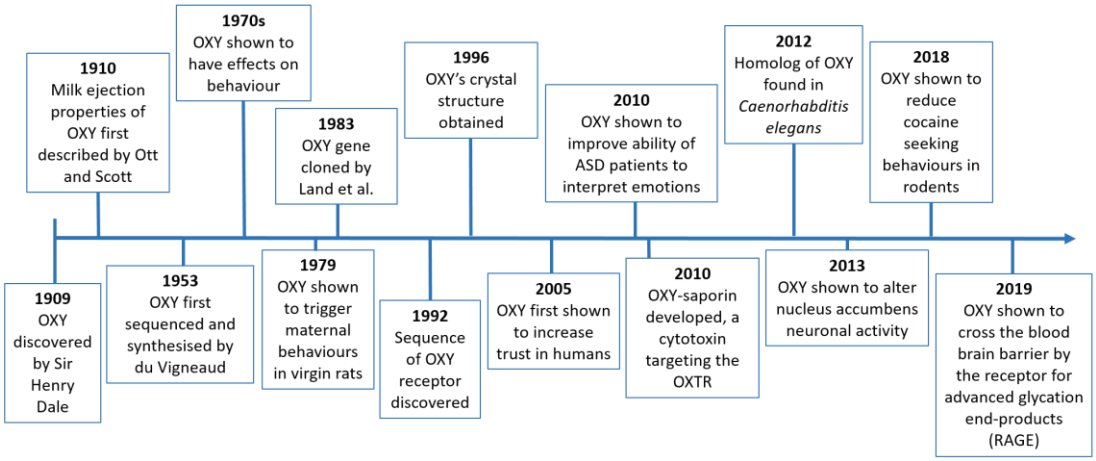

Figure 1.2: Key discoveries in OXY research since its discovery in 1909 by Nobel prize winner Sir Henry Dale (Dale, 1909), through to the present day (Baskin *et al.*, 2010; Beets *et al.*, 2012; Dölen *et al.*, 2013; Du Vigneaud *et al.*, 1953; Garrison *et al.*, 2012; Guastella *et al.*, 2010; Kimura *et al.*, 1992; Kohtz *et al.*, 2018; Kosfeld *et al.*, 2005; Land *et al.*, 1983; Ott & Scott, 1910; Pedersen & Prange, 1979; Rose *et al.*, 1996; Singh & Hofer, 1978; Weber *et al.*, 2018; Yamamoto *et al.*, 2019).

1.2.2 Oxytocin synthesis and release

Both OXY and vasopressin are primarily synthesised in the paraventricular nucleus (PVN) and the supraoptic nuclei (SON) of the hypothalamus. OXY is produced by two different cell types in the hypothalamus; parvocellular and magnocellular, which can be distinguished through their differing cell body diameter (15 µm and 25 µm respectively) and structure (magnocellular neurons have thicker axons with numerous varicosities, while parvocellular neurons are thin with few varicosities) (Dölen, 2015; Ludwig & Leng, 2006). While magnocellular neurons are found in both the SON and PVN, parvocellular neurons are found only in the PVN. As OXY is a neuropeptide it can act as both a hormone and a neurotransmitter; reflected in the three mechanisms of OXY release (Figure 1.3).

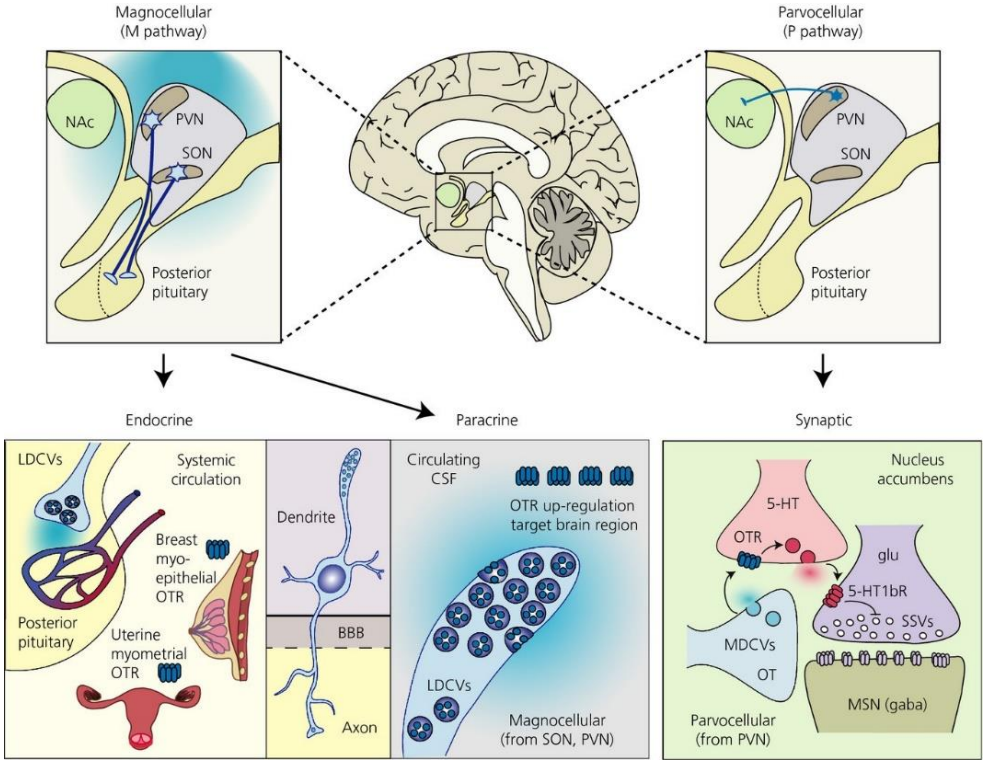

Figure 1.3: Three mechanisms of OXY release from magnocellular and parvocellular neurons of the PVN and SON; giving rise to endocrine, paracrine, and synaptic signalling of OXY. Top; the expression and projections of parvocellular and magnocellular neurons in the PVN and SON. Bottom; endocrine (left), paracrine (middle), and synaptic (right) methods of OXY transmission. 5-HT, 5-hydroxytryptamine (serotonin); 5-HT1bR, 5-HT1B receptor subtype; glu, glutamate; MSN, medium spiny neuron; OT, OXY; OTR, OXY receptor; SSVs, small synaptic vesicles. Image taken from Dölen (2015).

Magnocellular neurons are responsible for the endocrine function of OXY, releasing OXY into the peripheral circulation through projections to the posterior pituitary. In magnocellular neurons OXY is stored in large dense core vesicles (LDCV), which are distributed throughout their axons, dendrites, and soma (Dölen, 2015; Jurek & Neumann, 2018). OXY is synthesised as a 'prohormone'; consisting of the OXY peptide attached to a carrier protein, neurophysin (Mitre *et al.*, 2018). The neurophysin carrier protein is critical to ensure OXY is targeted and stored within neurosecretory granules. Once within the neurosecretory granules OXY is cleaved to form the OXY nonapeptide (Brownstein *et al.*, 1980). The posterior pituitary is a circumventricular organ; a structure in the brain that has highly permeable capillaries, unlike other brain regions which have a BBB (Felten *et al.*, 2016). The highly fenestrated capillary

bed in the posterior pituitary means magnocellular neuron axon terminals are in close contact to neurohypophysial capillaries. Therefore, exocytosis of OXY from these LDCVs, triggered by increased intracellular calcium, releases OXY directly into the peripheral circulation (Dölen, 2015; Jurek & Neumann, 2018; Ludwig & Leng, 2006). Physiological effects of peripheral OXY include myometrial contraction in labour and milk let down (Jurek & Neumann, 2018). To enable this neuroendocrine role the posterior pituitary has an extremely high concentration of OXY; 1000x higher than in the rest of the brain (Ludwig & Leng, 2006), totalling 0.5-1µg in rats and 28µg in humans (Leng & Ludwig, 2016).

Magnocellular neurons also give rise to paracrine signalling. Dendritic release of OXY from LDCVs releases OXY directly into the cerebral spinal fluid (CSF) and third ventricle, where OXY can diffuse through the brain having modulatory effects (Jurek & Neumann, 2018; Ludwig & Leng, 2006). Dendritic release of OXY is dependent upon local increases in intracellular calcium, either by entry of extracellular calcium, or following release from intracellular stores (Ludwig *et al.*, 2016). Interestingly, although action potentials can propagate into the dendrites and trigger exocytosis (Bains & Ferguson, 1999), dendritic OXY release can also occur independently of action potentials (Ludwig *et al.*, 2005; Ludwig *et al.*, 2002). For example, alpha melanocyte-stimulating hormone can bind to melanocortin 4 receptors on OXY magnocellular neurons, causing the release of calcium from intracellular stores and subsequent dendritic OXY release, but simultaneously inhibiting the electrical activity of the neuron, preventing OXY release into the periphery from axon terminals (Sabatier *et al.*, 2003). Within the CSF OXY has a much longer half-life than in the blood (20 minutes in the CSF, compared to 5 minutes in the blood); allowing for more sustained action of OXY in the CSF and brain following dendritic release (Leng & Ludwig, 2008; Mens *et al.*, 1983). Areas such as the ventromedial nucleus have dense OXTR expression but contain very low levels of OXY fibres. Therefore, these receptors are likely activated following paracrine OXY release from magnocellular neurons from the SON and PVN (Leng & Sabatier, 2017). Dendritic release of OXY can also potentiate its own release, through acting back on OXTRs expressed on dendrites, enabling dendritic release to be self-sustaining (Striepens *et al.*, 2011). This allows

OXY to have its actions over a sustained period that outlasts the events that initiate its release. For example, in sheep high OXY concentrations in the CSF are present for up to several hours after they give birth, contributing to the formation of social bonds with their offspring, which is critical in the first two hours (Kendrick, 2000).

While magnocellular neurons trigger paracrine and endocrine signalling, parvocellular neurons are primarily responsible for OXY synaptic release, where OXY exerts its affects as a neurotransmitter following release from medium dense core vesicles (MDCV) in axon terminals. Exocytosis of OXY occurs following depolarisation of terminals by action potentials, opening voltage sensitive calcium channels, resulting in rapid rises in intracellular calcium, triggering exocytosis (Ludwig & Leng, 2006). Parvocellular PVN OXY neurons have long range axonal projections which project to numerous regions of the brain, including the NAc, ventral tegmental area (VTA), hippocampus, spinal cord, and amygdala, where they make monosynaptic connections with other neurons (Figure 1.4) (Dölen, 2015; Hung *et al.*, 2017; Ludwig & Leng, 2006; Stoop, 2012). For example, OXY release from parvocellular neurons that terminate in the VTA activate mesolimbic dopamine neurons (Hung *et al.*, 2017). Magnocellular neurons also contribute to synaptic OXY release in a few specific areas, such as the arcuate nucleus (Ludwig & Leng, 2006). In contrast to endocrine and paracrine OXY release, synaptic OXY release is rapid and releases much lower concentrations than during paracrine and endocrine release (Dölen, 2015).

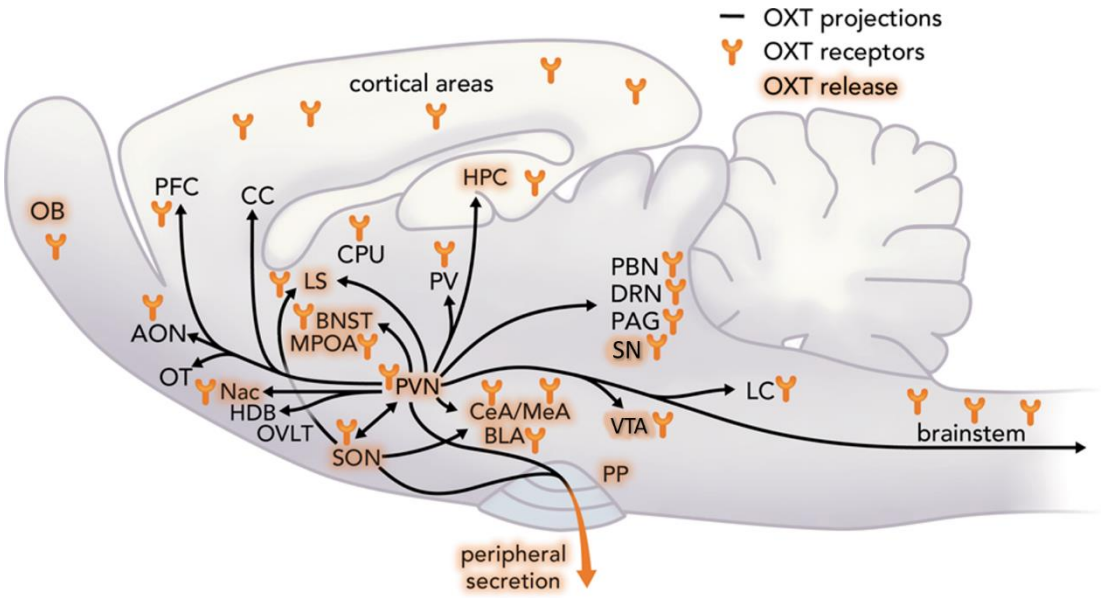

Figure 1.4: Diagram representing a sagittal slice of the rat brain demonstrating OXTR expression, and long range oxytocinergic projections from the PVN and SON (black lines) to numerous regions of the brain. Those highlighted in orange are regions in which OXY release has been directly measured through microdialysis. Abbreviations: AON anterior olfactory nucleus, BLA basolateral amygdala, BNST bed nucleus of the stria terminalis, CC cingulate cortex, CeA central amygdala, CPu caudate putamen, DRN dorsal raphe nucleus, HDB nucleus of the horizontal limb of the diagonal band, HPC hippocampus, LC locus coeruleus, LS lateral septum, MeA medial amygdala, MPOA medial preoptic area, NAc nucleus accumbens, OB olfactory bulb, OT olfactory tubercle, OVLT organum vasculosum laminae terminalis, PAG periaqueductal gray, PBN parabrachial nucleus, PFC prefrontal cortex, PP posterior pituitary, PV periventricular nucleus of the thalamus, PVN paraventricular nucleus, SON supraoptic nucleus, SN substantia nigra, VTA ventral tegmental area. Image adapted from Jurek and Neumann (2018) and Grinevich *et al.* (2016).

The BBB is highly effective at preventing the majority of circulating OXY from re-entering the brain following release from the posterior pituitary (Ludwig & Leng, 2006). However, Yamamoto *et al.* (2019) recently demonstrated that a receptor expressed on neurovascular units in the brain of humans and rodents can enable the transport of circulating OXY into the brain, across the BBB (Cheng *et al.*, 2005; Yamamoto & Higashida, 2020; Yamamoto *et al.*, 2019). The receptor is known as the Receptor for Advanced Glycation End-products (RAGE) (Yamamoto & Higashida, 2020; Yamamoto *et al.*, 2019). Therefore, circulating OXY in the blood can re-enter the brain, influencing OXY paracrine and synaptic transmission. However, OXY has a lower affinity for RAGE than it does for the OXTR (Yamamoto *et al.*, 2019). Under

normal physiological conditions with very low levels of circulating OXY the RAGE is unlikely to be engaged, meaning OXY does not re-enter the brain. However, during childbirth, lactation, or following systemic OXY administration the levels of peripheral OXY in the blood are significantly increased. This enables OXY to bind to RAGE and be subsequently transported across the BBB (Yamamoto *et al.*, 2019). As such, Yamamoto *et al.* (2019) demonstrated significant increases in OXY in the third ventricle and PVN following s.c. OXY administration in mice. The precise mechanisms through which RAGE transports OXY across the BBB are unknown, however it is likely to involve endocytosis and transcytosis (Figure 1.5) (Yamamoto *et al.*, 2019).

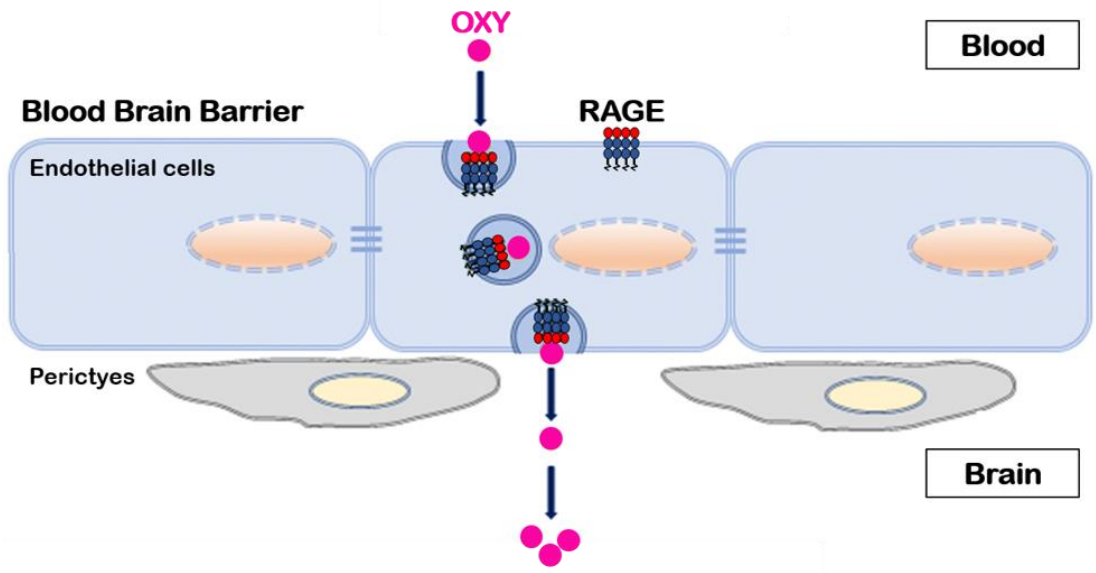

Figure 1.5: Transcellular transportation of OXY across the BBB by RAGE. Although the precise mechanisms through which OXY is transported across the BBB by RAGE is currently unknown, it is thought to involve a vesicular trafficking system, using endocytosis and transcytosis mechanisms. Image adapted from Leerach *et al.* (2021).

1.2.3 The oxytocin receptor

1.2.3.1 Central expression

OXTRs are extensively expressed throughout the brain of both animals and humans, with marked differences in expression between species (Gimpl & Fahrenholz, 2001; Wang *et al.*, 2013; Young *et al.*, 1996). It is thought differential expression of OXTRs are what contribute to the natural diversity and variations in social behaviours both within, and between, species (Keebaugh *et al.*, 2015; Olazábal & Sandberg, 2020; Ross *et al.*, 2009b).

In rats OXTRs are highly expressed in multiple brain regions, including the olfactory system, the mesolimbic system, the hypothalamus, and the spinal cord (Dumais *et al.*, 2013; Smith *et al.*, 2017a; Smith *et al.*, 2019). Notably, higher densities are often found in areas of the brain associated with social behaviours and reward (Smith *et al.*, 2017a). Indeed, OXTRs are expressed in all seven nodes of the rat social behaviour neural network (SBNN); an interconnected set of brain regions thought to regulate social behaviour in rats (Newman, 1999; Smith *et al.*, 2019).

As well as OXTR expression varying between species, expression also varies throughout development, and between the sexes (Smith *et al.*, 2017a). Age differences in OXTR densities in rats have been observed when looking at OXTR expression throughout development. Juveniles, defined as 22-35 day-old rodents, have significantly higher OXTR density in fifteen brain areas, including the olfactory nucleus, hypothalamus, and hippocampus, when compared to adult rats (defined as 55 days old and above). In contrast, adult rats have higher OXTR density in ten brain regions, including the perirhinal cortices and medial amygdala (Lukas *et al.*, 2010; Smith *et al.*, 2017a). Interestingly, the regions in which juveniles have higher OXTR density are directly implicated in mediating socially rewarding behaviours, as shown through increased social conditioned place preference and partner preference following OXTR activation in these areas (Dölen *et al.*, 2013; Smith *et al.*, 2017a; Song *et al.*, 2016). In contrast, the regions in which adults have higher OXTR density are

implicated in the processing of social odour cues, facilitating social recognition (Dumais *et al.*, 2016; Gur *et al.*, 2014; Lukas *et al.*, 2013). This suggests the role of OXTRs may change throughout development, switching from a predominant role in social reward to a primary role in social recognition during ageing.

Prominent differences in OXTR expression are also observed between the sexes. Sex differences are present during the juvenile period and become more pronounced at adulthood (Smith *et al.*, 2017a). In rats females have a higher OXTR density in areas such as the perirhinal cortex, while males have higher levels in the nucleus accumbens (NAc) and medial amygdala (Dumais & Veenema, 2016; Smith *et al.*, 2017a). Differences in OXTR expression between sexes suggests behaviours modulated by OXTR activity may exhibit sexual dimorphism. For example, intracerebroventricular (i.c.v.) OXY enhances social recognition in male but not female rats (Benelli *et al.*, 1995; Engelmann *et al.*, 1998). In addition, social defeat-induced social avoidance in male rats can be reversed through i.c.v. OXY administration, but not in female rats (Lukas *et al.*, 2011). The sex differences in OXTR expression between males and females can be attributed, at least in part, to modulation by circulating gonadal hormones (Dumais & Veenema, 2016; Smith *et al.*, 2017a). For example, gonadectomy in both male and female adult rats reduces OXTR expression in regions such as the Islands of Calleja and ventromedial hypothalamus, and administration of testosterone or oestrogen following gonadectomy can return OXTR expression back to normal levels (Tribollet *et al.*, 1990). Furthermore, oestrogen can increase the affinity of OXY for the OXTR in the medial preoptic area, and increase OXTR expression in the ventromedial hypothalamus (Caldwell *et al.*, 1994). Consistent with this, in female rats OXTR mRNA expression in the ventromedial hypothalamus fluctuates during the oestrous cycle (Bale *et al.*, 1995). Therefore, regions of the brain that are sensitive to gonadal hormones, such as the ventromedial hypothalamus, are likely to have differing levels of OXTR expression between males and females (Patchev *et al.*, 1993).

Environmental factors can also influence OXTR expression. Early-life stress, such as maternal separation (MS), causes significant changes in social behaviours later in life and is a risk factor for the onset of psychiatric disorders in man (Bales & Perkeybile,

2012; Lukas *et al.*, 2010). Exposing rats to MS for three hours daily for a fortnight post-weaning significantly alters OXTR expression in multiple forebrain regions during different stages of development (Lukas *et al.*, 2010). Changes include reduced OXTR binding in the striatum at adulthood, and increased OXTR binding in the agranular cortex during both the juvenile and adult period (Lukas *et al.*, 2010). Typical changes in OXTR densities throughout development are thought to be vital in the onset of normal social behaviours, thus changes in OXTR densities are likely to account for the aberrant changes in social behaviour often seen following MS (Lukas *et al.*, 2010).

1.2.3.2 Activation and signalling

The OXTR is a class I G-protein coupled receptor (GPCR) comprising of seven, highly conserved, transmembrane α-helices; connected by three intracellular and three extracellular loops (Gimpl & Fahrenholz, 2001) (Figure 1.6). The seven transmembrane domains (TMDs) of GPCRs are highly conserved, suggesting there are common residues within these regions critical for receptor activation and signalling (Zingg & Laporte, 2003). Across all GPCRs agonist binding induces conformational changes, and receptor activation requires specific changes in the orientation of TMD III and VI, exposing a G-protein binding site (Zingg & Laporte, 2003). Conserved residues within the TMDs play vital roles in the activation and transduction of the receptor. For the OXTR the asparagine residue in transmembrane II (D85) is critical (Gimpl & Fahrenholz, 2001); exchanging the OXTR D85 with other residues leads to significantly impaired agonist binding and signal transduction (Wheatley *et al.*, 1998). Peptide ligands, such as OXY, bind close to the receptor surface, forming interactions with the extracellular loops and the N-terminal tail (Figure 1.6). Specifically, the arginine residue at position 34 (R34) in the N-terminal tail is critical in the high affinity binding of OXY to the human OXTR; removal of R34 causes a 2000-fold reduction in the affinity of OXY for the OXTR (Wesley *et al.*, 2002). Other OXTR residues important in receptor activation and selectivity include the phenylalanine at position 103 (F103) in the first extracellular loop of the OXTR (Chini *et al.*, 1995). These residues are believed to interact with the C-terminus tail of OXY, where vasopressin and OXY differ

at position 8, allowing receptor specificity and high affinity for OXY (Zingg & Laporte, 2003). Other OXTR residues thought to be important in ligand interactions include tyrosine at position 209 (Y209) and phenylalanine at position 284 (F284), believed to interact with OXYs isoleucine at position 3 within the cyclical part of OXY. This is the second amino acid that differs between vasopressin and OXY, suggesting Y209 and F284 may also contribute to the ligand specificity of the OXTR (Zingg & Laporte, 2003).

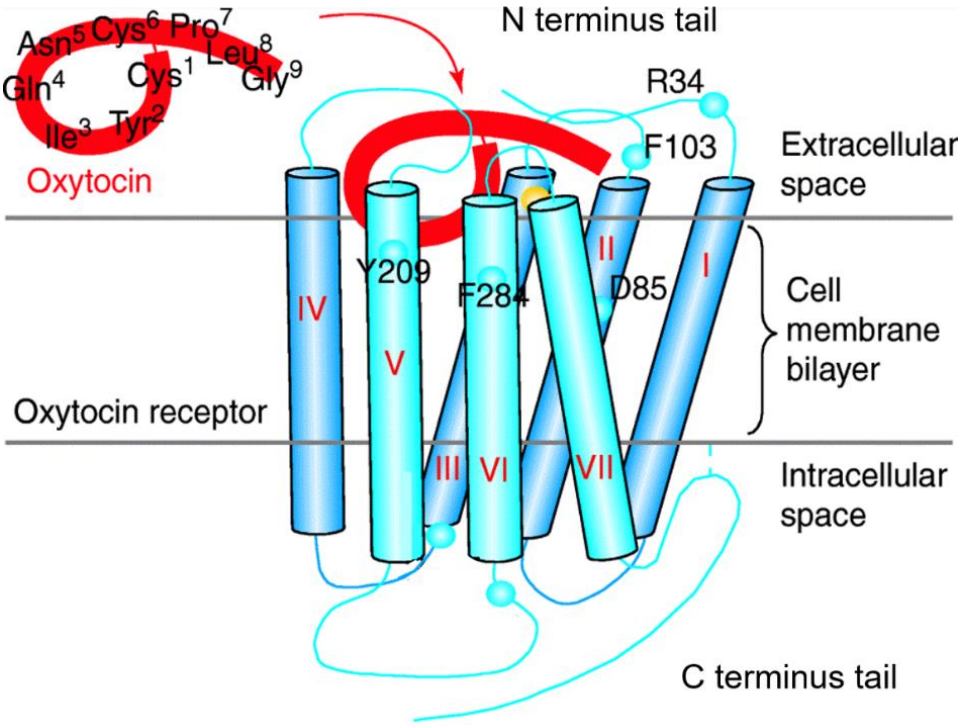

Figure 1.6: The structure of the human OXTR, illustrating seven transmembrane domains, intra- and extra-cellular loops, and the N- and C-terminal tails. The OXY binding site at the receptor is illustrated and thought to involve interactions with both the transmembrane domains and extracellular loops. Key residues involved in the activation of the OXTR, and OXY binding, are detailed: the arginine residue at positions 34 (R34), asparagine at position 85 (D85), phenylalanine residues at positions 103 and 284 (F103 and F284), and tyrosine at position 209 (Y209). Image adapted from Zingg and Laporte (2003).

The OXTR, as with all other GPCRs, is composed of Gα, Gβ and Gγ subunits. The OXTR is predominantly coupled to a $G\alpha_q$ subunit, which is ubiquitously expressed throughout the brain (Milligan, 1993). In the resting state the $G\alpha_q$ subunit contains a guanosine 5'diphosphate (GDP) unit. Ligand binding to the OXTR stimulates the

release of GDP from the $G\alpha_q$ subunit, and subsequent binding of guanosine 5'triphosphate (GTP). GTP binding causes the dissociation of the Gβγ subunit from the OXTR, resulting in conformational changes within the TMDs. This initiates an excitatory signalling pathway involving phospholipase C (PLC) and protein kinase C (PKC), enhancing neuronal firing (discussed in more detail in Chapter 2.1) (Gimpl & Fahrenholz, 2001; Stoop, 2012; Xiao *et al.*, 2017).

Although the OXTR is predominantly coupled to the $G\alpha_q$ subunit, evidence has also shown the OXTR can also couple to the $G\alpha_i$ subunit. In contrast to $G\alpha_q$ signalling, activation of $G\alpha_i$ coupled receptors leads to inhibition of adenylyl cyclase, reducing activation of cyclic adenosine monophosphate (cAMP) and intracellular calcium release (Kim *et al.*, 2016). Coupling to both $G\alpha_q$ and $G\alpha_i$ adds a layer of complexity to OXTR signalling. In human embryonic kidney-293 cells (HEK293) with stable OXTR expression $G\alpha_q$ coupling can stimulate cell growth, whist $G\alpha_i$ coupling inhibits cell growth (Busnelli *et al.*, 2012; Rimoldi *et al.*, 2003). In addition, $G\alpha_q$ and $G\alpha_i$ coupling have opposing effects upon neuronal excitability *in vitro* (Gravati *et al.*, 2010).

Interestingly, intracellular concentrations of OXY are thought to contribute to the coupling of the OXTR to either $G\alpha_q$ or $G\alpha_i$. Busnelli *et al.* (2012) demonstrated that $G\alpha_q$ signalling is activated by OXY with an EC_{50} of 2.16 nM, while in contrast, activation of the $G\alpha_i$ coupled OXTR requires an EC_{50} value of 11.5-91.8 nM. This suggests that $G\alpha_i$ coupled OXTRs are activated at higher OXY concentrations than $G\alpha_q$ coupled OXTRs, indicating the local concentration of OXY is crucial in determining OXTR coupling and subsequent physiological effects (Busnelli & Chini, 2018). Despite coupling of OXTRs to both $G\alpha_q$ and $G\alpha_i$ in the brain, OXTRs are likely to be predominantly $G\alpha_q$ coupled. The concentration of OXY in the brain is extremely low, around 1nm, and $G\alpha_q$ is expressed throughout the brain, unlike $G\alpha_i$ (Busnelli & Chini, 2018; Milligan, 1993). However, coupling to $G\alpha_i$ is likely to be present and important in specific regions of the brain and central nervous system (CNS). Indeed, OXTR- $G\alpha_i$ signalling has been demonstrated to have analgesic effects in the spinal cord of Wistar rats through administration of the bias OXTR-$G\alpha_q$ antagonist/OXTR-$G\alpha_i$ agonist atosiban (Eliava *et al.*, 2016).

Consistent with other Class I GPCRs, both persistent and acute agonist exposure leads to the desensitisation and internalisation of the OXTR *in vitro,* in a process mediated by β-arrestin and protein kinases (Berrada *et al.*, 2000; Smith *et al.*, 2006; Wilson *et al.*, 2005). During OXTR activation conformational changes expose PKC-consensus sites in the C-terminus tail of the OXTR, allowing phosphorylation of the OXTR by G-protein receptor kinases (GRKs) or PKC. Hasbi *et al.* (2004) showed GRK phosphorylation of the OXTR occurs within four seconds of ligand binding, and allows the binding of arrestin proteins, e.g. β-arrestin, to the OXTR (Gimpl & Fahrenholz, 2001). Recruitment of arrestin to the OXTR causes subsequent uncoupling of the G-protein subunits from the receptor (Berrada *et al.*, 2000; Smith *et al.*, 2006), causing receptor desensitisation, meaning the OXTR can no longer signal. The presence of β-arrestin allows the OXTR to interact with clatherin, allowing internalisation of the receptor through the formation of clatherin coated pits. Dynamin, a GTPase, then pinches of the clatherin coated pit, fully internalising the OXTR (Goodman *et al.*, 1996). Internalisation of the OXTR can either lead to recycling of the receptor to the cell membrane, which occurs after four hours in transfected HEK293 cells (Conti *et al.*, 2009), or degradation by lysosomes (Smith *et al.*, 2006). Recycling of the OXTR back to the cell membrane removes the β-arrestin protein, allowing the OXTR to recouple to G-proteins and enable signalling again. However, lysosomal degradation of OXTRs following internalisation causes downregulation of the OXTR (Conti *et al.*, 2009). Downregulation reduces the availability of the receptor on the cell surface, ultimately reducing signalling.

OXTR downregulation and desensitisation is an important process with significant clinical implications. Desensitisation and downregulation of the mu-opioid GPCR underlies the onset and maintenance of tolerance to mu-opioid receptor targeting analgesic medication, such as morphine and fentanyl (Bailey *et al.*, 2009). Therefore, prolonged or repeated OXY administration may lead to reduced OXTR expression. This would limit the clinical efficacy of OXTR targeting medication and interfere with other endogenous OXTR mediated processes. In mice, chronic OXY administration (twice daily, 14 days) reduces both the expression of social behaviours in a social interaction paradigm and OXTR expression throughout the brain (Huang *et al.*, 2014).

In addition, Bales *et al.* (2013) demonstrated acute intranasal OXY increased social behaviours in prairie voles, while chronic intranasal OXY (once daily, 21 days) impaired the formation of partner preference in prairie voles, and they attributed this reduction to possible receptor downregulation. However, Calcagnoli *et al.* (2015a) showed no OXTR desensitisation in the brain following acute or chronic intranasal OXY (once daily, 7 days) administration in male rats. Whether OXTR desensitisation occurs in the brain following OXY administration or not, it is an important aspect to consider when administering chronic OXY *in vivo*.

1.2.4 Behavioural effects in animals

Following early work indicating a clear role for peripheral OXY in parturition and lactation, research began to focus more on the behavioural effects of OXY, arising from central OXY containing neurons. The actions of OXY within the CNS are primarily mediated by OXTR receptor distribution. As previously stated, OXTRs are located extensively throughout the brain, from areas such as the hypothalamus and amygdala, to the prefrontal cortex (PFC) and NAc (Smith *et al.*, 2017a). The location in which OXTRs are located directly influences the role OXY has on behaviour. For example, OXTRs in the hippocampus primarily mediate learning and memory, whereas OXTRs in the PVN are involved in maternal and reproductive behaviours, although this is not exclusive (Viero *et al.*, 2010). OXY also has affinity at vasopressin 1a receptors (V1aR), therefore the expression of V1aRs in the brain also contributes to the behavioural effects of OXY and is discussed in more detail in section 1.2.5.

Amongst the first evidence indicating a role for OXY in the modulation of behaviour was the observation that central OXY could co-ordinate the onset of maternal behaviours immediately following parturition, resulting in mother-infant bonding. One of the pivotal papers demonstrating this was Pedersen and Prange (1979), showing i.c.v. OXY administered to virgin rats produced maternal behaviour towards pups within two hours. This has been further supported by studies showing oestrogen priming alongside OXY administration can increase the probability of virgin rats

exhibiting short-term spontaneous maternal care, and OXTR antagonism by CNS penetrant antagonists can block the onset of maternal behaviours in rats following parturition (Fahrbach *et al.*, 1984; 1985; van Leengoed *et al.*, 1987). Although OXY appears critical in the onset of maternal behaviours, it is unlikely to play a role in their maintenance, as administration of an OXTR antagonist five days post-partum does not block maternal behaviours (Fahrbach *et al.*, 1985). In support of a role for central OXY in maternal behaviour OXY knockout mice display reduced levels of licking and grooming compared to wild type mice, and mice expressing dysfunctional OXTRs in the forebrain have impaired maternal behaviours, including lack of nursing and nest building (Pedersen *et al.*, 2006; Wettschureck *et al.*, 2004). OXTRs expressed in brain regions such as the NAc have been heavily implicated in maternal behaviours, with higher levels of NAc OXTRs associated with an increased likelihood of exhibiting alloparental care; the act of displaying parenting behaviour in non-descendant young (Olazabal & Young, 2006). There is some evidence to suggest OXY may also be involved in the formation of pup to mother interactions. In fifteen day old rat pups OXTR antagonists can block the preference for maternal odours (Nelson & Panksepp, 1996), and both OXY and OXTR knockout pups emit significantly fewer ultrasonic vocalisations (emitted as part of maternal-pup interaction repertoire) following MS than wild type pups (Insel & Winslow, 1991).

Not only is OXY involved in the formation of mother-infant bonds, but it also plays a prominent role in the formation of reproductive relationships. Throughout OXY research prairie voles have become a popular choice to study the effects of OXY upon the formation of pair bonds; a social bond between two mating animals. Unlike many rodents, prairie voles are monogamous and are therefore used as a model to study the effects of OXY on pair bonding (Ross & Young, 2009). Williams *et al.* (1994a) demonstrated i.c.v. OXY induced pair bonding in female prairie voles, which can be prevented through pre-limbic cortex and NAc injections of an OXTR antagonist ($d(CH_2)_5$,[Tyr(Me)^2,Thr^4,Tyr-NH_2^9]OVT) (Young *et al.*, 2001). In addition, central OXY can trigger pair bonding in male prairie voles, which is prevented by i.c.v. administration of both an OXTR and V1aR antagonist ($d(CH_2)_5$,[Tyr(Me)^2,Thr^4,Tyr-NH_2^9]OVT and [$d(CH_2)^5$-[Tyr(Me)]AVP, respectively) (Cho *et al.*, 1999). As

monogamous voles have significantly higher OXTR density in the NAc than non-monogamous voles (Insel & Shapiro, 1992) this region has been implicated in the differential response. In addition, OXTR antagonists injected into the NAc can inhibit partner preference, and microdialysis has shown increased NAc OXY release during mating (Ross *et al.*, 2009a). While some confounding results have been observed, particularly surrounding the role of OXY in male pair bonding, it is generally accepted that the OXY and vasopressin system plays an important role in pair bonding (Donaldson & Young, 2008; Winslow *et al.*, 1993; Young & Wang, 2004).

OXY is also implicated in feeding and appetite. OXTR knockout mice are heavier than wild type mice, and OXTR antagonism in rodents increases meal sizes (Blouet *et al.*, 2009; Camerino, 2009). In addition, OXY administration can reduce feeding and improve gastric motility (Blevins & Baskin, 2015; Petring, 1989). Effects of OXY on feeding are likely due to OXY synthesis in the PVN, an area heavily involved in food regulation (Leibowitz *et al.*, 1981). OXY release in the nucleus tractus solitarius (NTS) from PVN OXY neurons can reduce food intake through increasing the satiety response to cholecystokinin (Baskin *et al.*, 2010). In a somewhat similar mechanism OXY can also reduce the intake of drugs of abuse through interactions with the mesolimbic system, increasing the feelings of reward associated with drugs of abuse (Weber *et al.*, 2018). OXY administration in rodents can reduce alcohol self-administration (King *et al.*, 2017; MacFadyen *et al.*, 2016), reduce morphine tolerance and withdrawal (Sarnyai & Kovács, 2014), and reduce methamphetamine and cocaine seeking behaviours (Weber *et al.*, 2018; Zhou *et al.*, 2014). The ability of OXY to enhance NAc dopamine activity and give feelings of reward is thought to prevent the need for further reward seeking, thus reducing the seeking and administration of drugs of abuse (Weber *et al.*, 2018). Other effects of central OXY include its analgesic effects through activation of inhibitory gamma-Aminobutyric acid (GABA) circuits in the spinal cord (Xin *et al.*, 2017), and antipsychotic-like effects; demonstrated through reversal of phencyclidine (PCP)-induced hyperactivity in rats (Kohli *et al.*, 2019) and facilitation of latent inhibition (Feifel *et al.*, 2015).

Another central effect of OXY, which has important implications upon dose selection, is the observation that high dose OXY (1-2mg/kg i.p.) has ‘sedative-like’ effects upon

locomotor activity (Hicks *et al.*, 2012; Hicks *et al.*, 2014; Klenerova *et al.*, 2009), which likely results from OXY activity in the nigrostriatal pathway (Angioni *et al.*, 2016). Lower doses of OXY (0.3mg/kg s.c. and below) have no effect on locomotor activity, therefore should be used when examining the behavioural effects of OXY (Kohli *et al.*, 2019). In addition to the aforementioned roles, central OXY also plays a prominent and important role in platonic and non-reproductive social behaviours, such as social recognition and social reward, and is to be discussed in detail in section 1.4.1 (Dölen *et al.*, 2013; Hung *et al.*, 2017; Raam, 2020).

1.2.4.1 Peripheral effects of OXY

In addition to the CNS effects of OXY, and its peripheral effects upon lactation and uterine contraction, OXY may also lower body temperature and heart rate. These are important factors to consider when delivering OXY by systemic administration, as the prosocial effects of OXY may also result in unwanted side effects through peripheral activity of OXY.

In male Wistar rats administration of both OXY and vasopressin (0.1-1mg/mg i.p.) cause dose-dependent reductions in body temperature and heart rate, and are prevented through V1aR antagonism (Costa-e-Sousa *et al.*, 2005; Hicks *et al.*, 2014). Similarly, Kohli *et al.* (2019) found a significant hypothermic effect of 0.3mg/kg s.c OXY in male Lister-hooded rats which was also attenuated through prior administration of the V1aR antagonist SR 49059. Therefore, it is likely the V1aR mediates the peripheral effects of high dose OXY. Due to the lower affinity of OXY at the V1aR, its effects at the V1aR may become apparent following high doses of peripheral OXY, making dose selection in behavioural studies extremely important.

1.2.5 Similarities with vasopressin

Like OXY, vasopressin is also a nonapeptide with a cyclic structure. Structurally, vasopressin only varies by two amino acids from OXY, and is also a neuropeptide involved in the regulation of social behaviour (Caldwell, 2017) (Figure 1.7). While the neutral isoleucine amino acid at position 3 within the structure of OXY is critical for OXTR activation, arginine, a basic amino acid in position 8 of vasopressin, is required for vasopressin receptor activation (Gimpl & Fahrenholz, 2001). The difference in polarity of these amino acids is believed to be responsible for the specificity of OXY and vasopressin for their respective receptors, described in detail in below (Barberis *et al.*, 1998; Gimpl & Fahrenholz, 2001).

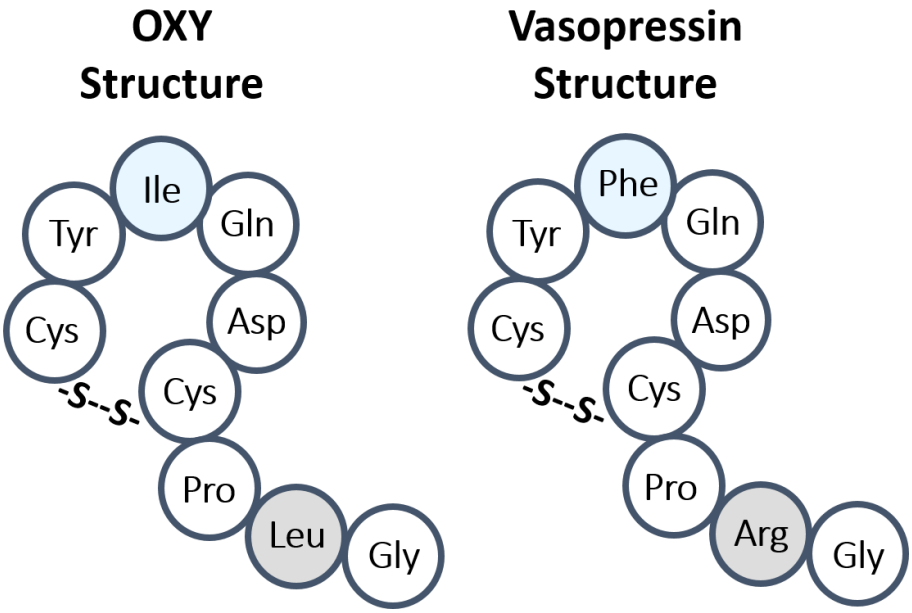

Figure 1.7: The amino acid structures of OXY and vasopressin, differing at positions and 3 and 8, with a disulphide bridge connecting the two cysteine residues; allowing the formation of a 6 amino acid cyclical stricture with a 3 amino acid tail (Gimpl & Fahrenholz, 2001).

Both OXY and vasopressin are thought to have arisen from an ancestral gene duplication during evolution. Both genes are on found the same chromosome (20 in humans), and are orientated in opposing transcriptional directions (Caldwell, 2017).

Homologs of OXY and vasopressin existed at least 700 million years ago (Donaldson & Young, 2008) and are still present in ancient organisms such as fresh water hydra (Caldwell *et al.*, 2008; Grimmelikhuijzen & Spencer, 1984). Analogues of OXY and vasopressin can be found across almost all vertebrates and invertebrates in at least one form (Donaldson & Young, 2008), and have very similar biological functions, including: involvement in sexual and nurturing behaviours, water balance control (Ukena *et al.*, 2008), and changing behaviours in response to environmental changes (Quintana & Guastella, 2020). An example of the crucial role OXY and vasopressin homologs play in even simple organisms has been elegantly demonstrated by simple learning paradigms in flat worms (*Caenorhabditis elegans*). Flat worms can associate particular environments with aversive properties, whilst roundworms that lack an OXY/vasopressin homolog are unable to display the same level of associative learning (Beets *et al.*, 2012). Other behavioural effects of OXY and vasopressin homologs in non-mammalian species include egg laying in snails (Van Kesteren *et al.*, 1995), reproductive behaviours in earthworms (Satake *et al.*, 1999), and pair bonding and aggression in birds (Kelly & Goodson, 2014; Klatt & Goodson, 2013). Both OXY and vasopressin homologs show strong functional and structural stability throughout evolution, reinforcing how important these peptides are in social functioning and reproduction across a huge, varying range of species.

1.2.5.1 Vasopressin receptors

In contrast to OXY which has only one known receptor in mammals, there are three forms of vasopressin receptors: the V1aR, the vasopressin 1b receptor (V1bR), and vasopressin 2 receptor (V2R) (Song & Albers, 2018). While the V1aR and V1bRs are both expressed in the brain and periphery, the V2R is only expressed peripherally; predominantly in the kidney tubules where it mediates water reabsorption (Renaud, 2007). Although the V1bR is expressed in the brain, its expression is very limited and highly restricted to pyramidal cells in the CA2 region of the hippocampus (Pagani *et al.*, 2015; Young *et al.*, 2006). Therefore, behavioural effects arising from OXY and vasopressin activity in the brain are most likely to arise from either activity at OXTRs

or V1aRs. Like the OXTR, V1aRs and V1bRs are also coupled to the $G\alpha_q$ G-protein. In contrast, the V2 receptor is $G\alpha_s$ coupled; stimulating adenylyl cyclase and activating protein kinase A (Schöneberg *et al.*, 1998).

In addition to the structural similarities between vasopressin and OXY, their receptors are also structurally very similar, with approximately 25% of amino acids conserved between the mammalian receptors (Gimpl & Fahrenholz, 2001). Due to their high sequence homology it is possible for OXY to signal through the V1aR, and for vasopressin to signal through the OXTR. For example, hypothermic effects observed in rats following high dose s.c. OXY are reversed through a selective V1aR antagonist (SR 49059), while vasopressin can modulate social reward in Syrian hamsters through the OXTR in the VTA, but not V1aR activity (Kohli *et al.*, 2019; Song *et al.*, 2016). In terms of selectivity for their respective receptors it appears OXY has a significantly higher affinity for the OXTR over vasopressin receptors, while vasopressin has a similar affinity for both OXTRs and vasopressin receptors (Table 1.1). This means it is more likely to see vasopressin signalling through the OXTR, than OXY signalling through the V1aR. OXY and vasopressin agonists been developed, with varying levels of specificity between the receptors; some of which have clinical uses, for example in the treatment of diabetes insipidus and in the induction of labour (Table 1.1). The high sequence homology between OXY and vasopressin, and their receptors, has hindered the initial development of selective agonists and antagonists. However, recent progress has resulted in the availability of selective antagonists for these receptors, progressing OXY and vasopressin research (Table 1.2). Selective antagonists have since been used to determine the specific receptors responsible for different OXY mediated behaviours.

	OXTR (K_i(nM))		V1a (K_i(nM))		V1b (K_i(nM))		V2 (K_i(nM))		Clinical use?
	Human	Rat	Human	Rat	Human	Rat	Human	Rat	
OXY	0.8	0.6	120.0	71.0	>1000	294.0	3500.0	89.0	Used in the induction of labour, and control of postpartum haemorrhage
Vasopressin	1.7	1.7	1.1	2.6	0.7	0.3	1.2	0.4	Used in the treatment of diabetes insipidus
[Thr^4,Gly^7]OT	4.0		158.0						
Arginine vasotocin	0.4		3.2	3.2	10.0		6.3		
Carbetocin	7.1	1.9		7.2*		7.2*	>1000	61.3	Used to prevent excessive bleeding after childbirth following Cesarean section
d[Cha^4]AVP	20.0		79.4		0.2		316.2		
d[Leu^4,Dap^8] vasopressin		1259.0		3162.3		0.4		251.2	
d[Leu^4]LVP		63.1		>1000	0.2			100.0	
Desmopressin	200.0			2512.0		10.0		2.0	Used in the treatment of diabetes insipidus and bedwetting
Oxypressin	1.6			501.1			79.4		
WAY 267,464		51.6							

Table 1.1: The affinities of OXY and vasopressin at the human and rat V1aR, V1bR, V2 receptor and OXTR, displayed as K_i values (nM), as well as the affinities of some commonly used agonists. A smaller K_i value indicates a higher affinity for the receptor. *This paper did not discriminate between V1aR and V1bRs (Alexander *et al.*, 2019; Hicks *et al.*, 2012; Manning *et al.*, 2012; Passoni *et al.*, 2016; Ring *et al.*, 2010; Song & Albers, 2018)

	OXTR (K_i (nM))	V1aR (K_i (nM))	V1bR (K_i (nM))	V2 (K_i (nM))
[d(CH$_2$)^5-[Tyr(Me)]AVP	5.0	0.6	100.0	79.0
[Phaa^1,D-Tyr(Et)^2,Lys^6,des-Gly^9]AVP		0.8	31.6	251.0
Atosiban*	25.1	5.0	251.0	>1000
Compound 25	9.5	>1000.0		
Conivaptan		4.0		0.4
d(CH$_2$)$_5$,[Tyr(Me)^2,Thr^4,Tyr-NH$_2$^{9}]OVT	0.1	0.2		
d(CH$_2$)$_5$[D-Ile^2,Ile^4,Tyr-NH$_2$^{9}]AVP				0.3
d(CH$_2$)$_5$[Tyr(Me)$_2$,Thr^4,Orn^8,Phe(3I,4N$_3$)-NH$_2$^{9}]vasotocin	1.0	1.3	>1000.0	199.5
d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Tyr-NH$_2$^{9}]OVT	0.1	4.0	>1000	>1000
desGly-NH$_2$,d(CH$_2$)$_5$-[Tyr(Me)^2,Thr^4]OVT	0.3	1.3		
L-368,899	7.9			
Retosiban	1.0			
SR 49059	316.2	0.5	50.1	125.9
SSR149415	1.6	100.0	0.5	
Tolvaptan		12.6		0.4

Table 1.2: Commonly used antagonists that target the OXY and vasopressin receptors. A smaller K_i value indicates a higher affinity for the receptor. *While atosiban acts as an antagonist at the $G\alpha_q$ coupled OXTR, it acts as an agonist at the $G\alpha_i$ coupled OXTR (Alexander *et al.*, 2019; Eliava *et al.*, 2016; Manning *et al.*, 2012; Manning *et al.*, 2008).

Despite high sequence homology between the OXTR and V1aR it is important to note that their expression in the rodent brain is very different (Smith *et al.*, 2017a; Smith *et al.*, 2019) (Figure 1.8). Although there are some regions in which they are co-expressed, such as all seven nodes of the SBNN, they are expressed at different densities (Smith *et al.*, 2019). Co-expression of OXTR and V1aRs in certain brain regions provides the possibility that crosstalk between the OXY and vasopressin systems could occur, potentially enabling OXY and vasopressin to act promiscuously to influence behaviour.

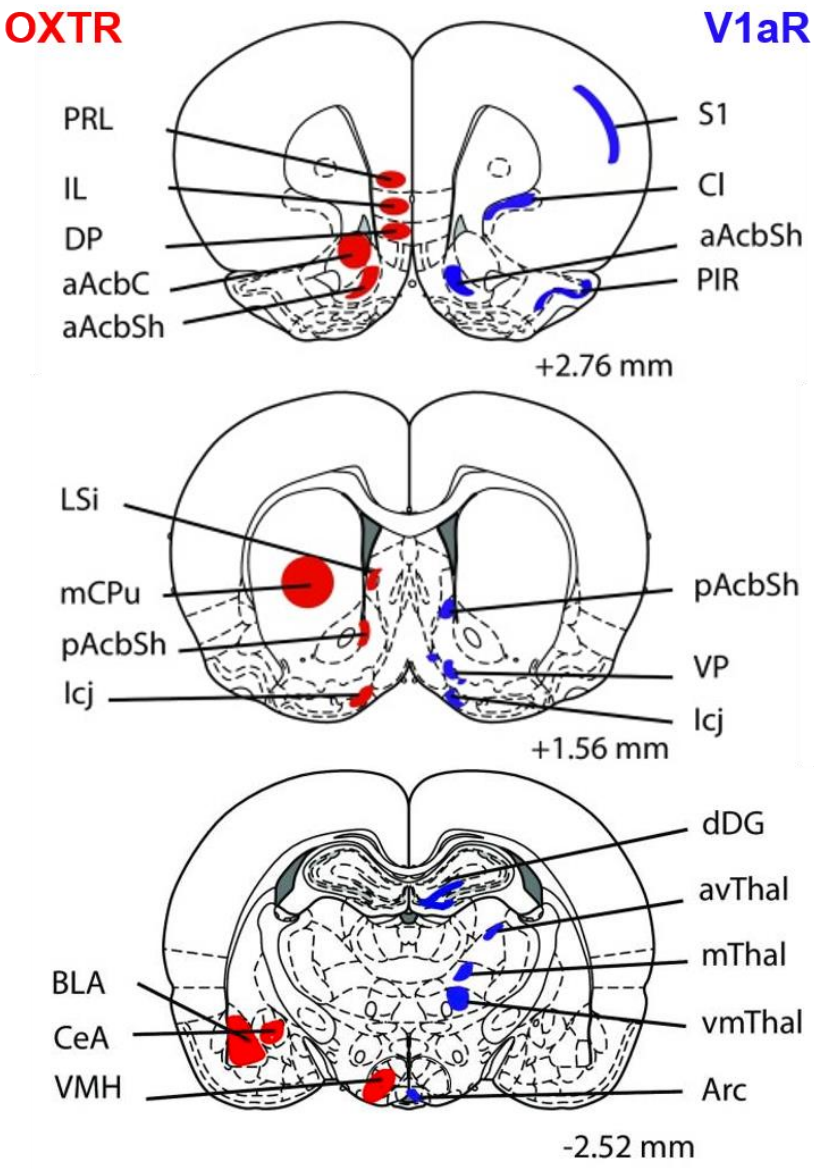

Figure 1.8: OXTR and V1aR expression in three different coronal sections of an adult male rat brain, showing the variations in OXTR (red) and V1aR (blue) expression. Receptor expression measured through autoradiography and distance from bregma shown in mm. Image adapted from Smith *et al.* (2017a). Abbreviations: aAcbC anterior nucleus accumbens core, aAcbSh anterior nucleus accumbens shell, Arc arcuate nucleus of hypothalamus, avThal anteroventral thalamic nucleus, BLA basolateral amygdala, CeA central amygdala, Cl claustrum, Ddg dorsal dentate gyrus, DP dorsal peduncular cortex, Icj islands of Calleja, IL infralimbic cortex, LSi intermediate part of the lateral septal nucleus, mCPu medial caudate putamen, mThal medial thalamic nucleus, pAcbSh posterior nucleus accumbens shell, PIR piriform cortex, PRL prelimbic cortex, S1 primary somatosensory cortex, VMH ventromedial nucleus of hypothalamus, vmThal medial thalamic nucleus, VP ventral pallidum.

1.2.5.2 Vasopressin synthesis and release

Similar to OXY, vasopressin is also synthesized in a distinct set of neurons in the magnocellular and parvocellular neurons of SON and PVN, and can undergo endocrine, paracrine, and synaptic release (Ludwig & Leng, 2006). Following synthesis in magnocellular neurons vasopressin is axonally transported in LDCVs to the posterior pituitary, where it is released into the peripheral circulation and has effects on renal function (Buijs *et al.*, 2021). As with OXY, vasopressin can also undergo both dendritic release from magnocellular neurons, resulting in diffusion through the brain to sites distal to release, and synaptic release from magnocellular and parvocellular long range projections (Ludwig, 1998; Ludwig & Leng, 2006; Meyer-Lindenberg *et al.*, 2011; Smith *et al.*, 2019). While vasopressin and OXY are synthesised and released from distinct populations of PVN and SON neurons, there is some evidence to suggest that some PVN magnocellular neurons can co-express both vasopressin and OXY under certain physiological conditions (Otero-García *et al.*, 2016). For example, during lactation neurons that co-express vasopressin and OXY produce additional vasopressin, hypothesized to compensate for the loss additional fluids associated with lactation (Mezey & Kiss, 1991; Otero-García *et al.*, 2016). The expression of vasopressin neurons in the brain that contribute to synaptic release are different to that of OXY (Figure 1.9). However, as with receptor expression, there are some regions in the rat brain which receive both OXY and vasopressin neuronal projections, albeit at different densities, such as all seven nodes of the SBNN (Smith *et al.*, 2019).

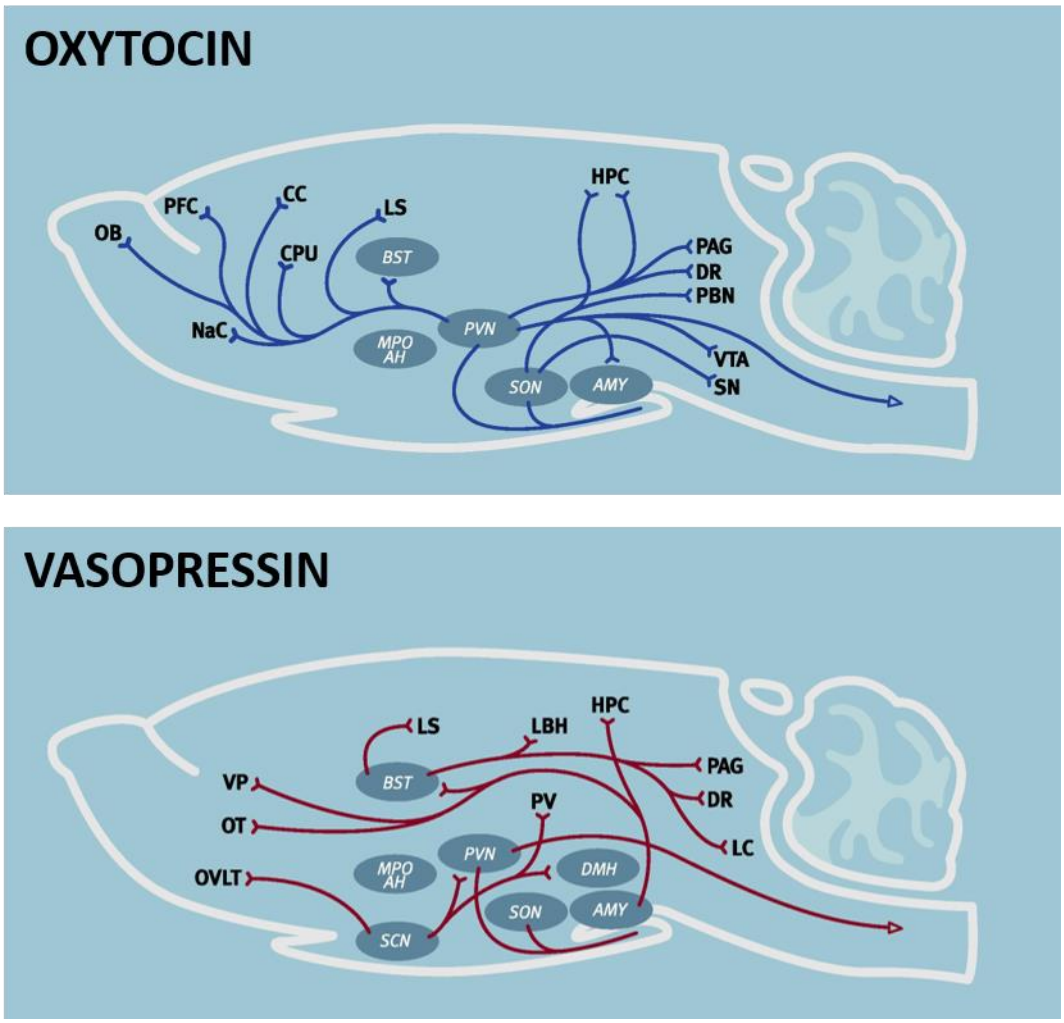

Figure 1.9: Diagrammatic representation of oxytocinergic and vasopressin projections in the rodent brain, arising from the PVN and SON of the hypothalamus, demonstrating differences in the neuronal expression of OXY and vasopressin projections. Represented is a compilation of major OXY and vasopressin projections from several rodent species including rat, mouse, and hamster. Exact vasopressin and OXY projections can vary to some extent between species and from those depicted above. Abbreviations: AMY amygdala, BST bed nucleus of the stria terminalis, CPU caudate putamen, CC Cingulate cortex, DR dorsal raphe, HPC hippocampus, LS lateral septum, LC locus coeruleus, MPO AH medial preoptic area of the anterior hypothalamus, NAc nucleus accumbens, OB Olfactory bulb, OT olfactory tubercle, OVLT organum vasculosum laminae teriminalis, PBN parabrachial nucleus, PVN paraventricular nucleus, PAG periaqueductal grey, PFC prefrontal cortex, SN substantia nigra, SCN suprachiasmatic nucleus, SON supraoptic nucleus, VP ventral pallidum and VTA ventral tegmental area (VTA). Image from (Song & Albers, 2018).

The expression of OXY and vasopressin projections in the brain contributes to the ability of OXY and vasopressin to signal through both their own, and each other's, receptors (Ludwig & Leng, 2006; Smith *et al.*, 2019). Most sites that receive dense

vasopressin projections have high V1aR expression, likewise, most brain regions receiving dense OXY innervation have high OXTR expression. However, there are some regions where there is a mismatch between receptor expression and neuronal innervation (Ludwig & Leng, 2006; Smith *et al.*, 2019). For example, regions such as the posterior medial amygdala receive dense vasopressin neuron innervation but have low V1aR expression. However, they have dense OXTR expression. In contrast, areas such as the periaqueductal grey have dense OXY neuron expression alongside dense V1aR expression, but sparse OXTR expression (Smith *et al.*, 2019). This mismatch in density patterns between OXY fibres and OXY receptors, and vasopressin fibres and V1aRs, in some regions of the brain likely contributes to promiscuous action of OXY at the V1aR and vice versa.

In addition to vasopressin parvocellular and magnocellular neurons, the PVN also contains a population of smaller parvocellular neurons that co-release vasopressin and corticotrophin-releasing hormone (CRH) in the hypophyseal-portal bed; a system of blood vessels connecting the hypothalamus with the anterior pituitary (Buijs *et al.*, 2021). CRH release contributes to the synthesis and release of adrenocorticotropic hormone from the anterior pituitary, subsequently stimulating the release of cortisol and glucocorticoids from the adrenal cortex (Buijs *et al.*, 2021). Another important population of vasopressin neurons in the brain are those in the suprachiasmatic nucleus (SCN), which help mediate circadian rhythms (Ludwig & Leng, 2006). Within the SCN vasopressin is both synthesised and released in a circadian pattern and has an excitatory role through activity at V1aRs; increasing SCN activity and increasing SCN outputs (Buijs *et al.*, 2021; Ingram *et al.*, 1998; Reghunandanan & Reghunandanan, 2006). The circadian activity of SCN vasopressin neurons results in daily fluctuations of CSF vasopressin levels, which start to rise during early morning (Buijs *et al.*, 2021). Although the precise role of vasopressin in the control of circadian rhythms is unknown, it is not thought to be critical, but rather serve more of a modulatory role (Buijs *et al.*, 2021; Kalsbeek *et al.*, 2010).

1.3. Social behaviour

1.3.1 Overview

As the focus of this thesis is to examine the neural mechanisms that contribute to the prosocial effects of OXY, due to its potential to be used in the treatment of social dysfunction, this section explores social behaviour in more detail. Social behaviours are any form of communication or interaction between two conspecifics and have been observed in microorganisms as well as humans and animals (Chen & Hong, 2018; Crespi, 2001). Social cognition and social behaviours are highly complex processes that allow us to acquire, process, store, and interpret information about people/animals around us, and are critical for survival (Patin & Hurlemann, 2015). Social behaviours are needed for the formation of relationships, the survival of offspring, reproduction, and general health and wellbeing. Disruptions to these processes can be detrimental. In humans this can result in interpersonal problems and social deficits, including social withdrawal, which in turn worsens other psychiatric symptoms (Porcelli *et al.*, 2019). Social deficits are a primary symptom in a range of neuropsychiatric disorders, including schizophrenia, ASD, Alzheimer's disease, and MDD (NICE, 2014; Porcelli *et al.*, 2019). The transdiagnostic nature of social deficits across multiple disorders makes the neural mechanisms underlying typical and atypical social cognition of great interest and highlights the need for successful treatments to ameliorate these social deficits.

1.3.2 Neuropsychiatric disorders with social deficits

Social dysfunction is a primary diagnostic criteria for numerous neuropsychiatric disorders, including schizophrenia, ASD, and MDD, and is often one of the first symptoms observed in these disorders (Bilderbeck *et al.*, 2019). It is unknown whether the social dysfunction observed in multiple disorders have a common neural mechanism, or whether despite similar symptoms, they have different biological

causes. Disorders with social deficits also often share some cognitive deficits, which are likely to also contribute to interpersonal behaviours (Bowie *et al.*, 2008). OXTR single nucleotide polymorphisms have been associated with social deficits in disorders such as schizophrenia and ASD and are discussed in more detail in section 1.4.2.

Schizophrenia, a psychiatric disorder affecting 1 in 100 of the world's population (Royal College of Psychiatrists, 2018), has prominent deficits in social cognition. The severity of patient's negative symptoms, including social deficits, are predictors of functional recovery in schizophrenia. Only 13.5% of patients with schizophrenia achieve full recovery; recovery defined as improvements in both 'clinical and social domains' sustained for over 2 years (Jaaskelainen *et al.*, 2013). Social cognitive deficits are strongly associated with a poorer prognosis, including a greater likelihood of hospital admission and longer stays in hospital (Aleman *et al.*, 2017). Patients with schizophrenia suffer impairments across many aspects of social cognition, from their perception of facial expressions to aberrant emotional responses and empathy (Green *et al.*, 2015). These impairments can lead to the misinterpretation of social cues, often resulting in social withdrawal and isolation. In addition, social deficits are thought to exacerbate other symptoms of schizophrenia, such as paranoia (Millan & Bales, 2013). Despite changes in social cognition often preceding the onset of psychotic symptoms, social deficits in schizophrenia remain constant throughout the course of the disease and are often left untreated by existing drugs; antipsychotics show no efficacy in improving social impairments (Kucharska-Pietura & Mortimer, 2013; Roberts *et al.*, 2010).

ASDs are also characterised by prominent impairments in social cognition, such as impaired interpretation and understanding of social behaviours, as well as atypical communication (Barlati *et al.*, 2020; Chisholm *et al.*, 2015). As with schizophrenia, these symptoms can often result in social withdrawal and isolation. Although viewed as separate disorders, levels of social cognitive dysfunction are very similar across schizophrenia and ASD diagnoses, with no significant differences seen between schizophrenia and ASD groups in numerous clinical tasks assessing multiple aspects of social cognition (Fernandes *et al.*, 2018). This raises the possibility that the social

deficits in both disorders may share a common mechanism, and interventions that may work to treat social dysfunction in one disorder could likely work in the other.

Other disorders that show deficits in social cognition include MDD, attention-deficit-hyperactivity-disorder (ADHD), and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Besag & Vasey, 2019; Bilderbeck *et al.*, 2019; Jankovic, 2008). With changes in social behaviour being transdiagnostic, and often one of the first symptoms to arise across a spectrum of disorders, the neurobiological causes of these changes may give great insights into the aetiology of these disorders. Disrupted social cognition can have great impacts on quality of life and functional recovery, highlighting the need for research into potential treatments for social deficits.

1.3.3 Preclinical models used to assess social behaviour

1.3.3.1 Behavioural models

Rodents are highly social animals with well documented patterns of social behaviour. There are numerous rodent paradigms that can be utilised to advance our understanding of social behaviour and enable the identification of the neural substrates involved. Studying social behaviour in rodents can also be a useful paradigm to monitor social changes that may arise from environmental and pharmacological manipulations (Fernández *et al.*, 2018; Meaney & Stewart, 1981).

There are many preclinical paradigms that can be used to look at different clinically relevant aspects of social behaviour, from social motivation and social recognition, to partner preference and alloparental care, detailed in Table 1.3. Numerous preclinical models of schizophrenia, both pharmacological and neurodevelopmental, produce deficits in social behaviour in a variety of these tasks, supporting their face validity for studying symptoms such as social withdrawal in schizophrenia (Jones *et al.*, 2011). However, it is important to note that no single paradigm used in rodents captures the complex, multidimensional nature of human social behaviour.

Model		Details
Reproductive	**Alloparental care**	This is the display of parenting behaviours towards a non-descendant young. It allows the study of maternal behaviours in the absence of physiological changes associated with parturition and pregnancy (Ross & Young, 2009). To measure alloparental care pups can either be placed into home cages with adult rodents, or both can be placed in an experimental apparatus and behaviours monitored (Garner *et al.*, 2016; Perry *et al.*, 2016).
	Partner preference	Allows the study of pair bonding, an indicator of social monogamy. Typically involves an arena with three chambers: one neutral chamber, one with a familiar opposite sex 'partner', and the other with an unfamiliar opposite sex conspecific, with the test subject able to move freely between the chambers. The time that the test rodent spends in each chamber is measured, as well as the behaviours, affiliative, or aggressive, displayed (Carp *et al.*, 2016).
Non-reproductive	**Social interaction**	Social interaction refers to behaviours that occur between two animals of the same species. The task can provide an index of social motivation; the desire to interact. For this task, two novel weight and treatment (if applicable) matched animals are placed into an arena and social interaction behaviours quantitatively measured, including both playful and aggressive behaviours such as anogenital sniffing, body sniffing, and boxing. It is imperative the two conspecifics have never met before to ensure comparable results. Animals are often housed singly for 24 hours prior to undertaking the task to enhance interaction (Kohli *et al.*, 2019; Wilson & Koenig, 2014).
	Social recognition	This task determines the ability of rodents to differentiate between a novel and a familiar conspecific which it has previously been exposed to, i.e. a litter or cage mate. The time and/or number of each component exploratory behaviour in each individual is recorded. Increased exploration of the novel conspecific is used as evidence of social discrimination and recognition. There are multiple ways in which this task can be designed, such as using juveniles in place of a conspecific, or allowing rodents to interact or restraining the non-test animals in mesh cage (Millan & Bales, 2013).
	Social Stress	Also known as the resident/intruder paradigm, this test is the standard model for assessing aggression and social stress. This task involves introducing an experimental mouse or rat into the territory/home cage of an aggressive conspecific of a higher weight. Rodents are left to interact for a given time period, with the behaviours recorded. This interaction generally results in

		social conflict, leading to social defeat of the experimental male. It is important to have a predefined definition of social defeat, such as the display of submissive behaviour, to ensure the welfare of the animals. The protocol can be repeated depending on the experimental protocol, for example for models of chronic social stress the experimental rat can be kept in the residents cage, but separated by a wire screen, or the procedure can be repeated recurrently; to model recurrent social stress (Koolhaas *et al.*, 2013; Patel *et al.*, 2019).
	Social preference/ sociability	This compares the rodent's preference between a conspecific and a socially neutral stimuli and is a good measure of social reward. This task has two identical chambers, one containing a social stimuli (conspecific) or a non-social stimuli (a toy), and measures the motivation to engage in social interaction (Millan & Bales, 2013). Similar to the classic 'conditioned place preference' task, often used to measure rewarding properties of drugs of abuse.

Table 1.3: Commonly used preclinical tasks used to monitor both reproductive and non-reproductive social behaviours in rodents (Millan & Bales, 2013; Ross & Young, 2009).

When using preclinical models to study social behaviour of relevance to neuropsychiatric disorders it is important to make a distinction between reproductive and non-reproductive social behaviours, and ensure the preclinical paradigm used is translatable to the human condition. In rodents most research looking at social behaviours has typically focussed on reproductive and maternal social relationships, and it is only in the last decade more research has been conducted into the neural circuits underlying same-sex, platonic interactions (Dölen *et al.*, 2013; Gunaydin *et al.*, 2014; Hung *et al.*, 2017; Kohli *et al.*, 2019; Ramos *et al.*, 2013; Williams *et al.*, 2020). It is the neural circuitry that underlies same-sex, non-reproductive interactions that is of most relevance when studying social behaviours in relation to human disorders with aberrant social functioning. Therefore, rodent models looking at reproductive and maternal behaviours should be avoided when studying social behaviours of relevance to psychiatric disorders, and when examining the therapeutic potential of novel treatments for social deficits as seen in schizophrenia and ASD (Fernández *et al.*, 2018; Gunaydin *et al.*, 2014).

The social interaction task (Table 1.3), to be used throughout the experimental chapters of this thesis, has high construct validity for determining social deficits in models of schizophrenia. The amygdala, hippocampus, and PFC have all been implicated in specific roles in both social interaction and negative symptoms of schizophrenia (Wilson & Koenig, 2014). Additionally, both dopaminergic and glutamatergic systems have been implicated in both schizophrenia aetiology and social interaction (Carlsson *et al.*, 2001; Wilson & Koenig, 2014). Although a simple behavioural model the social interaction task is strongly representative of naturally occurring rodent social interactions; rodents are left to freely explore and interact with one another, giving the task high ethological validity. Furthermore, social interaction is disrupted in many rodent models that aim to replicate the genetic, neurological, or developmental aspects of schizophrenia, making the social interaction task translationally relevant to schizophrenia (Hida *et al.*, 2013; Sams-Dodd, 1998; 1999).

1.3.3.2 Ultrasonic vocalisations

Much like human social interaction, those between rodents are not solely based on physical touch and contact. Rodents can communicate through the emission and perception of ultrasonic vocalisations (USVs) (Kagawa *et al.*, 2017; Matsumoto *et al.*, 2016). USVs are thought to be a social communication signal for both juvenile and adult rats, with rodents emitting USVs during a huge variety of social encounters, from social play and mating to aggression (Brudzynski, 2015; Burgdorf *et al.*, 2008). Analysis of emitted USVs in both natural situations and following pharmacological and environmental manipulations can give great insights into the neural mechanisms underlying social behaviours and social communication in rodents.

There are multiple types of USVs produced by rats, and the type of calls emitted are reflective of their affective state. In adult rats 50 kilohertz (kHz) calls represent a positive affective state and are associated with rewarding stimuli, and have been suggested to be an evolutionary homolog of laughter in humans (Panksepp, 2007).

Increased emission of 50 kHz calls occurs during positive social encounters, such as social play, rat play with human hands (tickling), and in response to playback of 50 kHz calls (Brudzynski, 2015; Panksepp & Burgdorf, 2000; Wöhr & Schwarting, 2009). In addition, increases in 50 kHz calls are also seen during rewarding processes such as self-administration of sweet foods, such as sucrose (Browning *et al.*, 2011). Interestingly, rats with a higher natural production of 50 kHz calls make more social contacts than those with lower emissions of prosocial calls, demonstrating a clear association between 50 kHz calls and social behaviour (Brudzynski, 2015). 50 kHz calls can be further classified into three different subtypes based upon call features, such as duration and modulation of frequency (Figure 1.10). Although all 50 kHz calls are associated with positive affective states, they are thought to be emitted in different contextual situations. For example, frequency modulated (FM) 50 kHz calls appear to be emitted in highly rewarding situations, such as sexual interactions, while 50 kHz calls of a constant frequency with no modulation are thought to be emitted in social contact situations and feeding behaviours, and serve more of a communicative purpose (Brudzynski, 2015; Burgdorf *et al.*, 2008).

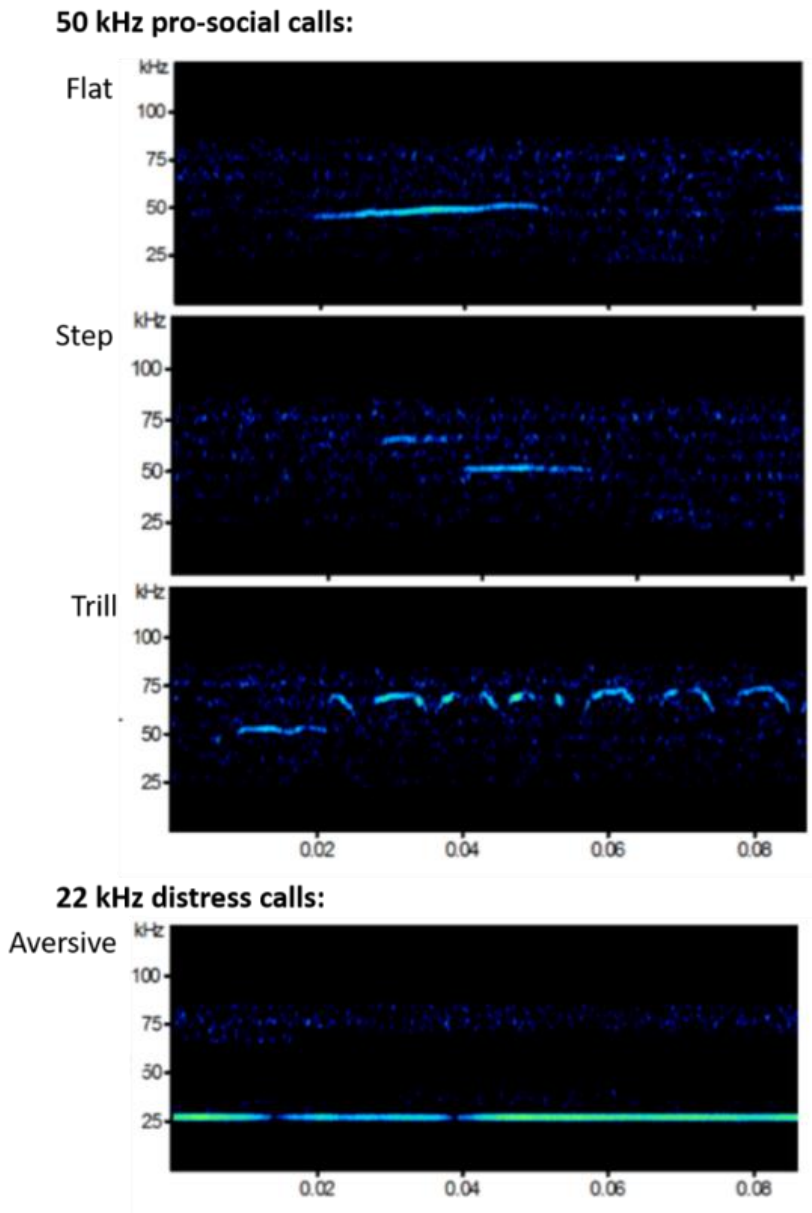

Figure 1.10: Pattern analysis of rat 50 kHz prosocial USVs and 22 kHz distress USVs. Flat calls have frequency changes of less than 5 kHz, however an upward or downward direction in the call could result in a start and end frequency greater than 5 kHz. Step calls contain a minimum of one short element that is more than 5 kHz higher or lower than the main call. Trill calls have a minimum of one longer frequency modulation than step calls and/or a zig zag shape or a series of inverted-u shapes (X axis time scale in seconds). 22 kHz calls are identifiable by a flat, low frequency, with a longer duration than 50 kHz calls (Brudzynski, 2015).

Contrary to the production of 50 kHz calls, production of 22 kHz calls reflect a negative affective state, and are typically called 'distress' calls (Brudzynski, 2015). 22 kHz calls are produced in response to a range of aversive stimuli, including: mild foot

shocks (Wöhr *et al.*, 2005), loud startling acoustic stimuli (Kaltwasser, 1990), the presence of a predator such as a cat (Blanchard *et al.*, 1991), encounters with dominant rats (Assini *et al.*, 2013), and unexpected puffs of air (Brudzynski & Holland, 2005). In addition, 22 kHz calls are also produced during isolation and chronic pain (Calvino *et al.*, 1996; Francis, 1977). Whilst 50 kHz calls have been seen following the administration of drugs with rewarding properties, withdrawal from addictive agents such as opiates, alcohol, and benzodiazepines result in 22 kHz calls being emitted (Covington & Miczek, 2003; Mutschler & Miczek, 1998). Playback of 22 kHz calls to rats causes behavioural changes such as freezing responses and reduced locomotor activity, suggesting 22 kHz calls are alarm calls and evoke a fear response in rats (Brudzynski & Chiu, 1995). 22 kHz calls can also be classified into two further subtypes: long and short. Although both of these calls are associated with distress and aversion, long calls are thought to be expressed when there is an external threat or danger, such as a predator, while short calls represent internal distress or discomfort, for example during drug withdrawal (Barker *et al.*, 2010; Brudzynski, 2015).

Rat USVs are sensitive to pharmacological manipulation, giving an insight into the neural mechanisms involved in USV production. Both systemic and NAc micro-injections of dopamine agonists cause 50 kHz call emission in rats, suggesting NAc dopamine may be involved in the production of prosocial 50 kHz USVs (Burgdorf *et al.*, 2001; Thompson *et al.*, 2006). Administration of drugs associated with feelings of reward, such as cocaine and amphetamines, also increase the production of 50 kHz prosocial calls (Burgdorf *et al.*, 2001; Ma *et al.*, 2010; Simola *et al.*, 2012). Further to this, in alcohol dependent rats the number of 50 kHz calls emitted positively correlated with the amount of alcohol drunk (Buck *et al.*, 2014). In contrast, administration of carbachol increases the production of 22 kHz aversive and 'distress' calls (Brudzynski & Bihari, 1990), as does administration of drugs with aversive effects, such as lithium chloride and naloxone (Burgdorf *et al.*, 2001).

Given USVs are reflective of affective state in rodents, and the strong association between rewarding stimuli and 50 kHz emission, the neural mechanisms underlying 50 kHz prosocial calls may give an insight into the neural basis of social behaviours

and emotion, advancing our understanding of psychiatric disorders with aberrant emotional processing and social cognition. Emission of 50 kHz calls during social interaction was therefore investigated in the current thesis as a potentially relevant index of changes in social communication.

1.4. Oxytocin in social behaviour

1.4.1 Preclinical evidence in rodents

Despite an abundance of work looking at the effects of exogenous OXY administration on reproductive and maternal social behaviour in rodents, there is a surprising lack of research into its effects on non-reproductive social behaviours between two animals of the same sex. Our understanding of the role the OXY system plays in non-reproductive social behaviours has largely been advanced through the availability of OXY and OXTR knockout mice. These models have demonstrated robust deficits in social interaction and recognition; clearly implicating the oxytocinergic system in these social behaviours (Table 1.4) (Ferguson *et al.*, 2000; Pobbe *et al.*, 2012; Sala *et al.*, 2013; Sala *et al.*, 2011).

Knockout	Author	Behavioural findings
OXTR (-/-)	Pobbe *et al.* (2012)	Robust decreases in social interaction and social recognition
	Sala *et al.* (2011); Sala *et al.* (2013)	Impaired social recognition and reduced social interaction
	Dhakar *et al.* (2012)	Elevated levels of aggression to intruders and greater c-Fos reactivity in the medial amygdala compared to wild type
	Hattori *et al.* (2015)	Greater aggression to intruders of the same strain and impaired social recognition
OXY (-/-)	DeVries *et al.* (1997)	Reduced duration of aggressive behaviours between two males
	Ferguson *et al.* (2000)	Impaired social memory, but intact spatial memory
	Winslow *et al.* (2000)	Increased aggression towards intruders compared to wild type
	Crawley *et al.* (2007)	Normal social recognition and normal sociability
	Lazzari *et al.* (2019)	Reduced aggression and increased social interaction compared to wild-type

Table 1.4: Studies investigating the effect of both OXY and OXTR knockout on non-reproductive social behaviours in mice**.**

Central OXY administration has also been utilised to determine the role of OXY in social behaviour. Clear anti-aggressive and prosocial effects of OXY have been observed in male rats following both acute and chronic i.c.v. OXY administration (Calcagnoli *et al.*, 2013). In addition, i.c.v. OXY can restore deficits in social preference that arise from social defeat in rats (Lukas *et al.*, 2011). Central administration of OXTR antagonists (desGly-NH_2,d$(CH_2)_5$[Tyr(Me)^2,Thr^4]OVT and vasotocin) have also demonstrated a prominent role for OXY in social behaviours. I.c.v. administration of OXTR antagonists can impair social memory when administered immediately after memory acquisition, reduce social interaction between novel male rats and mice, and impair social recognition (Lukas *et al.*, 2011; Lukas *et al.*, 2013; Samuelsen & Meredith, 2011). In addition, i.c.v. infusion of the OXTR antagonist, tocinoic acid, can attenuate 3,4-methylenedioxymethamphetamine (MDMA)-induced increases in social behaviour in Wistar rats, although it had no effect when administered alone (Thompson *et al.*, 2007).

Although there is controversy and debate over the extent of BBB penetration, systemic OXY administration has been widely utilised to examine the effects of OXY on social behaviour, and intranasal, intraperitoneal (i.p.), and s.c. OXY administration have all been shown to increase brain OXY levels (Neumann *et al.*, 2013; Yamamoto *et al.*, 2019). Justifying this approach in the current thesis, both s.c. and i.p. OXY can increase social interaction between two male rats (Kent *et al.*, 2016; Kohli *et al.*, 2019; Ramos *et al.*, 2013). In addition, in rats i.p. OXY increases social huddling in response to threat stimuli and enhances social preference (Bowen & McGregor, 2014; Ramos *et al.*, 2016). Following intranasal administration OXY can increase social interaction between two male Sprague Dawley rats, primarily increasing sniffing and following behaviours (Kent *et al.*, 2016). Similarly, Calcagnoli *et al.* (2015a) demonstrated increased social interaction between two novel male rats, as well as reduced aggression following intranasal OXY. Furthermore, Gulevich *et al.* (2019) demonstrated reduced aggression in rats deemed to be naturally aggressive following five days intranasal OXY administration. Intranasal OXY can also restore social deficits that arise from a single prolonged stressor in male rats, used as a model for post-traumatic stress disorder (Wang *et al.*, 2019).

OXY is also thought to play a role in development and contributes to the expression of normal social behaviours in adulthood (Lukas *et al.*, 2010; Nardou *et al.*, 2019). During development GABAergic activity shifts from being excitatory to inhibitory, due to reduction in the concentration of intracellular chloride during parturition (Tyzio *et al.*, 2014; Zhang *et al.*, 2020). It is thought OXY activity may contribute to this shift, through increasing the stability of the potassium-chloride transporter KCC2 and upregulating its activity, in order to maintain low intracellular chloride (Leonzino *et al.*, 2016; Tyzio *et al.*, 2006; Tyzio *et al.*, 2014). Pre-treating maternal rats with an OXTR antagonist before parturition abolishes the excitatory to inhibitory GABA shift and produces some behavioural changes in their off-spring associated with ASD models, such as increased USV emission upon maternal separation (Tyzio *et al.*, 2014). However, this paper did not determine whether these changes persisted beyond the first developmental stage, or whether abolishing the excitatory to inhibitory GABA shift impacted on platonic social interaction. It is of interest this

excitatory-inhibitory shift is abolished in two rodent models of ASD (valproate and fragile X) (Roux *et al.*, 2018; Tyzio *et al.*, 2006), both of which have profound deficits in social behaviour (Bambini-Junior *et al.*, 2011; de Esch *et al.*, 2015; Gantois *et al.*, 2013; Mines *et al.*, 2010; Nicolini & Fahnestock, 2018). Administration of bumetanide, which potentiates GABA activity and alters the balance of excitatory-inhibitory activity in the brain, can reduce the core symptoms of ASD in both children and animal models of ASD (Tyzio *et al.*, 2014; Zhang *et al.*, 2020). Therefore, OXY likely plays an important role in development, with deficits leading to impaired social behaviour, as well as contributing to the aetiology of ASD.

1.4.2 Clinical evidence

Clinical OXY research has been slow given the fact OXY does not easily cross the BBB and i.c.v. administration in humans is not ethically viable. The discovery that intranasal administration of neuropeptides can achieve direct access to the CSF sparked significant interest in the OXY field and has paved the way for numerous clinical studies investigating the effect of intranasal OXY on social cognition and behaviour (Born et al., 2002). However, the nasal epithelium and BBB are highly effective at preventing large quantities of OXY entering the brain following intranasal administration. As such, supramaximal doses of OXY are often administered to counteract the poor penetration (Leng & Ludwig, 2016). The first study demonstrating a prosocial effect of OXY in humans was by Kosfeld *et al.* (2005). This paper showed intranasal OXY increased the willingness to trust others in a money game and was supported by another group later that year (Kosfeld *et al.*, 2005; Zak *et al.*, 2005). Furthermore, Baumgartner *et al.* (2008) demonstrated those treated with OXY remain trusting even after having their trust betrayed on multiple occasions. However, a recent, large, double-blinded placebo-controlled replication of Kosfeld's 2005 study found no effect of OXY on trusting behaviours (Declerck *et al.*, 2020). Other OXY research in healthy individuals has shown both males and females judge faces to be more attractive and trustworthy following OXY administration (Theodoridou *et al.*, 2009), and Mikolajczak *et al.* (2010) reported OXY treated

participants rate happy faces as more trustworthy than participants who received placebo.

OXY has also been implicated in feelings of empathy. Elevated levels of OXY are seen in those watching videos of emotional scenes compared to those watching emotionally neutral scenes (Barraza & Zak, 2009), and intranasal OXY can enhance emotional empathy by altering visual attention towards emotional faces, as opposed to other contextual cues (Le *et al.*, 2020). However, other groups have found little effect of OXY on feelings of empathy in healthy individuals (Bartz *et al.*, 2019). Potential anxiolytic actions of OXY have also been reported from clinical studies (Dou *et al.*, 2021). Numerous functional magnetic resonance imaging (fMRI) studies have shown reduced amygdala activation in response to fearful faces following intranasal OXY (Domes *et al.*, 2007; Gamer *et al.*, 2010; Kirsch *et al.*, 2005). More recently, Radke *et al.* (2017) demonstrated intranasal OXY administration to healthy males reduces amygdala activation in response to threatening social stimuli, again supporting a potential anxiolytic effect of OXY. As with preclinical work, social recognition has also been studied in humans following OXY administration. Although findings are inconsistent, generally intranasal OXY is thought to facilitate the recognition of human facial expressions and emotions (Guastella *et al.*, 2008a; Lischke *et al.*, 2012; Rimmele *et al.*, 2009). For example, Savaskan *et al.* (2008) found intranasal OXY increased the identification of angry and neutral faces, while in contrast, Marsh *et al.* (2010) only found a positive effect of intranasal OXY upon the identification of happy faces. Similarly, Guastella *et al.* (2008b) found healthy males that had received intranasal OXY were more likely to recall previously seen pictures of happy faces compared with angry and neutral faces.

The relationship between OXY and psychiatric disorders has also been explored. In females with schizophrenia higher levels of plasma OXY are associated with less severe positive symptoms, as well as increased prosocial behaviours (Rubin *et al.*, 2010). In male patients with schizophrenia a correlation between CSF OXY levels and negative symptoms has also been observed (Sasayama *et al.*, 2012). In addition, some genetic links have also been identified. Two single nucleotide polymorphisms (SNP) (rs53576 and rs2740204) within the OXTR gene are associated with schizophrenia,

where carriers exhibit fewer social skills (Montag *et al.*, 2013). The rs53576 and rs2254298 OXTR SNPs have also been associated with ASD (Jacob *et al.*, 2007; Wu *et al.*, 2005). With the abundance of preclinical evidence, genetic associations, and work in healthy human populations, numerous randomised control trials (RCTs) have studied the effect of OXY in schizophrenia and ASD patient populations to determine whether OXY has any therapeutic effects upon social deficits.

Despite extensive work examining the effects of intranasal OXY upon social deficits in patient groups, findings have been inconsistent. A meta-analysis of seven double-blind, placebo-controlled, RCTs found that intranasal OXY did not differ from placebo in regards to positive and negative symptoms, however it was able to reduce scores on the Positive and Negative Syndrome Scale (PANSS) general psychopathology subscale in patients with schizophrenia, which includes items such as depression and anxiety (Oya *et al.*, 2016). However, a later meta-analysis of the same RCTs, plus an additional RCT published after Oya *et al.* (2016), found daily intranasal OXY did not improve positive symptoms, negative symptoms, or general psychopathology in patients with schizophrenia, as measured by the PANSS (Williams & Bürkner, 2017). A further meta-analysis by Zheng *et al.* (2019) also found no effect of intranasal OXY on positive, negative, and general symptom scores, although they did find a positive effect of high dose (80 international units (IU)) intranasal OXY; improving general psychopathology and positive symptoms (Zheng *et al.*, 2019). Consistent with this, a 2021 meta-analysis looking at the effect of intranasal OXY on schizophrenia negative symptoms found no effect of treatment, though there was moderate effect of high dose intranasal OXY (40-80 IU) on the improvement of negative symptoms. However, this effect disappeared after the exclusion of one study (Sabe *et al.*, 2021). A recent meta-analysis by Martins *et al.* (2021) also examined the effect of repeated intranasal OXY on the core symptoms of schizophrenia and ASD. This group also found no significant effect of intranasal OXY treatment, asides from a small effect on general psychopathology in schizophrenia patients, again measured by the PANSS (Martins *et al.*, 2021). Interestingly, they found increased variance in the response of schizophrenia negative symptoms to intranasal OXY, suggesting there maybe subgroups of patients with schizophrenia that may successfully respond to OXY

treatment and improve social deficits, while other subgroups are non-responsive. This would explain some of the inconsistent findings across numerous RCTs.

When looking at the effect of intranasal OXY on specific aspects of social cognition in patients with neurodevelopmental disorders, such as schizophrenia, as opposed to general negative symptoms, two meta-analyses have found intranasal OXY has large effects on 'high-level' social cognition (theory of mind and metalizing; both of which involve inferring and understanding the emotions of others) (Bürkner *et al.*, 2017; Keech *et al.*, 2018). This suggests OXY may improve certain aspects of social cognition in specific subgroups of populations; making it hard to draw consistent conclusions about the effects of intranasal OXY in clinical populations. The variances in findings could also be attributed to a variety of other reasons. Firstly, the poor penetration of OXY across the BBB and nasal epithelium may prevent consistent doses of OXY being administered across groups. To overcome this conjugation of OXY to novel cell-penetrating peptides has been trialled in rats, aiming to improve OXY penetration of the nasal epithelium and BBB (Wong et *al*., unpublished). However, it is unknown whether OXY remains biologically active following conjugation to cell-penetrating peptides. Therefore, the first chapter in this thesis focuses on whether OXY conjugates are biologically active and have the potential to be used for the remainder of the behavioural work in this thesis; examining the neural mechanisms that underlie the behavioural effects of systemic OXY. Secondly, OXYs effects on behaviour may be moderated by contextual or individual factors. For example, the trusting effects of OXY are diminished if the other person is not portrayed as trusting (Declerck *et al.*, 2010), and OXY differentially effects both males and females on social perception tasks (Fischer-Shofty *et al.*, 2013). Finally, a 2016 review concluded that clinical studies looking at intranasal OXY are generally statistically underpowered, thus meaning it is possible published findings do not represent the true effects of intranasal OXY on behaviour (Walum *et al.*, 2016).

1.5. Rationale and aims of the thesis

Disorders with social deficits, such as schizophrenia, currently have no effective treatment that targets the social symptoms of these disorders. Social deficits have great effects on patients' abilities to integrate into society and have huge impacts on functional recovery. There is a huge unmet clinical need for the development of drugs to treat these symptoms, as well as gaps in our understanding of the neural mechanisms that contribute to both healthy and aberrant social behaviour.

OXY has shown promise in both clinical and preclinical studies; increasing social recognition, reward, and interaction. In addition, OXY can have antipsychotic-like effects in preclinical models, making it a prime candidate for the treatment of schizophrenia, either alone or as an adjunct with antipsychotics. However, numerous preclinical studies have used i.c.v. OXY administration, lacking translational relevance. The neural mechanisms through which OXY has these behavioural effects are also poorly understood. Therefore, primary studies in this thesis will determine whether a novel OXY conjugate is biologically active, and whether it can be used to enhance intranasal OXY administration, which avoids the systemic effects of OXY. Subsequent chapters will use selective antagonists and a selective toxin to determine the receptors and brain regions involved in the prosocial and antipsychotic-like effects of systemic OXY administration. Thus, the overall aims of this thesis are to gain a deeper understanding of the neural mechanisms which modulate OXYs prosocial and antipsychotic-like effects in rats following systemic administration.

Chapter 2: Development of an Oxytocin Receptor Bioassay

2.1 Introduction

Both preclinical and clinical research has shown intranasal OXY administration can enhance prosocial behaviours, gaining interest for its use as a potential adjunctive therapy for treating social deficits in disorders such as schizophrenia and ASD. Intranasal administration is significantly less invasive than other methods of OXY administration, making it a highly favourable clinical option. However, both the nasal epithelium and BBB are highly effective at preventing the penetration of large molecules (>400 Daltons), hindering the therapeutic utility of intranasal OXY (Dal Monte *et al.*, 2014; Ermisch *et al.*, 1985; Fisher *et al.*, 1987; McMartin *et al.*, 1987; Meisenberg & Simmons, 1983). Whether OXY enters the brain following intranasal administration remains controversial, with studies showing as little as 0.002% of intranasally administered OXY successfully reaching the brain (Leng & Ludwig, 2016; Mens *et al.*, 1983). In both clinical and preclinical research supramaximal doses of OXY are administered in an attempt to elevate brain OXY in regions implicated in social behaviour, such as the VTA and NAc (Dolen *et al.*, 2013; Smith *et al.*, 2017b). Recent studies in rats have administered intranasal doses as high as 20µg OXY in an attempt to elicit behavioural changes; 20 times the total OXY content of the rat posterior pituitary (Joushi *et al.*, 2021; Leng & Ludwig, 2016; Neumann *et al.*, 2013; Yang *et al.*, 2019). For intranasal OXY to become a viable clinical option in the treatment of social deficits the efficacy of intranasal administration needs improving, to allow more targeted and efficient delivery of OXY to the brain. For intranasally administered drugs to successfully reach the brain they must first cross the nasal epithelium (Kamei & Takeda-Morishita, 2015). Therefore, one method to improve intranasal OXY administration is the conjugation of OXY to cell-penetrating peptides, to increase penetration of the nasal epithelium. Glycosaminoglycan (GAG)-binding enhanced transduction (GET; P21-LK15-8R) is a novel cell penetrating peptide that

can enhance small molecule transduction across epithelial layers, such as the nasal epithelium (Dixon *et al.*, 2016).

GET carrier peptides electrostatically conjugate to OXY to form an OXY-GET conjugation, which aims to enhance penetration across membranes such as the nasal epithelium and the BBB. P21, a 21 amino acid sequence from heparin-binding epidermal growth factor, interacts with cell membrane heparan sulphates to increase cell interaction, while the arginine-rich GET domain mediates high-efficiency transduction (Thiagarajan *et al.*, 2017). Following interactions with cell membrane heparan sulphates, the cationic arginine-rich domain of GET interacts with anionic phospholipids of cell membranes (Layek *et al.*, 2015). This causes invagination of the cell membrane; leading to endocytosis of the conjugated cell-penetrating peptide (Rehmani & Dixon, 2018). Endocytosis is the first step of transcytosis; the transfer of molecules from one side of the cell to the other, i.e. apical to basolateral (Rehmani & Dixon, 2018). However, the precise mechanisms through which GET conjugations cross the cell membrane is unknown, including the processes that mediate vesicular transfer and exocytosis. Although the mechanisms are unknown, multiple variants of GET peptides have been shown to increase the transduction of insulin across the intestinal epithelium and alveolar epithelium (Liang & Yang, 2005; Patel *et al.*, 2009; Rehmani & Dixon, 2018). In addition, cell-penetrating peptides have also demonstrated successful insulin delivery across the nasal epithelium to the brain following intranasal administration, with the insulin-complex accumulating in areas such as the olfactory bulb and brainstem (Kamei & Takeda-Morishita, 2015). Thus, conjugation of OXY to GET (OXY-GET) shows promise as a potential mechanism to improve the uptake and delivery of intranasal OXY to the brain, thereby increasing CNS penetration and behavioural effects. However, a previous *in vivo* test in rats demonstrated no significant effect of either intranasally administered OXY, or OXY-GET, on locomotor activity (LMA) or body temperature (Wong et.al, unpublished). Having an assay to monitor *in vitro* OXTR activation following the application of drugs can allow us to determine the biological activity of unknown compounds, such as the OXY-GET conjugations, and compare these to selective receptor agonists with known receptor affinity and efficacy. Determining whether the OXY-GET compounds are

biologically active and able to stimulate the OXTR will give greater insights into the potential therapeutic utility of OXY-GET conjugates.

To measure *in vitro* OXTR activation calcium fluorimetry assays can be utilised due to the rapid rise in intracellular calcium that occurs following $G\alpha_q$ coupled OXTR activation. Calcium is a ubiquitous intracellular messenger that controls, or influences, almost every cellular process. Following rises in cytosolic calcium a variety of cellular events are initiated. In neurosecretory cells rapid rises in intracellular calcium control cell excitability, modulating firing patterns and neurotransmitter release (Delaney & Lin, 2020). In contrast, calcium release over a longer period of time can promote gene transcription (Augustine, 2001; Berridge, 1995; Greer & Greenberg, 2008). Prolonged high concentrations of free intracellular calcium are cytotoxic, therefore the resting intracellular calcium concentration in cells is tightly controlled and remains low; maintained through activity of the plasma membrane calcium ATPase and the sodium/calcium exchanger (Bagur & Hajnóczky, 2017). Due to the tight control of intracellular calcium it is a very sensitive cellular indicator; small changes in concentration in either direction have large effects upon the cell. Following $G\alpha_q$ coupled OXTR activation inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) are produced (Gimpl & Fahrenholz, 2001), activating IP_3 receptors on the endoplasmic reticulum; in turn causing the release of calcium into the cytosol (Kania *et al.*, 2017). Thus, calcium fluorimetry assays can be utilised to quantify OXTR-activated rises in intracellular calcium.

Calcium-sensitive fluorescent dyes, such as Fluo-4 acetoxymethyl (AM), are used to detect intracellular calcium. The lipid-soluble AM ester group enables diffusion across the cell membrane (Caers *et al.*, 2014). Once in the cytoplasm esterases cleave the AM group, leaving a negatively charged fluorophore inside the cell. Upon OXTR activation, and subsequent intracellular calcium release, positively charged calcium ions bind to the negative fluorophore. When Fluo-4 is bound to calcium, excitation at 485 nanometres (nm) causes a fluorescent emission at 525nm. Thus, the measurement of emissions at 525nm can be used as a quantitative index of the rise in cytoplasmic calcium (Figure 2.1) (Caers *et al.*, 2014). Calcium responses observed following OXY application are transient; typically reaching a peak after a few seconds,

then steadily declining. However, the time to the peak emission is variable, and dependent on the degree of receptor activation; higher agonist concentrations typically achieving peak responses more rapidly. Generally, the peak emission is used as a reflection of receptor activation; a greater peak in emission reflecting greater receptor activation (Charlton & Vauquelin, 2010).

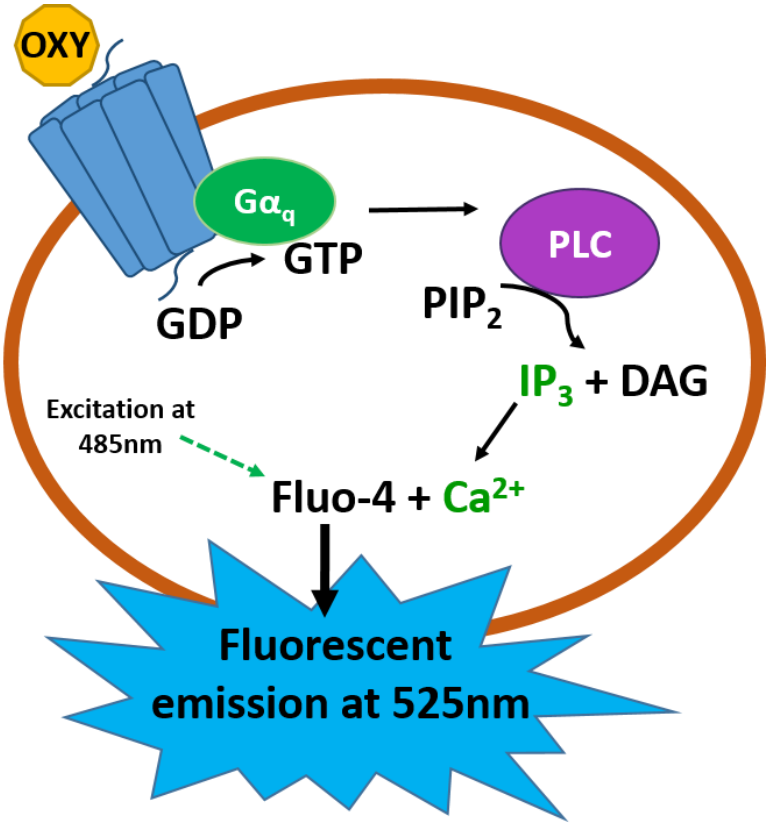

Figure 2.1: Schematic diagram of the intracellular cascade of events leading to Fluo-4 emission. OXY activation of the $G\alpha_q$ coupled OXTR causes a conformational change, converting GDP to GTP. Binding of GTP dissociates the $G\alpha_q$ subunit from the OXTR, activating PLC, which in turn hydrolyses phosphatidylinositol bisphosphate (PIP_2) to DAG and IP_3. IP_3 then activates IP_3 dependent channels on the endoplasmic reticulum, causing rapid release of calcium into the cytoplasm. Binding of calcium to the negatively charged Fluo-4 molecule causes a change in fluorescent emission, from 480nm to 525nm, as measured by the FlexStation 3. Image adapted from Caers *et al.* (2014).

Determining whether OXY-GET conjugations can activate the OXTR *in vitro* may provide an explanation as to why no *in vivo* effect of the OXY-GET conjugations was observed. Intranasal administration is a clinically preferred route of administration,

therefore determining whether OXY-GET conjugations can agonise the OXTR will help determine whether intranasal OXY-GET application is a viable method for future studies in this thesis, aiming to determine the neural mechanisms of OXYs behavioural effects.

2.2 Aims

The aim of this chapter was to A) develop a sensitive *in vitro* calcium fluorimetry assay to determine biological activity at the OXTR and to B) determine whether OXY-GET conjugations are able to activate the OXTR and can be utilised in future *in vivo* studies to determine the neural mechanisms of OXY.

2.3 Materials and methods

2.3.1 Cell culture

Multiple cell lines expressing the functional OXTR were initially selected and trialled to determine the best *in vitro* method to evaluate OXTR activity. HEK293T cells stably expressing the OXTR were obtained from The Centre of Membrane Proteins and Receptors, University of Nottingham, School of Life Sciences. Cells were cultured in high glucose Dulbecco's Modified Eagle Medium serum (DMEM; Sigma Aldrich, Poole, UK) supplemented with 10% foetal calf serum (FCS).

Michigan Cancer Foundation-7 (MCF-7) cells were obtained from the Mongan Laboratory, University of Nottingham, School of Veterinary Medicine and Science. Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium with L-glutamine and sodium bicarbonate (Sigma Aldrich, Poole, UK), supplemented with 10% FCS.

Hs 578T cells were obtained from the Grabowska Laboratory, University of Nottingham, School of Medicine. Cells were cultured in DMEM, supplemented with 10% FCS and 0.01mg/mL bovine insulin (Sigma Aldrich, Poole, UK). Both MCF-7s and Hs 578T cells were selected due to their endogenous expression of the OXTR (Amico *et al.*, 2002; Copland *et al.*, 1999).

All cells were grown at 37°C with 5% CO_2 in a humidified atmosphere and passaged regularly once 80% confluent. Twenty-four hours prior to undertaking the calcium fluorimetry assay cells were seeded into a black walled, clear bottomed, 96 well plate at a density of 80,000 cells per well, in a volume of 100µL. Cells were left to grow overnight.

2.3.2 Drug preparation

All drugs were added (20µL drug to 100µL cell suspension) to cells from a 96 well compound plate. Drugs were made up to six times the final concentration to account for a 1 in 6 dilution upon administration to cells.

OXY acetate (Bachem, St Helens, UK) was diluted from powder in assay buffer (1X Hanks Balanced Salt Solution (HBSS), 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) with 0.1% BSA) and serial dilutions performed to give required concentrations based on the known EC_{50} of OXY in HEK293 cells, ~2.1nM (Tahara *et al.*, 2000).

GET peptides (10mM) and heparin (10mg/mL) were obtained from Dr. James Dixon, University of Nottingham, School of Pharmacy. Both were diluted in assay buffer to the desired concentration. For OXY-GET peptides GET was always conjugated to 0.1µM OXY, regardless of the GET concentration.

Carbachol was used as a positive control to activate the muscarinic M_3 receptor, endogenously expressed in HEK293T cells and MCF-7 cells (Vetter & Lewis, 2010). As with the OXTR, the muscarinic M_3 receptor is also coupled to the $G\alpha_q$ G-protein and

activates intracellular calcium release upon activation (Robert *et al.*, 2020). Carbachol (Sigma Aldrich, Poole, UK) was diluted from solid in assay buffer.

The OXTR antagonist, (2*S*)-2-Amino-*N*-[(1*S*,2*S*,4*R*)-7,7-dimethyl-1-[[[4-(2-methylphenyl)-1-piperazinyl]sulfonyl]methyl]bicyclo[2.2.1]hept-2-yl]-4-(methylsulfonyl)butanamide (L-368,899), was obtained from Tocris Bioscience, Bristol, UK, and the V1aR antagonist (2*S*)-1-[[(2*R*,3*S*)-5-Chloro-3-(2-chlorophenyl)-1-[(3,4-dimethoxyphenyl)sulfonyl]-2,3-dihydro-3-hydroxy-1*H*-indol-2-yl]carbonyl]-2-pyrrolidinecarboxamide (SR49059) and V2 receptor antagonists N-[4-[(7-Chloro-2,3,4,5-tetrahydro-5-hydroxy-1H-1-benzazepin-1-yl)carbonyl]-3-methylphenyl]-2-methylbenzamide (Tolvaptan) were obtained from Sigma Aldrich, Poole, UK. All antagonists were initially diluted from solid in 5% dimethyl sulfoxide (DMSO, Sigma Aldrich, UK) and assay buffer. Further dilutions were undertaken with assay buffer. The DMSO concentration that the cells were subjected to was antagonist concentration dependent, but never exceeded 0.005%. All antagonists were selected due to specificity at their respective receptors, and doses were selected based around their known K_i (see Table 1.2), obtained from The Concise Guide to Pharmacology: G protein-coupled receptors 2017/2018 (Akerlund *et al.*, 1999; Alexander *et al.*, 2017; Jasper *et al.*, 1995; Yamamura *et al.*, 1998).

Cells were incubated with antagonist for 40 minutes prior to the addition of agonists, allowing equilibrium between the receptor and antagonist to be attained. Concentrations and incubation times were based upon previous literature (Pont *et al.*, 2012).

2.3.3 Calcium fluorimetry assay

To assess whether the OXY-GET peptides were biologically active a calcium fluorimetry assay was used to measure intracellular calcium responses following administration of selected test peptides. The Fluo-4 No Wash Calcium Assay Kit F36206 (ThermoFisher Scientific, UK) was used to measured calcium mobilisation on the FlexStation 3 machine (Molecular Devices, UK) because the fluorescent dye, Fluo-

4 AM, has high affinity for calcium, therefore allowing the detection of subtle changes in calcium concentration.

One bottle of Fluo-4 NW dye mix was equilibrated to room temperature and combined with 12mL of assay buffer and 120µL probenecid stock solution, contained within the Fluo-4 kit; giving a final probenecid concentration of 2.5mM. Presence of probenecid prevents the active transport of Fluo-4 dye out of the cells (Caers *et al.*, 2014). Medium was removed from the cell culture 96 well plate. Care was taken to only aspirate media and not to disrupt adhered cells. 100µL of the Fluo-4 calcium assay buffer was added to each well and incubated at 37°C for 40 minutes prior to commencing the assay.

Agonists were added via a computer using the FlexStation 3 and Softmax Pro Software (Molecular Devices, UK). Emitted fluorescence was measured every 1.6 seconds for 90 seconds. 20µL of drug (or assay buffer in control wells) was added after 16 seconds. Emission was measured at 525nm in either duplicates or triplicates, with excitation at 485nm. Each experiment repeated at least three times, unless otherwise stated.

2.3.4 Data analysis

All data is shown as mean with standard error of the mean (SEM), unless otherwise stated. Maximal emission was calculated as the maximum minus the minimum raw fluorescent units (RFU), as obtained from the Softmax Pro Software readout (Figure 2.2).

Where stated, data in dose-response curves were normalised, using the control OXY data to define 0% and 100%. Data was normalised in order to increase the accuracy of EC_{50} estimates (Weimer *et al.*, 2012). Data were fitted to sigmoidal dose-response curves by GraphPad Prism (Version 7.04 for Windows), and curves were fitted to a Hill Slope of 1. A Hill Slope of 1 was selected as the antagonists used in this chapter, SR 49059, L-368,899 and tolvaptan, are thought to display independent binding at

the OXTR and are not known to display any positive or negative receptor cooperativity (Alexander *et al.*, 2019; Manning *et al.*, 2012; Manning *et al.*, 2008).

One-way analysis of variance (ANOVA) was selected to analyse data; aiming to determine whether there were any statistically significant differences between the five independent treatment groups.

Prior to analysis it was confirmed that all one-way ANOVA test assumptions were satisfied: one dependent variable that is measured at the continuous level (fluorescence, RFU), one independent variable that consists of two or more categorical, independent groups (treatment), independence of observations, no significant outliers, homogeneity of variance (using Levene's test), and the dependent variable is normally distributed for each group of the independent variable (using Shapiro-Wilk's test). Following the one-way ANOVA, if there was a significant interaction, Dunnett's post-hoc test was performed. Dunnett's post-hoc was selected to determine which different treatment groups (OXY-GET conjugations) significantly differed from the single control group (OXY alone). Both the one-way ANOVA and subsequent post-hoc tests were performed on GraphPad Prism. $p<0.05$ was considered statistically significant.

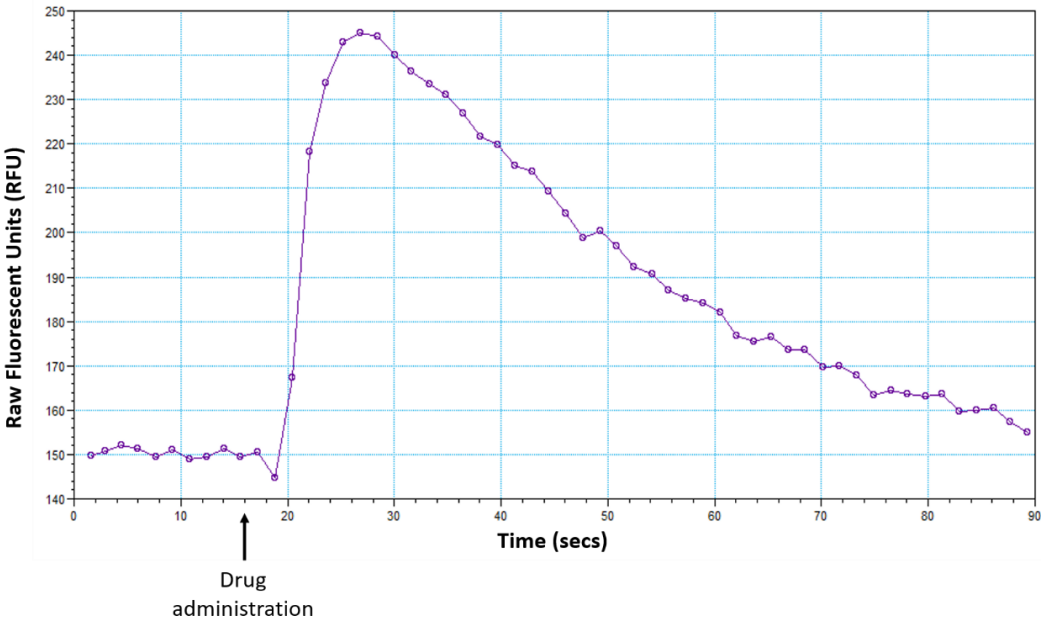

Figure 2.2: Representative trace obtained from the FlexStation 3 and Softmax Pro Software, showing a rapid rise in fluorescent emission following drug administration, caused by increased cytosolic calcium, which reaches a peak after a few seconds and then steadily declines.

2.4 Results

2.4.1 Selecting a cell line

Preliminary studies developed the calcium assay on both transiently and stably transfected HEK293 cells overexpressing the OXTR. A large response to carbachol was seen in all HEK293 cells (EC_{50} = 8.45 x 10^{-7} M), confirming a functional calcium assay signalling response could be obtained. No response was seen to OXY on any strain of HEK293 cells (see representative data, Figures 2.3A and 2B).

Breast cancer tumour MCF-7 cells were therefore selected for further use due to endogenous OXTR and muscarinic M_3 receptor expression (Ito *et al.*, 1996; Negroni *et al.*, 2010). A dose-related rise in intracellular calcium was evoked by both carbachol (EC_{50} = 1.47 x 10^{-5}M) and OXY (EC_{50} = 1.17 x 10^{-6}M). However, the carbachol response was much smaller and less potent than seen in HEK293 cells, possibly due to low endogenous expression of the muscarinic receptors and OXTRs (Figures 2.3C and 2D).

Due to the small response observed in MCF-7 cells, Hs 578T cells were subsequently obtained; another breast cancer tumour cell line also known to endogenously express the OXTR, with published evidence suggesting an operant calcium signal (Gimpl & Fahrenholz, 2001). A potent response was seen following OXY administration in Hs 578T cells (EC_{50} = 2.6 x 10^{-8}M), with double the maximum emission seen in MCF-7 cells, thus were selected for use throughout the rest of the *in vitro* calcium assay (Figures 2.3E and 2F).

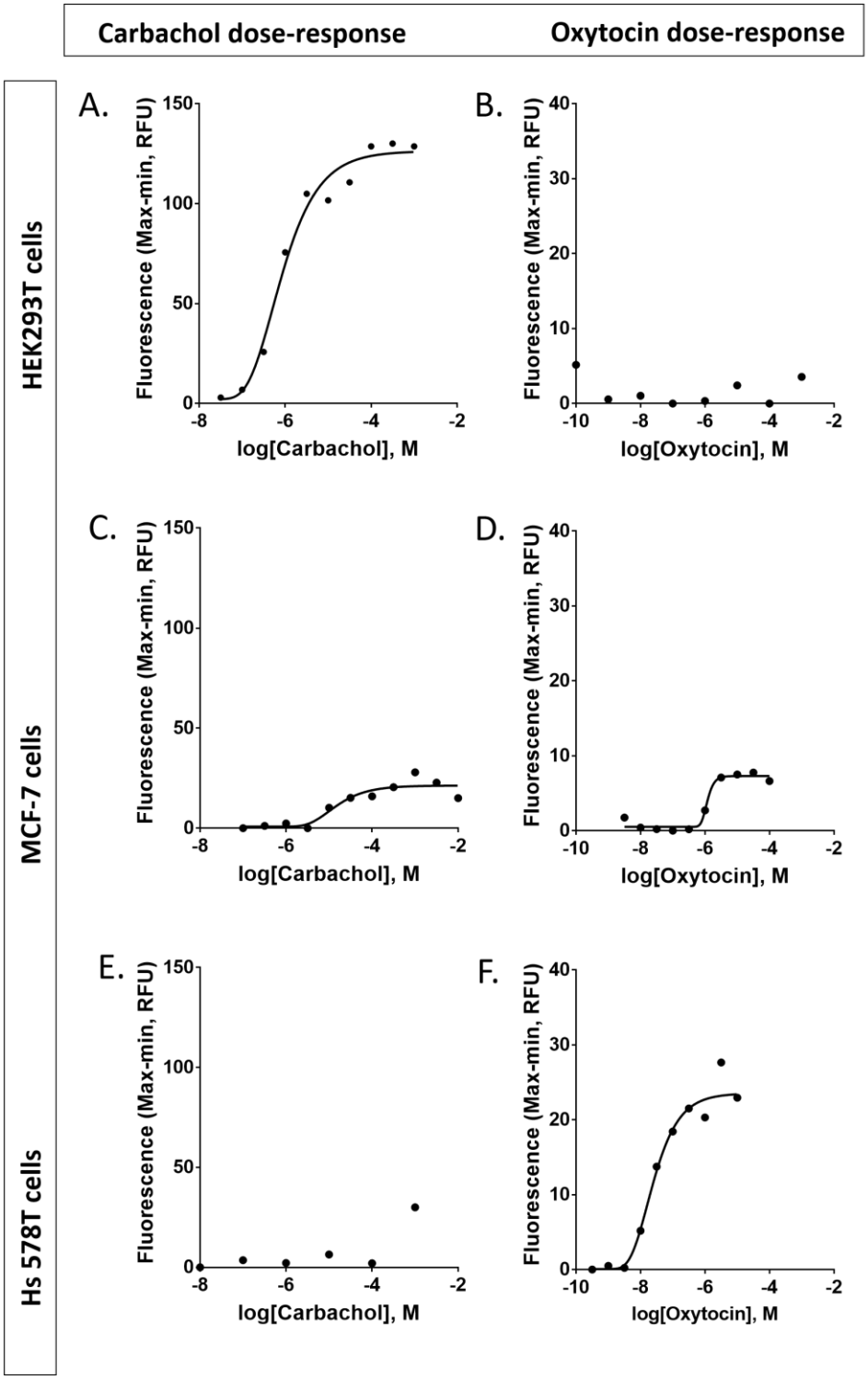

Figure 2.3: OXY and carbachol dose-response curves obtained in three different cell lines, with Hs 578T cells demonstrating the greatest response to OXY (N=1, mean of triplicate recordings). **(A)** A large and dose-related to carbachol was seen in HEK293T cells overexpressing the OXTR (EC_{50} = 8.45 x10^{-7} M). **(B)** No response to OXY was seen in OXTR expressing HEK293T cells. **(C+D)** Small responses to both carbachol (left, EC_{50} = 1.47 x 10^{-5}M) and OXY (right, EC_{50} = 1.17 x 10^{-6}M) were seen in MCF-7 cells. **(E+F)** No response to carbachol was seen in Hs 578T cells at the highest dose of carbachol, but a large and potent response to OXY was seen (EC_{50} = 2.6 x 10^{-8} M). Data shown as raw fluorescent units (RFU) from the Flexstation3 readout, measured at 525nm.

2.4.2 Confirmation of OXY response

To ensure the response to OXY observed in Hs 578T cells was due to OXTR activation a highly selective OXTR antagonist was used, L-368,899 (Alexander *et al.*, 2019; Williams *et al.*, 1994b). L-368,899 produced a dose-related and significant rightward shift in the dose-response curve (One-way ANOVA, $F_{(3, 142)}$=24.48, p<0.001), typical of a competitive antagonist and as expected with L-368,899 (Figure 2.4A). The EC_{50} value for OXY alone was 228 ± 50nM.

With OXY also having affinity at both V1a and V2 receptors (Alexander *et al.*, 2019; Bichet D *et al.*, 2017), highly selective V1a and V2 receptor antagonists (SR 49059 and tolvaptan, respectively) were used to determine if activity at either, or both, of these receptors were contributing to the OXY-induced calcium response. Doses were selected based upon the K_i of both antagonists (Alexander *et al.*, 2017). No effect of either SR 49059 or tolvaptan was seen on the OXY-induced calcium response, confirming our calcium response was due to OXTR activation (One-way ANOVA of EC_{50} values, $F_{(2, 8)}$=1.01, p=0.406) (Figure 2.4B).

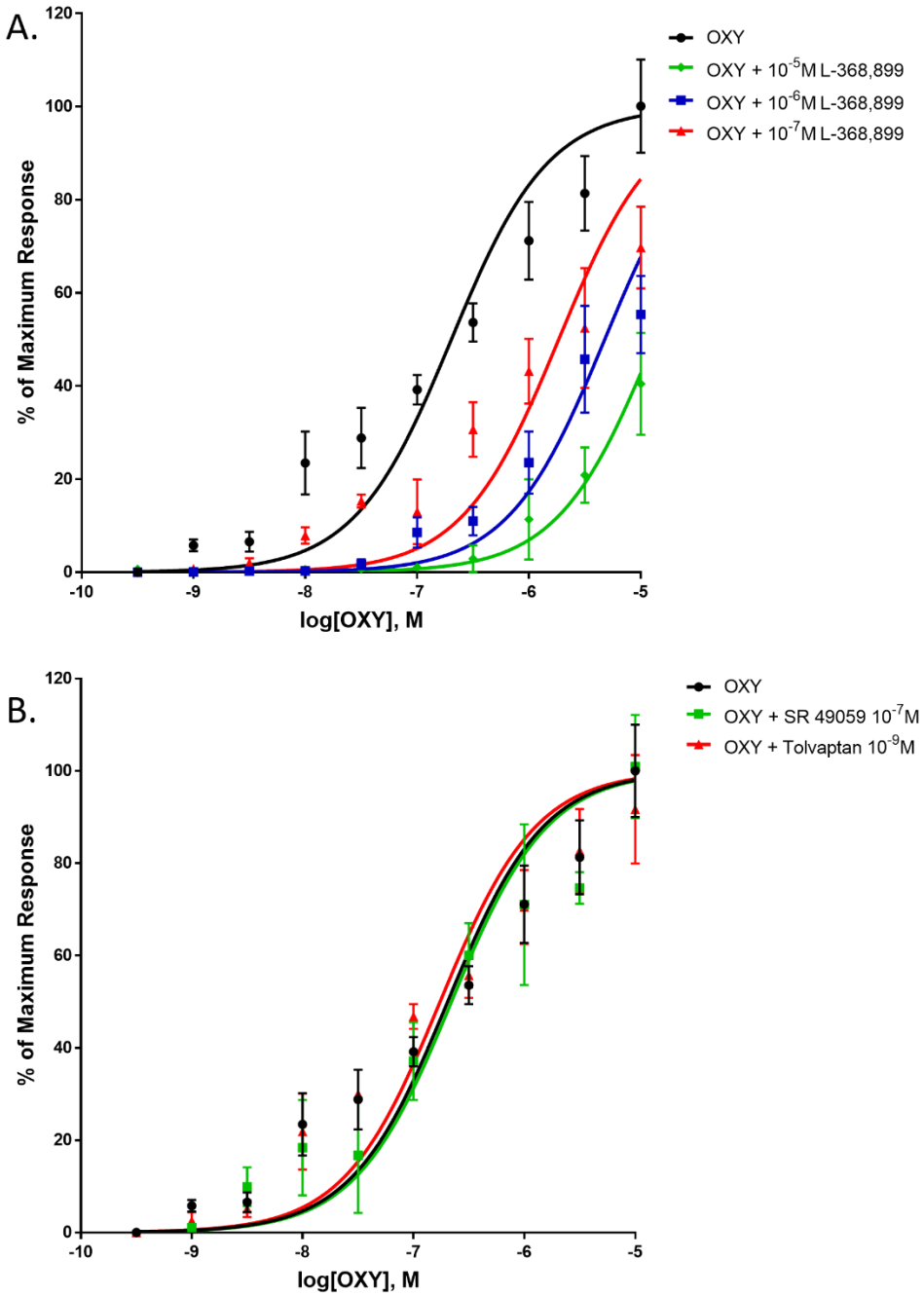

Figure 2.4: OXY dose response curves in the presence and absence of selective vasopressin and OXTR antagonists (mean ± SEM, n=3). **(A)** OXY dose response curves in the presence of increasing concentrations of L-368,899, a selective OXTR antagonist. Increasing concentrations of L-368,899 caused significant rightward shifts in the dose response curve. One-way ANOVA revealed EC_{50} ± SEM values were significantly different between treatment groups ($F_{(3, 11)}$=445.6, p<0.001). Dunnett's post-hoc revealed all concentrations of L-368,899 significantly increased the EC_{50} values when compared to OXY alone (p<0.001). **(B)** OXY dose response curves in the presence of V1aR and V2 receptor antagonists, SR 49059 and Tolvaptan. There was no significant effect of either antagonist on EC_{50} values, and no shift in dose response curves (One-way ANOVA of EC_{50} values, $F_{(2, 8)}$=1.01, p=0.406). Data normalised to the OXY control fitted to a sigmoidal dose-response curve with a Hill Slope of 1.

2.4.3 OXY-GET activity at the OXTR

Following confirmation of OXTR activity in Hs 578T cells, calcium responses after addition of both OXY (0.1µM) and OXY 0.1µM conjugated to increasing concentrations of the GET peptide were assessed. OXY-GET 1 refers to a ratio of 0.1µM OXY conjugated to 5nM GET, with each increased OXY-GET conjugation referring to a 10-fold increase in the concentration of GET conjugated to 0.1µM OXY; OXY-GET 5 being 0.1µM OXY conjugated to 50µM GET.

Increasing concentrations of GET conjugated to 0.1µM OXY significantly reduced the peak calcium response ($F_{(5,21)}$=12.33, p<0.001, Figure 2.5A). GET alone had no effect on calcium release at any concentration; indicating no endogenous OXTR activity (Figure 2.5B).

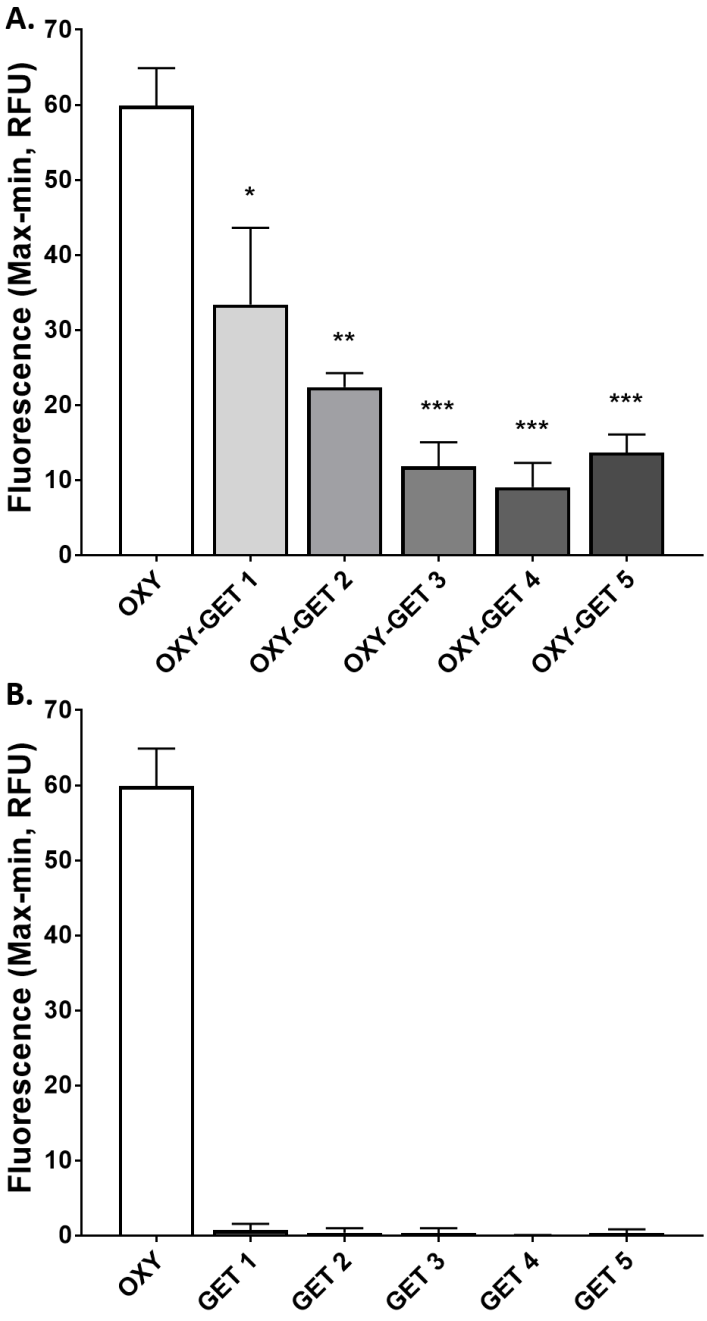

Figure 2.5: The effect of GET, and GET conjugated to 0.1µM OXY, on fluorescence emission following drug application (mean + SEM, n=3-5). A) The effect of increasing concentrations of GET conjugated to 0.1µM OXY on fluorescence emission following drug application. Increasing concentrations of GET conjugated to 0.1µM OXY significantly reduced the calcium response. One-way ANOVA analysis of OXY-GET concentrations showed a significant difference in peak emissions between treatment groups ($F_{(5,21)}$=12.33, p<0.001). There was a significant progressive reduction in peak fluorescence following conjugation of 0.1µM OXY to increasing concentrations GET, when compared to OXY alone. *** p<001. ** p<0.01 and * p<0.05 compared to 0.1µM OXY; Dunnett's post-hoc. B) The effect of increasing concentrations of GET on fluorescent emission following application. No fluorescence was observed following application of GET alone to OXTR expressing cells.

Heparin prevents the conjugation of OXY to GET by displacing OXY and preferentially electrostatically binding to GET (Dixon *et al.*, 2016). The effect of OXY-GET on evoked calcium release was repeated in the presence of 10µg/mL heparin. As expected, heparin reversed the GET-induced attenuation of the OXY response, returning the calcium response back to the level of OXY alone (Figure 2.6), with no effect of heparin on the OXY response.

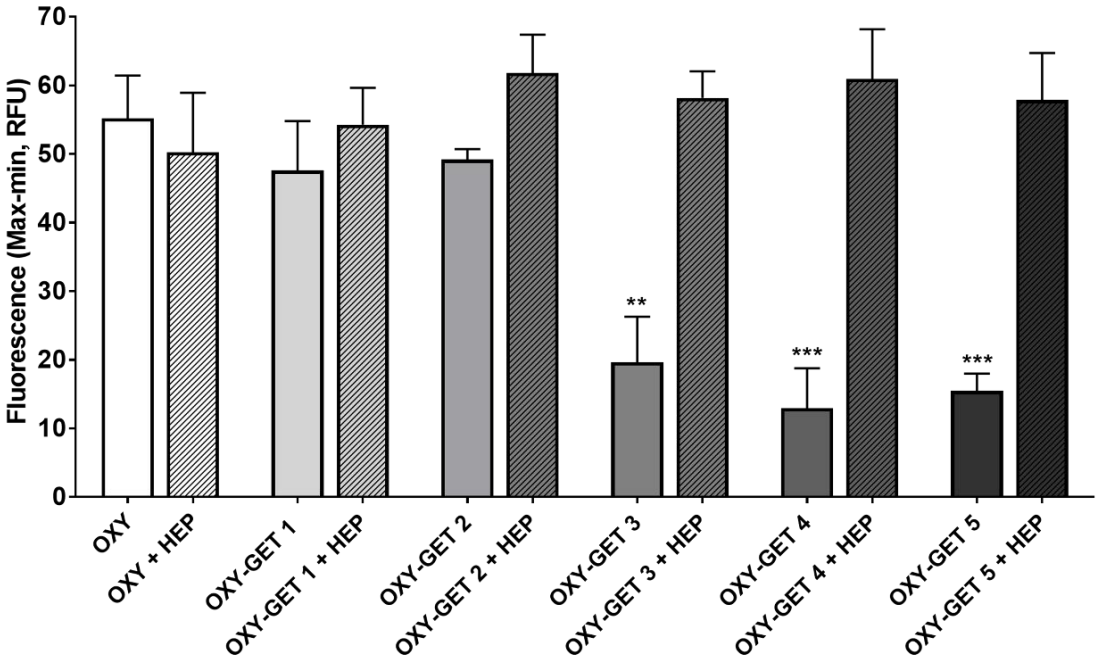

Figure 2.6: OXY-GET and OXY responses in the presence and absence of 10µg/mL heparin (HEP) (mean + SEM, n=4). One-way ANOVA showed a significant difference between treatment groups ($F_{(11,32)}$=9.281, p<0.001), with Dunnett's post-hoc showing the presence of heparin alongside OXY-GET 3, OXY-GET 4 and OXY-GET5 prevented GET conjugation from significantly reducing the fluorescence emission following application. ** p<0.01 and *** p<0.001 compared to 0.1µM OXY.

2.5 Discussion

These results demonstrated that Hs 578T cells produced a significant transient increase in intracellular calcium upon application of OXY, with the response being attenuated through the application of the selective OXTR antagonist L-368,899;

demonstrated by a significant parallel rightward shift in the dose-response curve. Although OXY can have concomitant action at vasopressin receptors, due to high sequence homology of both the peptides and their receptors, no effect of the selective V1aR and V2 receptor antagonists were seen on the calcium response to OXY (Gimpl & Fahrenholz, 2001; Rae *et al.*, 2021). This confirmed that the calcium response observed following OXY application was due the specific activation of the OXTR. This is consistent with previous literature, with Hs 578T cells showing no expression of either V1aR or V2 receptors (Copland *et al.*, 1999). Confirmation with both OXY and vasopressin receptor antagonists allowed us to be confident in concluding we had a sensitive assay to measure *in vitro* biological activity at the OXTR. Our observed OXY EC_{50} of 186 ± 50nM was 100-fold higher than previous literature conducting OXY calcium fluorimetry assays in Hs 578T cells (Schiffmann & Gimpl, 2018). However, this group were using a calcium dye with a lower affinity for calcium, reducing sensitivity to small changes in calcium concentrations, and were manipulating serum ionic concentrations, making direct comparisons difficult (Schiffmann & Gimpl, 2018).

This assay was subsequently used to determine the biological activity of the OXY-GET peptides at the OXTR. Increasing concentrations of GET conjugated to 0.1µM OXY significantly reduced the calcium response when compared to OXY alone; suggesting the GET peptides were inhibiting OXTR activation. This reduction in biological activity could explain the lack of behavioural response seen *in vivo* (Wong *et al*., unpublished). GET peptides have previously been shown to enhance transduction across cell membranes through encapsulation of the target peptide (Thiagarajan *et al.*, 2017), in this case OXY. Biological activity of GET conjugations have not previously been assessed. GET encapsulation of OXY could be hindering the binding of the conjugate to the OXTR and reducing receptor activation, by blocking the key area of the OXY peptide needed for receptor activation; the isoleucine amino acid at position 3 (Gimpl & Fahrenholz, 2001). For OXTR activation the cyclical part of OXYs structure must interact with the second transmembrane domain of the OXTR, where the amino acid critical for activation, Asp-85, is located (Gimpl & Fahrenholz, 2001). If the GET peptide was preventing the cyclical part of OXY from interacting with the Asp-85

residue of the OXTR, this would prevent receptor activation, thus explaining the reduced response seen upon OXY conjugation to GET.

The presence of heparin alongside OXY-GET conjugations showed the reversal of the OXY-GET attenuated response, increasing OXTR activation back to levels seen with OXY alone. Heparin prevents coupling of OXY to GET; thus, providing indirect evidence that OXY does conjugate to the GET molecule, something we previously had no evidence for. However, separating the OXY-GET conjugation once in the brain to allow receptor activation would be difficult, if impossible, to replicate *in vivo*.

Limitations of our calcium fluorimetry assay to assess biological activity include the lack of information about receptor affinity and efficacy. Our assay can only provide information regarding the levels of receptor activation. To gain greater information about the pharmacological profile of OXY-GET conjugates, other *in vitro* assays, such as ligand binding assays and bioluminescence resonance energy transfer, should be developed for the OXTR.

2.6 Summary and Future Work

It was concluded we had developed a sensitive assay to assess biological activity at the OXTR, and that conjugation of OXY to GET reduces OXTR activation. Future studies, undertaken by Sara Wong, will determine if the OXY-GET conjugation can enhance the transduction across a cell membrane preparation akin to the nasal epithelium. However, for the purpose of this thesis it was concluded that intranasal administration of OXY-GET conjugates is not a viable method to administer OXY *in vivo* and cannot be used to determine the neural mechanisms of OXY, due to the lack of OXTR activation. Thus, s.c. OXY was selected as the route of administration for the remainder of the *in vivo* experimental work in this thesis, which will aim to decipher the neural mechanisms underlying OXYs prosocial and 'antipsychotic-like' effect in rats.

Chapter 3: Elucidating the receptors responsible for oxytocin attenuation of PCP-induced hyperactivity and oxytocin enhanced social interaction

3.1 Introduction

Oxytocin (OXY) has affinity at both central and peripheral OXTR and V1aRs and is well documented to enhance social behaviour following acute and repeated central administration. Despite incredibly low BBB penetration, acute peripheral OXY administration can also alter social behaviour in rodents (Kohli *et al.*, 2019; Ramos *et al.*, 2013).

For peripheral OXY to enter the brain it must cross the BBB. As described in chapter 1.2.2, the RAGE is expressed on the cell surfaces of endothelial cells within neurovascular units in the brain of humans and rodents, and serves as a transporter of OXY to the brain across the BBB (Cheng *et al.*, 2005; Yamamoto & Higashida, 2020; Yamamoto *et al.*, 2019). *In vitro* models of the BBB have shown dose dependent transport of OXY across cultured endothelial cells by RAGE, and *in vivo* studies have shown knockout mice lacking RAGE do not have an increase in CSF OXY following s.c. administration, as seen in wild type mice (Munesue *et al.*, 2021; Yamamoto *et al.*, 2019). Following s.c. OXY administration Yamamoto *et al.* (2019) also found increased OXY in the third ventricle and PVN. Evidence for peripherally administered OXY to act centrally is also supported by microdialysis studies. In rats s.c OXY increases NAc dopamine, and i.p. OXY potentiates methylphenidate induced dopamine release in the NAc shell (Kohli *et al.*, 2019; Lee *et al.*, 2019a), while in mice s.c OXY significantly increases OXY in the amygdala, PVN, and CSF (Yamamoto *et al.*, 2019).

Previous research has shown peripherally administered OXY can act at both OXTRs and V1aRs in rats. High dose OXY (>0.3mg/kg) causes hypothermia when administered s.c. and i.p., and hypoactivity when administered i.p. (Hicks *et al.*, 2012; Hicks *et al.*, 2014; Klenerova *et al.*, 2009; Kohli *et al.*, 2019). While the CNS penetrant

selective OXTR antagonist L-368,899 can attenuate OXY-induced hypoactivity, the V1aR selective antagonist SR 49059 is able to reverse OXY-induced hypothermia, with no effect of L-368,899 (Klenerova *et al.*, 2009; Kohli *et al.*, 2019). In addition, regional c-Fos expression arising from a high dose of peripheral OXY administration (1mg/kg, i.p.) can be prevented through V1aR antagonism; clearly demonstrating the cross signalling between these receptors at high OXY doses (Hicks *et al.*, 2016). With affinity at both OXTRs and V1aRs it is likely that both receptor systems are contributing to the behavioural effects observed following peripheral OXY administration.

It is of significance that peripherally administered OXY can modulate social behaviour in rats. 0.1 mg/kg s.c. OXY increases social interaction in male Lister-hooded rats, primarily through increased anogenital and body sniffing (Kohli *et al.*, 2019). Similarly, 5µg i.p. OXY can also increase social interaction in male Sprague Dawley rats, again predominantly through increased sniffing behaviours (Kent *et al.*, 2016). In addition, Ramos *et al.* (2013) found 0.5mg/kg i.p. OXY reduced anogenital sniffing but increased lying side-by-side in male Long-Evans rats. Interestingly, increased adjacent lying was reversed by the co-administration of the V1aR antagonist, SR 49059 (Ramos *et al.*, 2013). In contrast, co-administration of the OXTR antagonist Compound 25 (C25) had no effect; suggesting OXY was likely mediating these social behaviours, at least in part, by V1aR activation. The same dose of OXY (0.5mg/kg i.p) also increases social huddling in rats in response to threat stimuli, such as cat fur (Bowen & McGregor, 2014). Similarly, pre-treatment with the selective V1aR antagonist SR 49059 reversed OXYs effect on social huddling, suggesting the V1aR mediates OXYs prosocial response to threat (Bowen & McGregor, 2014).

Activity at OXTRs can also modulate social behaviour in rats. Ramos *et al.* (2016) demonstrated 0.5mg/kg i.p. OXY can increase social preference in male Long-Evans rats, and pre-treatment with an OXTR antagonist (C25), but not V1aR antagonist (SR 49059), can prevent OXY-induced social preference. In addition, i.c.v. infusion of a selective OXTR antagonist (desGly-NH_2,d$(CH_2)_5$[Tyr(Me)^2,Thr^4]OVT) in male Wistar rats reduces social exploration of a novel conspecific, with no effect of a V1aR antagonist (Lukas *et al.*, 2011). Furthermore, OXTR knockout mice have impaired social memory (Ferguson *et al.*, 2000; Lee *et al.*, 2008; Takayanagi *et al.*, 2005).

During social behaviour rodents emit USVs as a means of social communication (Burgdorf *et al.*, 2008) which are reflective of their affective state (Brudzynski, 2015). Frequency modulated (FM) 50 kHz calls are thought to reflect a positive affective state, with rats emitting these during rewarding behaviours such as social play and mating (Brudzynski, 2015; Wöhr *et al.*, 2017). Flat 50 kHz calls without FM are still classified as prosocial, although thought to serve more of a social co-ordination function (Brudzynski, 2015). Thus, recording and analysing rat USVs during social tasks can give a greater insight into social behaviour and an objective measure of social communication. Peripheral s.c. OXY administration causes dose-dependent effects upon 40 kHz 'distress' calls emitted by maternally separated rat pups (Insel & Winslow, 1991). 1µg s.c. OXY increased call emission, whilst 100µg decreased the amount of calls emitted by separated pups; implicating the OXTR system in USVs (Insel & Winslow, 1991). However, the only study looking at the effect of OXY on 50 kHz prosocial calls in adult rats found no effect of 0.1mg/kg s.c. OXY, despite an increase in social behaviour (Kohli *et al.*, 2019). The V1aR also affects rat USV emission; central infusion of a V1aR antagonist reduced both social play and 50 kHz call emission in male Wistar rats (Lukas & Wohr, 2015). However, administration of synthetic vasopressin did not further increase 50 kHz USVs or social play. The above suggests both receptors are likely involved in USV emission, yet the precise mechanisms are yet to be understood. More recently, Potasiewicz *et al.* (2020) found decreased social behaviour and reduced emission of 50 kHz USVs during social encounters of rats who were prenatally exposed to methylazoxymethanol acetate (MAM); used as a rodent model for schizophrenia. MAM rats were also found to have reduced concentrations of OXY, vasopressin, OXTRs, and V1aRs in the hypothalamus, as well as reduced OXY and OXTRs in the PFC (Potasiewicz *et al.*, 2020). Taken together, this again suggests that the OXY/vasopressin system is likely to be at least partially involved in the neural mechanisms underlying social behaviour and social communication.

As well as effects on social behaviour, peripheral OXY (0.1mg/kg s.c.) reduces PCP-induced hyperactivity in a locomotor activity assay (Kohli *et al.*, 2019). PCP, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, is associated with producing

schizophrenia-like symptoms in healthy humans, such as hallucinations and cognitive deficits, and can trigger relapse in patients with schizophrenia (Yui *et al.*, 1999). NMDA receptor (NMDA-R) hypofunction has long been associated with the aetiology of schizophrenia (Balu, 2016; Coyle, 2012). For example, anti-NMDA-R antibodies have been observed in patients with schizophrenia which significantly reduce the expression of the NMDA-R (Tong *et al.*, 2019), and genetic variants of NMDA-R genes are associated with an increased risk of schizophrenia (Balu, 2016; Coyle, 2012). Furthermore, D-serine, required for NMDA-R activation, is significantly reduced in the CSF of patients with schizophrenia (reviewed in Balu (2016)).

In rodents, PCP primarily alters horizontal motor behaviour and can induce stereotypic behaviours such as repetitive head movements (Hackler *et al.*, 2010; Sturgeon *et al.*, 1979). These dysfunctional locomotor behaviours are analogous to stereotyped behaviours seen in patients with schizophrenia (Morrens *et al.*, 2006). PCP-induced hyperactivity in rodents is associated with limbic striatal function, with PCP and other NMDA receptor (NMDA-R) antagonists causing increased NAc dopamine release in rodents (Takahata & Moghaddam, 2003). Limbic abnormalities in humans are associated with psychosis and thought disorders, with D_2 receptors in the mesolimbic system being the primary target of current antipsychotic medications (Grace & Gomes, 2019; Meltzer, 1991). As well as hyperactivity, acute PCP and other NMDA-R antagonists can disrupt prepulse inhibition (Bakshi *et al.*, 1994; Mansbach & Geyer, 1989), cause social withdrawal (Sams-Dodd, 1995), and cause working memory deficits in rodents (Egerton *et al.*, 2005; Verma & Moghaddam, 1996); similar to the effects of NMDA-R antagonists in humans (Adler *et al.*, 1998; Krystal *et al.*, 1999). Acute PCP can therefore resemble some of the psychotic, cognitive, and negative symptoms of schizophrenia. Similarities between the behavioural effects of NMDA-R antagonists in humans and rodents suggest similar neural mechanisms may cause these effects. As a consequence, reversal of PCP-induced hyperactivity in rodents has long been used to assess antipsychotic efficacy, with antipsychotics including: olanzapine, haloperidol, risperidone, cariprazine, clozapine, and aripiprazole all successfully reversing PCP-induced hyperactivity (Dedic *et al.*, 2019; Gleason & Shannon, 1997; Gyertyán *et al.*, 2011; Jentsch & Roth, 1999; Kehr *et al.*,

2018; Li *et al.*, 2010; Maurel-Remy *et al.*, 1995; Millan *et al.*, 1999). As well as altered limbic function PCP-hyperactivity is also associated with increased PFC dopamine and 5-hydroxytryptamine (5-HT; serotonin) release (Jentsch & Roth, 1999), and increased PFC glutamatergic activity (Takahata & Moghaddam, 2003), which all likely contribute to the behavioural effects of acute PCP. Although Kohli *et al.* (2019) is the only study to date to demonstrate OXY reversal of PCP-hyperactivity, other preclinical work has shown antipsychotic-like effects of OXY through the restoration of pharmacologically disrupted prepulse inhibition of acoustic startle (Feifel & Reza, 1999). As with OXY effects on social behaviour, it is unknown how OXY is altering this antipsychotic-like effect, and through which receptors it is acting.

It is clear both the OXTR and V1aR contribute to the behavioural effects observed following OXY administration. However, it is also worth noting fMRI data suggests that systemic OXY administration does not activate the same areas as i.c.v. administration (Ferris *et al.*, 2015). It may be possible that the mechanisms underlying OXYs behavioural effects differ according to the route of administration, and between sex and strain of rat. Peripherally administered OXY is significantly less invasive than centrally administered OXY, thus more likely to find clinical use. To fully understand the prosocial and antipsychotic-like effects of s.c. OXY it is imperative we understand the mechanisms and receptors through which it provokes these effects. The recent availability of selective antagonists for the V1aR and OXTR that can penetrate the BBB have been used herein to determine the receptors responsible for specific behaviours (Boccia *et al.*, 2007; Kohli *et al.*, 2019). As both V1aR and OXTRs have differential expression in the brain (Smith *et al.*, 2019), determining the receptors responsible for these behavioural changes can also help identify possible brain regions involved in the antipsychotic-like and prosocial effects of s.c. OXY.

3.2 Aims

The aim of this study was to determine the involvement of the OXTR and V1aR in the enhancement of social behaviour following peripheral s.c. OXY administration. We

also aimed to decipher the roles of the OXTR and V1aR in attenuation of PCP-induced hyperactivity following peripheral s.c. OXY administration.

3.3 Materials and Methods

3.3.1 Animals

120 male Lister-hooded rats (Aged 42-48 days; Charles River UK) were housed in groups of three or four in individually ventilated 'double decker' cages (462mm x 403mm x 404mm; Techniplast, UK) with their litter mates. Male rats were used in order to enable comparisons to previous literature (Kohli *et al.*, 2019), as some behaviours modulated by the OXTR exhibit sexual dimorphism (Benelli *et al.*, 1995; Engelmann *et al.*, 1998; Lukas *et al.*, 2011). Furthermore, the sensitivity of the OXTR to gonadal steroids means OXTR expression is likely to fluctuate throughout the oestrus cycle in female rats, potentially impacting the effects of OXY on behaviour (Bale *et al.*, 1995; Patchev *et al.*, 1993). Food and water were provided *ad libitum* (Teklad global 18% protein diet, Harlan, UK), and cages were maintained at consistent temperature and humidity (21˚C ± 2, 45% humidity ± 15) with a 12-hour light-dark cycle (lights on 07.00h). Rats were gently handled for seven days prior to beginning behavioural tests to reduce anxiety and enhance animal welfare (LaFollette *et al.*, 2017). All procedures were performed in the light phase and were conducted in line with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines, and with approval of the Animals (Scientific Procedures) Act 1986 and The University of Nottingham Animal Welfare and Ethical Review Body (AWERB). At the end of the study all rats were killed by the Schedule 1 (S1) method of euthanasia with carbon dioxide, and death was immediately confirmed with cervical dislocation.

3.3.2 Experimental Design

In order to reduce the risk of a type II error, an *a priori* power analysis calculation was conducted to estimate a sufficient sample size. In this analysis the sample size is calculated as a function of the power level, the pre-specified significance level, and the effect size. Power analysis was conducted with GPower 3.1 (Faul *et al.*, 2007), ensuring sufficient power to allow statistically significant findings to be obtained, if such differences exist, while still adhering to the 3R's principle. Based on previous studies in this lab a large effect was expected (Kohli *et al.*, 2019). For that reason, an eta squared (η^2, the measure of effect size for ANOVA models) of 0.14 was selected; indicating a large effect size (Cohen, 1988). The conventional value of power as 80% was selected, which considers a Type I error to be four times more serious than the cost of a Type II error, when the significance level is also set to a conventional value of 0.05 (Perugini *et al.*, 2018). Therefore, group sizes of n=10 would give a power of >80% to detect significant differences between groups, using a significance threshold of p=0.05 (when using a repeated measures, between factors ANOVA).

Within each litter all treatments were randomly allocated, ensuring all litters received all treatments. All treatment groups were also balanced across the 10 locomotor activity (LMA) arenas (in a random order) and the average body weight was checked for equivalence across all treatment groups. The experimenter was unaware of drug treatment.

3.3.3 Drugs

OXY acetate was purchased from Bachem (St. Helens, UK), the selective OXY antagonist L-368,899 from Tocris Bioscience (Bristol, UK), and the selective V1aR antagonist SR 49059 and PCP from Sigma Aldrich (Poole, UK). OXY and PCP were dissolved in sterile saline (0.154 M), while the antagonists and their respective vehicle were dissolved in saline containing 5% DMSO (Sigma Aldrich, UK). Drugs were administered at a volume of 1mL/kg s.c. (OXY) or 2mL/kg (i.p.; Antagonists and PCP).

The antagonists in this study were carefully selected for high affinity and selectivity for their respective receptors, and good BBB penetration (Alexander *et al.*, 2017; Manning *et al.*, 2012). Antagonist doses were selected based upon previous *in vivo* research, demonstrating an onset of less than 15 minutes and a duration of 2-4 hours in rodents (Hicks *et al.*, 2014; Ramos *et al.*, 2013; Smith *et al.*, 2013a). The dose of OXY (0.1mg/mg) was selected from a previous report that enhanced social behaviour and attenuated PCP-induced hyperactivity, without supressing LMA or causing hypothermia in Lister-hooded rats (Kohli *et al.*, 2019).

3.3.4 Locomotor Activity (LMA)

LMA assessment was used to measure reversal of PCP-induced hyperactivity; widely used to determine potential antipsychotic efficacy (Jentsch & Roth, 1999). LMA assessment was undertaken in computerised, individual, Perspex arenas (39 x 23.5 x 24.5cm) surrounded by a dual-level infrared beam system (San Diego instruments, California, USA) with a removable wire roof under low light conditions (40 LUX). The lower level contained 8 beams along the length, with an additional 4 across the width. The upper level contained 8 beams upon the length only. Ambulation was recorded in 5-minute time bins. One ambulatory count was defined as two consecutive lower-level infrared beam breaks. Rears were defined as a break of a single upper-level beam, and fine movements as the breaking of a single lower level beam.

Rats were placed into the LMA arena for 120 min; 30 min habituation followed by 90 min behavioural testing. Typically, rats placed in a novel environment will show increased levels of activity which decreases over time as rats become familiarised to the novel environment, therefore the habituation phase ensured rats had habituated to the arena prior to behavioural testing. Antagonist (L-368,899 at 2mg/kg, or SR 49059 at 1mg/kg) or vehicle (1mL/kg with 5% DMSO) were administered i.p. 15 minutes into the habituation phase, followed by s.c. OXY (0.1mg/kg) or saline (1mL/kg) 15 minutes later. PCP (5.6mg/kg) or saline (2mL/mg) were then administered i.p. 30 minutes later; 30 minutes into behavioural testing (Figure 3.1). Dosing schedules were based upon previous research in this laboratory, ensuring the peak effect of OXY and PCP occurred simultaneously (Kohli *et al.*, 2019). Following

recording animals were returned to their home cage. LMA and rearing events were calculated as mean + SEM of the cumulative counts across each consecutive 5-minute epoch, and the total counts for 20 minutes after PCP or vehicle (Kohli *et al.*, 2019; McIntosh *et al.*, 2013; Watson *et al.*, 2012; Watson *et al.*, 2016). LMA arenas were cleaned with soap and water, followed by 20% ethanol between each trial to remove scent, faecal pellets, and hairs to ensure a homogenous environment and the accuracy of recording.

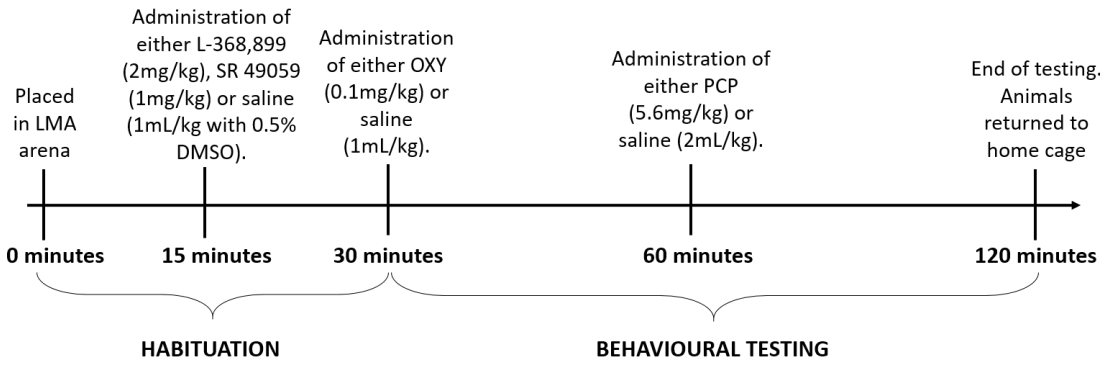

Figure 3.1: Timeline of LMA drug administration and behavioural recording, based on Kohli *et al.* (2019).

3.3.5 Social Interaction

Social interaction analysis can be used to determine the effect of drug administration on social behaviour in rodents, and can be used as a measure of negative symptoms of schizophrenia (Watson *et al.*, 2016; Wilson & Koenig, 2014). Six to seven days after LMA rats undertook the social interaction task. Two rats of matched weight and drug treatment, and from different cages and litters, were paired together for social interaction assessment so that their pair was novel to them. Rats were weight matched as close as possible to prevent dominance. Rats were singly housed overnight prior to undertaking social interaction to enhance behavioural interaction during the task (Niesink & van Ree, 1982). 60 minutes prior to undertaking the social interaction task rats received a dose of either L-368,899 (2mg/kg), SR49059 (1mg/kg) or saline (1mL/mg with 5% DMSO). Fifteen minutes later rats received a dose of either

OXY (0.1mg/kg) or saline (1mL/kg). Half an hour before undertaking social interaction rats were sprayed on the nape with either blue or red hair spray (Superdrug instant hairspray, Superdrug Stores, UK), to enable colour-tracking by Ethovision and independent scoring of behaviour for each rat in the pair. Forty-five minutes after receiving the OXY or vehicle dose paired rats were placed into an unfamiliar circular arena (75 cm diameter, 45 cm walls). Social interaction behaviours were recorded by software (Ethovision XT v8.5, Noldus) for 10 minutes in low light (40 LUX, Figure 3.2) to enhance the extent of interaction (Watson *et al.*, 2016).

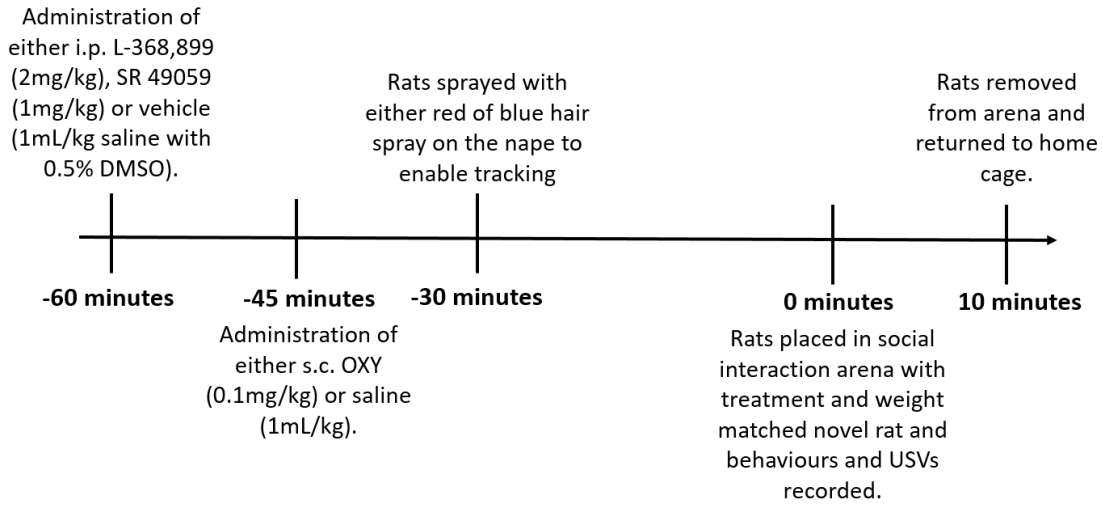

Figure 3.2: Timeline of the social interaction task and drug administration, based on Kohli *et al.* (2019).

Recorded prosocial behaviours were body sniffing, anogenital sniffing, following, crawling over and under, and lying side-by-side. The aggressive behaviours recorded were pinning, and boxing and biting. As the behaviour of each rat is dependent on that of its partner mean values for every component were calculated for each pair (Kohli *et al.*, 2019). Following recording rats were returned to their home cage. Between each trial the arena was cleaned with 20% ethanol to remove any scent, ensuring a homogenous environment.

From resultant video recordings behaviours were scored manually using the Ethovision software with the experimenter blinded to treatment. The experimenter was trained to score behavioural videos from an experienced individual, using previously obtained experimental videos from an unrelated study. To ensure inter-

rater reliability the scores obtained from practice videos were compared to scores obtained from experienced individuals, ensuring both scorers were within one percent of each other for the total duration and there were no discrepancies. To ensure intra-rater reliability on each day of behavioural scoring two random videos from the previous day of analysis were selected to be rescored, ensuring consistency during scoring.

3.3.6 Ultrasonic vocalisations (USVs)

USVs were recorded during the social interaction task using an electret microphone (Emkay, Avisoft Bioacoustics, Germany) linked to an ultrasound detection unit (Ultrasound Gate, customised model, Avisoft Bioacoustics, Berlin) that was placed above the arena. As rat USVs were dependent upon their partner, and it was not possible to identify which rat emitted the call, USV calls were analysed and reported for that of the pair.

To enable analysis USV recordings were digitalised and saved as a .wav file. Avisoft analysis software (SAS-Laboratory Pro, v. 4.38, Avisoft Bioacoustic, Berlin) extracted the time and frequency characteristics of every call. A spectrograph was created using the following parameters: 488 Hz frequency resolution, 512 FFT length, 75% frame size and 93.75% time window overlap. Cut off frequencies of 22 kHz (lower limit) and 85 kHz (upper limit) were used to reduce the level of background noise.

Once calls were identified, they were manually characterised into either 22 kHz aversive calls or 50 kHz pro-social calls. 50 kHz calls were subsequently further classified into either trill, flat or step calls based on their pattern analysis (Brudzynski, 2015). Flat calls had frequency changes of less than 5 kHz, however if there was an upward or downward direction in the flat call the difference in start and end frequency could be greater than 5 kHz. Step calls contained a minimum of one short element that was more than 5 kHz higher or lower than the main call. Trill calls had a minimum of one longer frequency modulation than step calls and/or a zig-zag shape or a series of inverted-u shapes (See Chapter 1.3, Figure 1.10).

3.3.7 Data analysis

Data were analysed using GraphPad Prism (Version 7.04 for Windows) and JASP (Version 0.12.1). To analyse the LMA data a 4-way repeated measures ANOVA was selected. This enabled the examination of the group differences over time, when groups are formed by a combination of the between-subject factors. Between subject factors were OXY, PCP, and antagonist, with time as the within-subjects variable. Before conducting the four-way ANOVA it was ensured that all the assumptions required for a 4-way repeated measures ANOVA were satisfied: one dependent variable that is measured at the continuous level, three within-subjects factors that consists of two or more categorical levels, no significant outliers, homogeneity of variance (using Levene's test), the dependent variable is normally distributed for each group of the independent variable (using Shapiro-Wilk's test), and sphericity of the data (using Mauchly's sphericity test). To determine which specific groups significantly differed from each other, if applicable, a Tukey's post-hoc was run. Tukey's post-hoc was selected as it compares all groups to each other and corrects for multiple comparisons; reducing the risk of type 1 errors (Lee & Lee, 2018; Moore *et al.*, 2012; Whitlock & Schluter, 2015).

To analyse the social interaction data a two-way ANOVA was selected. A two-way ANOVA was used to determine whether there was an interaction effect between two independent variables (OXY and antagonist) on a continuous dependent variable (social interaction duration). Similarly, a Tukey's post-hoc was selected to determine which specific groups significantly differed from each other, if applicable.

3.4 Results

3.4.1 LMA

3.4.1.1 Effect of OXY and an OXTR antagonist on LMA

To assess the impact of OXY on PCP-induced hyperactivity in the presence and absence of the OXTR antagonist L-368,899, rat activity was monitored in computerised, Perspex arenas for two hours; allowing both habituation to the novel environment and the assessment of LMA following drug administration. Two rats were excluded from LMA analysis; one due to it receiving the wrong drug combination during the trial, and the other because of an equipment fault during recording.

For analysis, data was divided into three sections and analysed separately: the habituation phase (0-30min, data not shown), the 30 min following OXY administration (30-60 min) and then the post-PCP phase (60-120 min). Mauchly's test of sphericity indicated the assumption of sphericity had been violated for all interactions (p<0.05), therefore a Greenhouse-Geisser correction was used. There was no significant difference between treatment groups in the habituation phase ($F_{(1.835, 113)}$=0.557, p=0.559; four-way repeated measures ANOVA), nor any effect of treatment on LMA prior to PCP administration ($F_{(4.135, 266)}$=1.105, p=0.402; four-way repeated measures ANOVA), therefore all treatment groups had the same level of basal activity before PCP administration. Data analysis subsequently focussed on the data accrued following PCP administration (60 min onwards).

ANOVA revealed a significant four-way interaction between time * antagonist * OXY * PCP ($F_{(3.44, 240.5)}$=2.896, p=0.029). There were also significant interactions between time * antagonist * OXY ($F_{(3.44, 240.5)}$=3.158, p=0.020), time * OXY ($F_{(3.44, 240.5)}$=2.734, p=0.037), time * PCP ($F_{(3.44, 240.5)}$=13.77, p<0.001), and a significant main effect of time ($F_{(3.44, 240.5)}$=39.55, p<0.001) (all details in Appendix 2). As expected, PCP significantly increased hyperactivity (p<0.001, Tukey's post-hoc) and this was significantly

attenuated by OXY; although this only reached significance at time point 75 and 80 min (p<0.05, Tukey's post-hoc). Administration of L-368,899 stopped OXY significantly attenuating PCP-induced hyperactivity, although it did not fully reverse this effect. Interestingly, administration of L-368,899 alongside PCP also appeared to attenuate PCP-induced hyperactivity, but this was not significantly different from PCP alone, and PCP was still able to significantly increase LMA at time point 75 (Figure 3.3). The effect of treatment on rears was also assessed, however four-way repeated measures ANOVA revealed no significant effect of treatment on the number of rears ($F_{(4.448, 311.3)}$=0.969, p=0.431, data not shown).

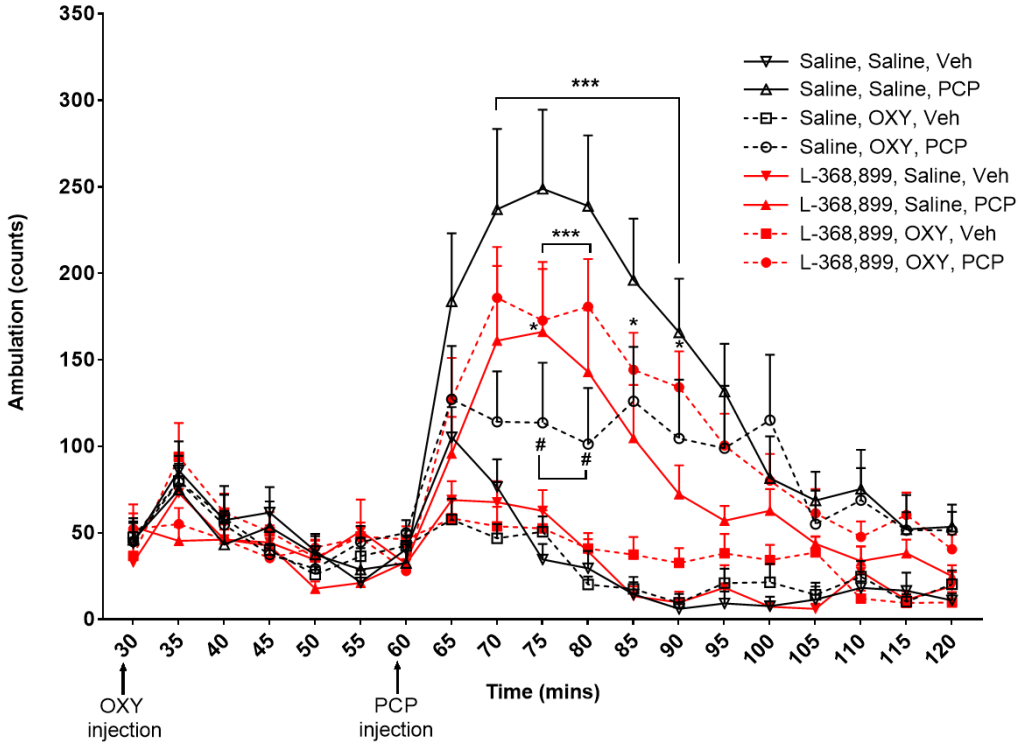

Figure 3.3: Time course of PCP-induced hyperactivity in the presence and absence of OXY and the selective OXTR antagonist L368-899 (mean + SEM, n=9 (saline_OXY_PCP and saline_OXY_veh) or 10 (all other groups) per group). L-368,899 was administered 15 minutes into the habituation phase (data not shown), 15 minutes prior to s.c. OXY administration. Four-way ANOVA revealed a significant four-way interaction between time * L-368,899 * OXY * PCP ($F_{(3.44,\ 240.5)}$=2.896, p=0.029), as well as significant interactions between time * L-368,899 * OXY ($F_{(3.44, 240.5)}$=3.158, p=0.020), time * OXY ($F_{(3.437, 240.5)}$=2.734, p=0.037), time * PCP ($F_{(3.437, 240.5)}$=13.77, p<0.001). PCP significantly increased LMA from time points 70-90min when compared to all control groups that did not receive PCP (p<0.001), and was attenuated by OXY administration, reaching significance 15-20 minutes after PCP injection (p<0.05). *** p<001. ** p<0.01 and * p<0.05 compared to saline_saline_veh and # p<0.05 compared to saline_saline_PCP, Tukey's post-hoc.

Further analysis specifically focussed on the cumulative ambulation twenty minutes post-PCP administration; where PCP was having its peak effect (Takahata & Moghaddam, 2003). A three-way ANOVA revealed a significant interaction between antagonist * OXY * PCP ($F_{(1,70)}$=3.89, p=0.05), and a significant two-way interaction between OXY * antagonist ($F_{(1,70)}$=5.164, p=0.026). There was also a significant main effect of PCP on LMA ($F_{(1,70)}$=47.96, p<0.001), such that PCP significantly increased LMA (p<0.001) and this was significantly attenuated by OXY administration (p<0.05, Tukey's post-hoc). Although administration of L-368,899 alone appeared to reduce PCP-hyperactivity (by 36 ± 12%), this did not reach statistical significance and LMA was not significantly less than that seen with PCP alone (p=0.16, Tukey's post-hoc). In contrast, L-368,899 prevented significant OXY-induced attenuation of PCP-hyperactivity, such that PCP could significantly increase activity (p<0.05, Tukey's post-hoc) and LMA was no longer significantly less than that seen with PCP alone. However, L-368,899 did not return LMA back to levels seen with PCP alone (Figure 3.4).

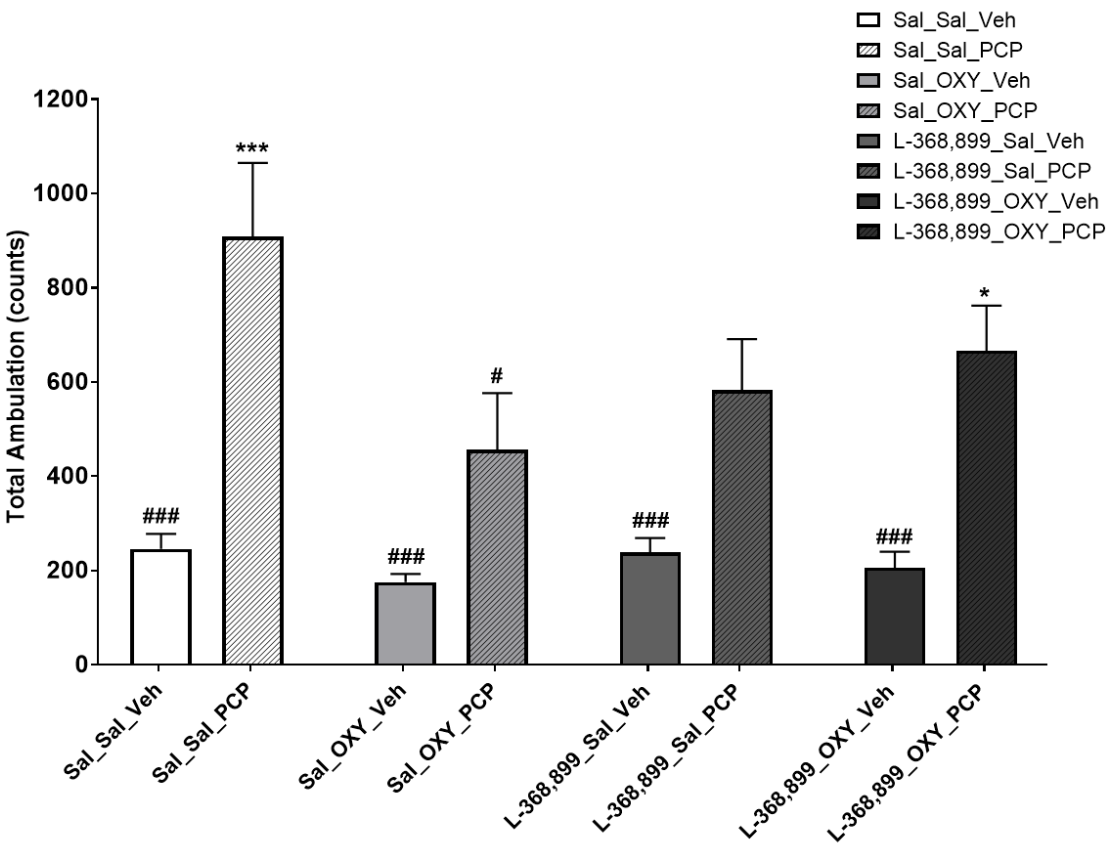

Figure 3.4: Total ambulation 20 minutes post-PCP administration in the presence and absence of the OXTR antagonist L-368,899 (mean + SEM, n= 9 (saline_OXY_PCP and saline_OXY_veh) or 10 (all other groups) per group). Three-way ANOVA revealed a significant three-way interaction between antagonist * OXY * PCP ($F_{(1,70)}$=3.89 p=0.05), a significant two-way interaction between OXY * antagonist ($F_{(1,70)}$=5.164. p=0.026), and a significant main effect of PCP on LMA ($F_{(1,70)}$=47.96, p<0.001). Tukey's post-hoc revealed PCP significantly increased LMA (p<0.001). OXY administration significantly attenuated PCP-induced hyperactivity (p<0.05). Administration of L-368,899 alongside OXY prevented OXY significantly reducing PCP-hyperactivity but did not return LMA back to the level of PCP alone. * p<0.05, *** p<0.001, compared to sal_sal_veh, # p<0.05, ### p<0.001 compared to sal_sal_PCP, Tukey's post-hoc.

3.4.1.2 Effect of OXY and a V1aR antagonist on LMA

The effect of OXY on PCP-induced hyperactivity was also examined in the presence and absence of the selective V1aR antagonist, SR 49059. Three rats were excluded from all LMA analysis: one due to human error and receiving the wrong drug combination during the trial and two due to an equipment fault mid assessment. Mauchly's test of sphericity indicated the assumption of sphericity had been violated for all interactions (p<0.05), therefore a Greenhouse-Geisser correction was used.

There was no significant difference between treatment groups in the habituation phase (0-30 min, $F_{(1.76, 112)}$=0.356, p=0.674; four-way repeated measures ANOVA), nor any effect of OXY on LMA prior to PCP administration ($F_{(3.73, 238)}$=0.223, p=0.916; four-way repeated measures ANOVA), therefore all treatment groups had the same level of basal activity before PCP administration. Data analysis subsequently focussed on the data accrued following PCP administration (60 min onwards).

Four-way repeated measures ANOVA revealed a significant main interaction between time * antagonist * OXY * PCP ($F_{(3.60, 248.6)}$=2.839, p=0.030) and significant interactions between time * antagonist * OXY ($F_{(3.60, 248.6)}$=4.216, p=0.004), time * PCP ($F_{(3.60, 248.6)}$=11.262, p<0.001), and a significant main effect of time ($F_{(3.60, 248.6)}$=34.91, p<0.001). Once again, PCP significantly increased LMA for 25 minutes post-administration (p<0.001, Tukey's post-hoc) in saline control rats, and in the presence of SR 49059 (at 75min, p<0.05, Tukey's post-hoc). OXY appeared to attenuate PCP-induced hyperactivity, such that PCP no longer significantly increased activity in the presence of OXY, however the OXY and PCP group was not significantly different from PCP alone at any time point (Figure 3.5). Administration of SR 49059 alongside OXY and PCP enabled PCP to significantly increase LMA again (at 75-80min, p<0.001, Tukey's post-hoc). The effect of treatment on rears was also assessed, however four-way repeated measures ANOVA revealed no significant effect of treatment on the number of rears ($F_{(3.62, 257.9)}$=0.347, p=0.828, data not shown).

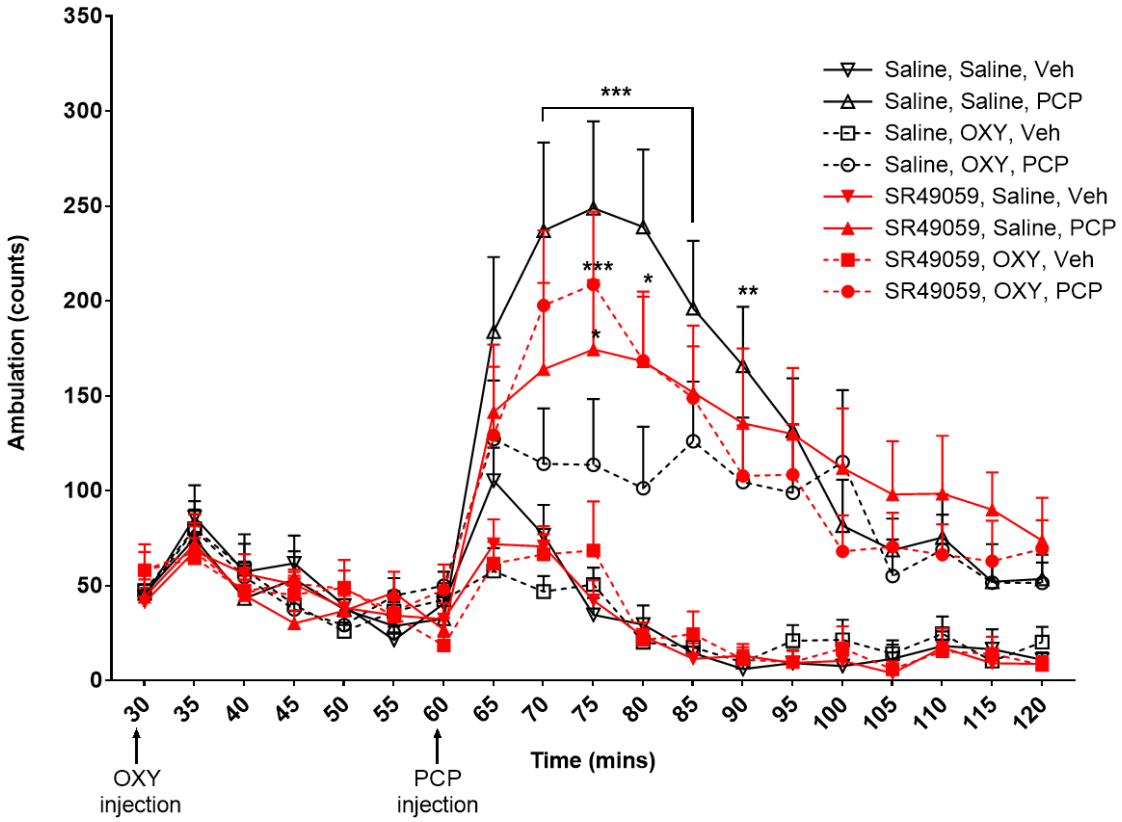

Figure 3.5: Time course of PCP-induced hyperactivity in the presence and absence of OXY and the selective V1aR antagonist SR 49059 (mean ± SEM, n=9 (SR 49059_OXY_veh, saline_OXY_PCP and saline_OXY_veh) or 10 (all other groups) per group.). Four-way repeated measures ANOVA revealed a significant four-way main interaction between time * antagonist * OXY * PCP ($F_{(3.60, 248.6)}$=2.839, p<0.05). There were also significant interactions between time * antagonist * OXY ($F_{(3.60, 248.6)}$=4.216, p<0.01), time * PCP ($F_{(3.60, 248.6)}$=11.262, p<0.01), and a significant main effect of time ($F_{(3.60, 248.6)}$=34.91, p<0.001). PCP administration significantly increased LMA for 25 minutes following administration (p<0.001). PCP was also able to significantly increase LMA in the presence of SR 49059, and SR 49059 + OXY. OXY administration attenuated PCP-hyperactivity, such that PCP could no longer significantly increase hyperactivity. *** p<0.001. ** p<0.01, * p<0.05, compared to saline_saline_veh, Tukey's post-hoc.

When looking at the total ambulation for 20 minutes following PCP administration, a three-way-ANOVA showed no significant three-way interaction between OXY * antagonist * PCP ($F_{(1,69)}$=2.24, p=0.142), however there was a significant two-way interaction between antagonist * OXY ($F_{(1, 69)}$=42.16, p=0.045) and a significant main effect of PCP ($F_{(1, 69)}$=4.179, p<0.001) (Figure 3.6). Post-hoc comparisons on the significant two-way interaction between antagonist * OXY revealed no significant effect of SR 49059 on OXY.

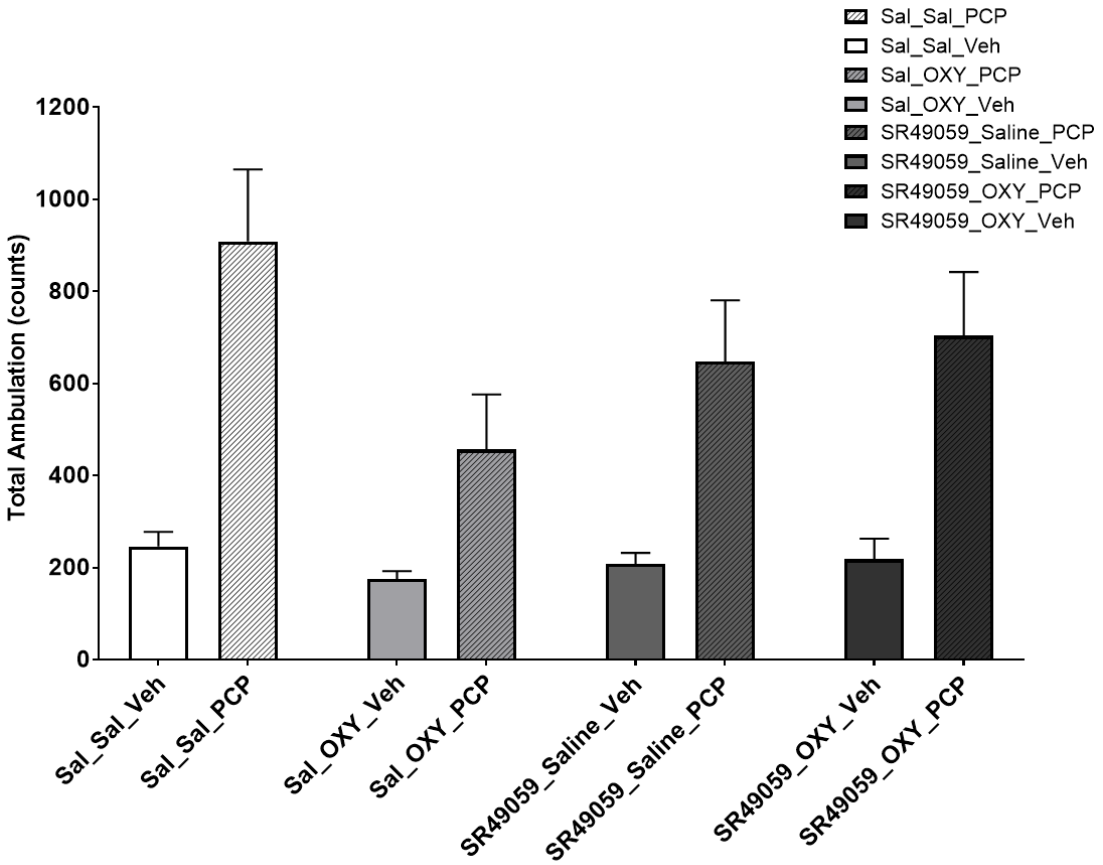

Figure 3.6: Total ambulation 20 minutes post-PCP administration in the presence and absence of the V1aR antagonist SR 49059 (mean + SEM, n=9 (SR 49059_OXY_veh, saline_OXY_PCP and saline_OXY_veh) or 10 (all other groups) per group.). Three-way-ANOVA showed a significant two-way interaction between antagonist and OXY ($F_{(1, 69)}$=42.16, p=0.045), and a significant main effect of PCP ($F_{(1, 69)}$=4.179, p<0.001), such that PCP appeared to increase LMA in all groups. Post-hoc comparisons on the significant two-way interaction showed no significant effect of SR 49059 on OXY.

3.4.2 Social interaction

3.4.2.1 Effect of OXTR and OXTR and V1aR antagonists on social behaviour

To determine the effect of OXY on social behaviour, and the role of the OXTR and V1aR antagonists, social interaction was assessed in an open-field arena. OXY and antagonist administration had no significant effect upon the distance moved during

social interaction ($F_{(2, 54)}$=0.066, p=0.936; detailed in Appendix 2). ANOVA analysis of the total duration of prosocial behaviours revealed a significant interaction between OXY * antagonist ($F_{(2, 54)}$=12.40, p<0.001), and significant main effect of both OXY ($F_{(1, 54)}$=33.49, p<0.001) and antagonist ($F_{(2, 54)}$=13.92, p<0.001). OXY significantly increased the total duration of prosocial behaviours (p<0.001, Tukey's post-hoc), which was prevented by L-368,899, such that OXY no longer significantly increased prosocial behaviours. In marked contrast, pre-treatment with SR 49059 had no effect on the OXY enhancement of social behaviour, which was still significantly greater than in saline + saline control rats (p<0.001, Tukey's post-hoc). Neither antagonist had any effect upon social behaviour when administered alone in the absence of OXY (Figure 3.7).

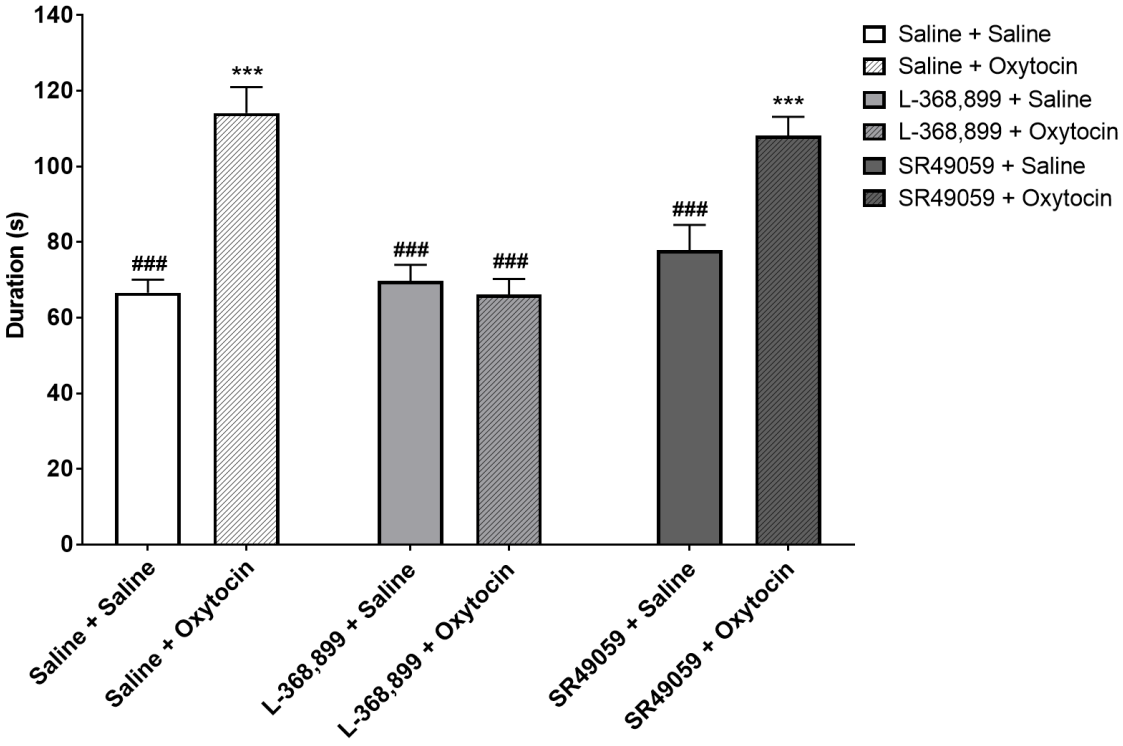

Figure 3.7: The effect of OXY, and OXTR and V1aR antagonism, upon the duration of prosocial behaviours during social interaction (mean + SEM, n=10/group). A two-way ANOVA revealed a significant interaction between OXY * antagonist ($F_{(2, 54)}$=12.40, p<0.001), and a significant main effect of both OXY ($F_{(1, 54)}$=33.49, p<0.001) and antagonist ($F_{(2, 54)}$=13.92 p<0.001). OXY significantly increased the total duration of prosocial behaviours (p<0.001, Tukey's post-hoc), which was completely prevented by L-368,899 but not by SR 49059. ***p<0.001 compared to vehicle controls (saline + saline) and ### p<0.001 compared to saline + OXY (Tukey's post-hoc).

Olfactory cues play a crucial role in rodent interactions, therefore the effect of OXY on both anogenital and body sniffing were examined (Wacker & Ludwig, 2012). A two-way ANOVA revealed a significant main effect of OXY ($F_{(1, 54)}$=4.148, p=0.046) on anogenital sniffing, but no effect of either antagonist (Figure 3.8A). When looking at body sniffing duration, there was a significant interaction between antagonist and OXY ($F_{(2, 54)}$=9.44, p<0.001), as well as a significant main effect of both OXY ($F_{(1, 54)}$=31.42, p<0.001) and antagonist ($F_{(2, 54)}$=15.38, p<0.001). OXY significantly increased body sniffing duration (p<0.001, Tukey's post-hoc) and was attenuated by the selective OXTR antagonist L-368,899, such that OXY no longer significantly increased body sniffing duration. No effect of the V1aR antagonist, SR49059, was observed (Figure 3.8B).

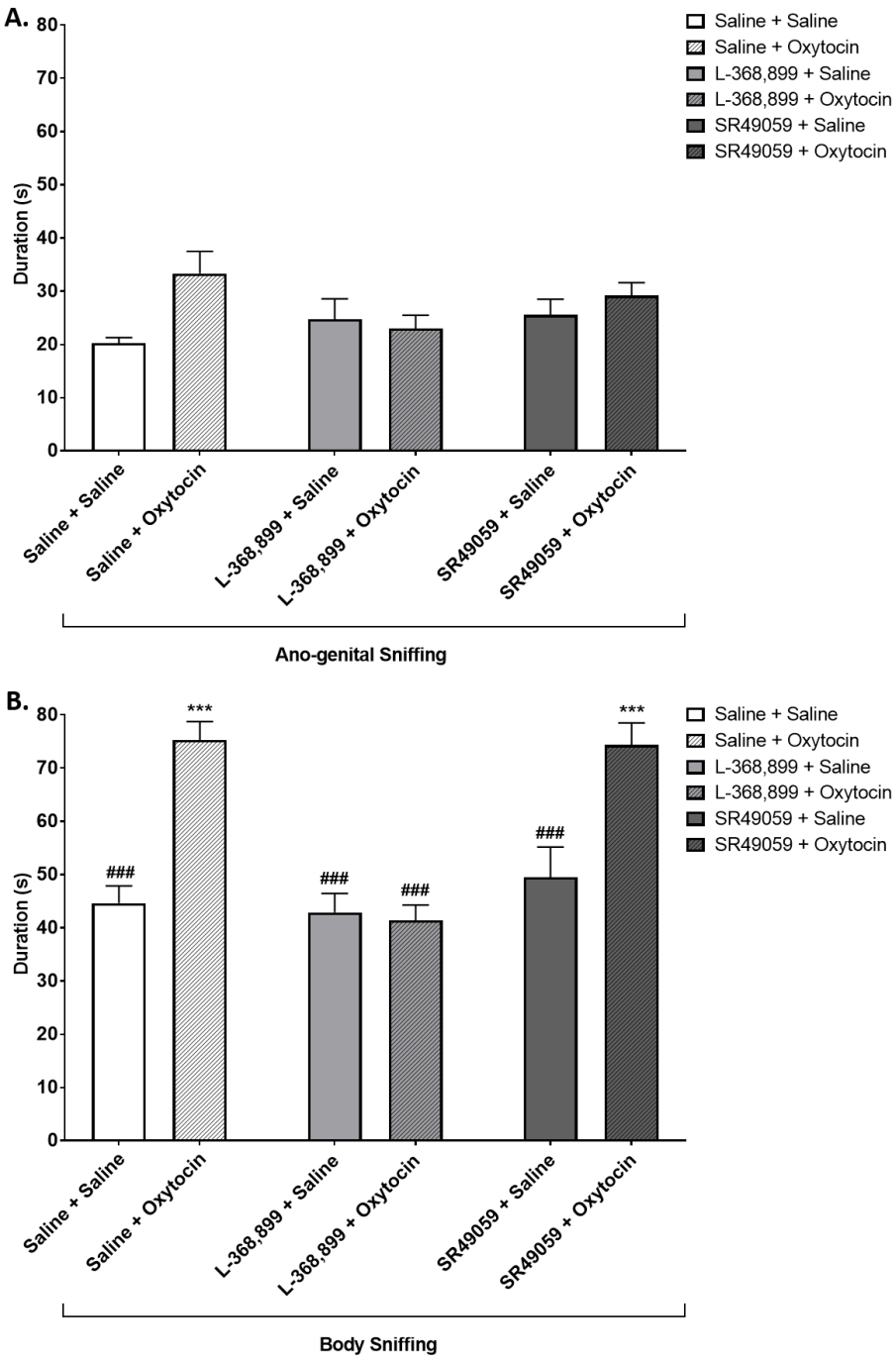

Figure 3.8: Effect of OXY, and OXTR and V1aR antagonists, on anogenital and body sniffing duration in social interaction (mean + SEM, n=10/group). A) A two-way ANOVA revealed a significant main effect of OXY ($F_{(1, 54)}$=4.148, p=0.046) on anogenital sniffing, but no effect of antagonist. B) A two-way ANOVA found a significant interaction between antagonist and OXY ($F_{(2, 54)}$=9.44, p<0.001), as well as a significant main effect of both OXY ($F_{(1, 54)}$=31.42, p<0.0001) and antagonist ($F_{(2, 54)}$=15.38, p<0.0001) on body sniffing duration. OXY significantly increased body sniffing (p<0.001; Tukey's post-hoc) and was attenuated by the selective OXTR antagonist L-368,899. SR 49059 had no effect on social behaviours. ***p<0.001 when compared to the control group (saline + saline) and ### p<0.001 compared to saline + OXY (Tukey's post-hoc).

In contrast, neither OXY nor either antagonist altered the duration or frequency of lying side-by-side and crawling over and under (data not shown). However, analysis of following duration showed a significant interaction between antagonist and OXY ($F_{(2,54)}$=4.716, p=0.013), and a significant main effect of OXY ($F_{(2, 54)}$=4.716, p=0.004) and antagonist ($F_{(2, 54)}$=4.304, p=0.018). OXY significantly increased the duration of following (p<0.01, Tukey's post-hoc) and pre-treatment with both SR 49059 and L-368,899 prevented an OXY-induced increase in following duration (Figure 3.9).

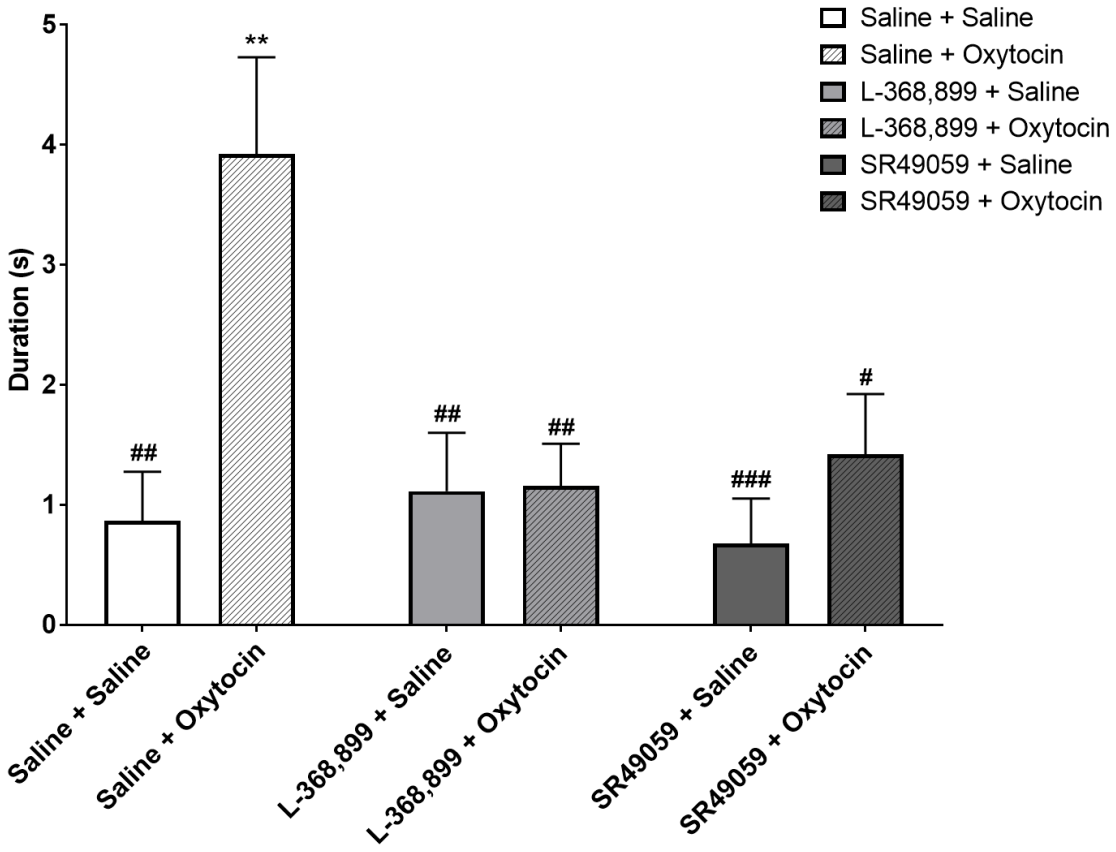

Figure 3.9: Effect of OXY, and OXTR and V1aR antagonism, on the duration of following in a social interaction paradigm (mean + SEM, n=10/group). A two-way ANOVA revealed a significant interaction between antagonist and OXY ($F_{(2, 54)}$=4.716, p=0.013), and a significant main effect of OXY ($F_{(2, 54)}$=4.716, p=0.004) and antagonist ($F_{(2, 54)}$=4.304, p=0.018). OXY significantly increased following duration (p<0.01; Tukey's post-hoc) and was prevented through administration of both antagonists. **p<0.01 from the vehicle control group (saline + saline), and # p<0.05, ## p<0.01, and ### p<0.001 compared to saline + OXY (Tukey's post-hoc).

The total duration of aggressive behaviours was also assessed (the sum of boxing and biting, and pinning) (Figure 3.10). ANOVA revealed no significant interaction between OXY * antagonist ($F_{(2, 54)}$=2.27 p=0.113). However, there was a significant main effect of OXY on the total duration of aggressive behaviours ($F_{(1, 54)}$=16.03, p<0.001), with OXY reducing aggressive behaviours in all groups, regardless of antagonist administration. No main effect of the antagonists upon aggressive behaviours was observed.

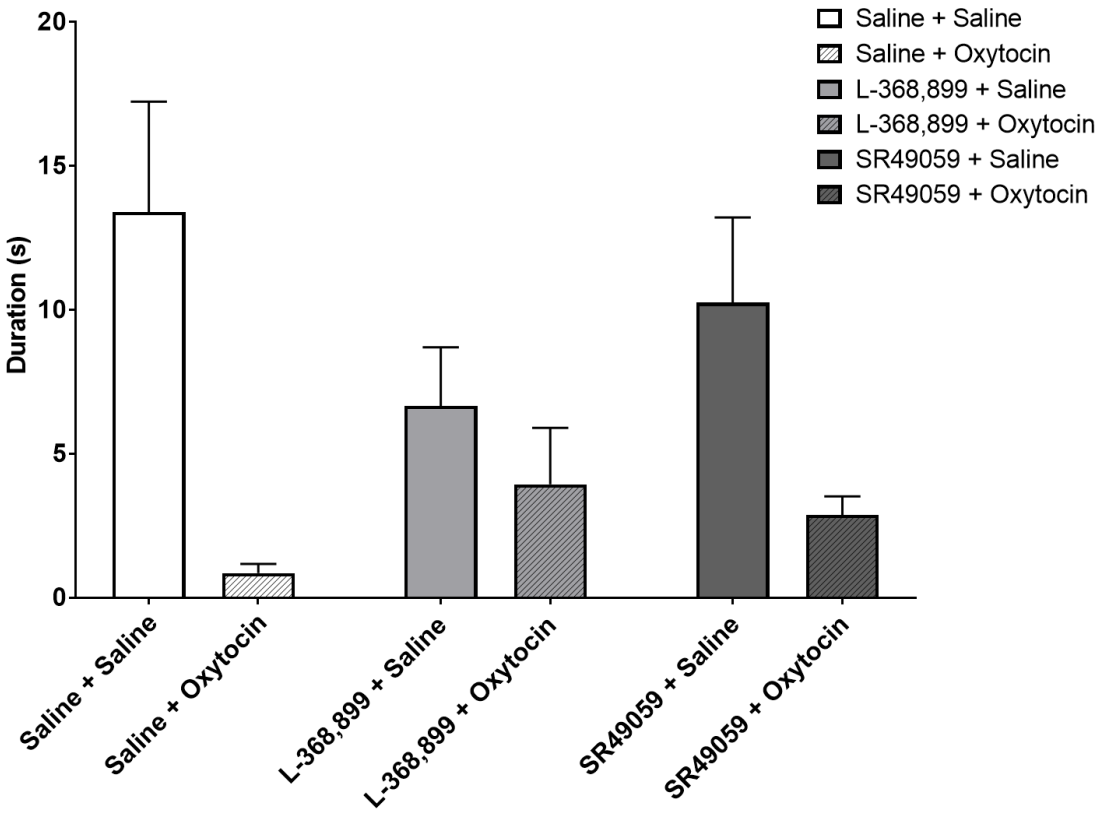

Figure 3.10: Effect of OXY, and OXTR and V1aR antagonism, on the duration of aggressive behaviours during social interaction (mean + SEM, n=10/group). Two-way ANOVA revealed no significant interaction between OXY * antagonist ($F_{(2, 54)}$=2.27 p=0.113). However, there was a significant main effect of OXY on the total duration of aggressive behaviours ($F_{(1, 54)}$=16.03, p<0.001), with OXY reducing the duration of aggressive behaviours in all groups, regardless of antagonist administration.

3.4.3 Ultrasonic Vocalisations

To determine if OXY had any effect on the emission of USVs during social behaviour, USVs were recorded during social interaction and analysed following recording with the experimenter blinded to treatment. One day of USV recordings were not recorded due to a fault with the recording equipment, therefore n=7 (saline + OXY and SR 49059 + OXY) or 8 (all other groups). No 22 kHz calls were seen in any of the files recorded. 50 kHz calls were classified into three subtypes: flat, step and trill, and data was $\log_{10}$ transformed to meet the assumption of normality. A two-way ANOVA for each call type revealed no significant effect of treatment on the number of calls emitted during social interaction (Figure 3.11) ($F_{(1,\ 35)}$=2.89, p=0.10; $F_{(1,\ 35)}$=0.56, p=0.46 and $F_{(1,\ 35)}$=3.46, p=0.071 for flat, trill, and step calls respectively).

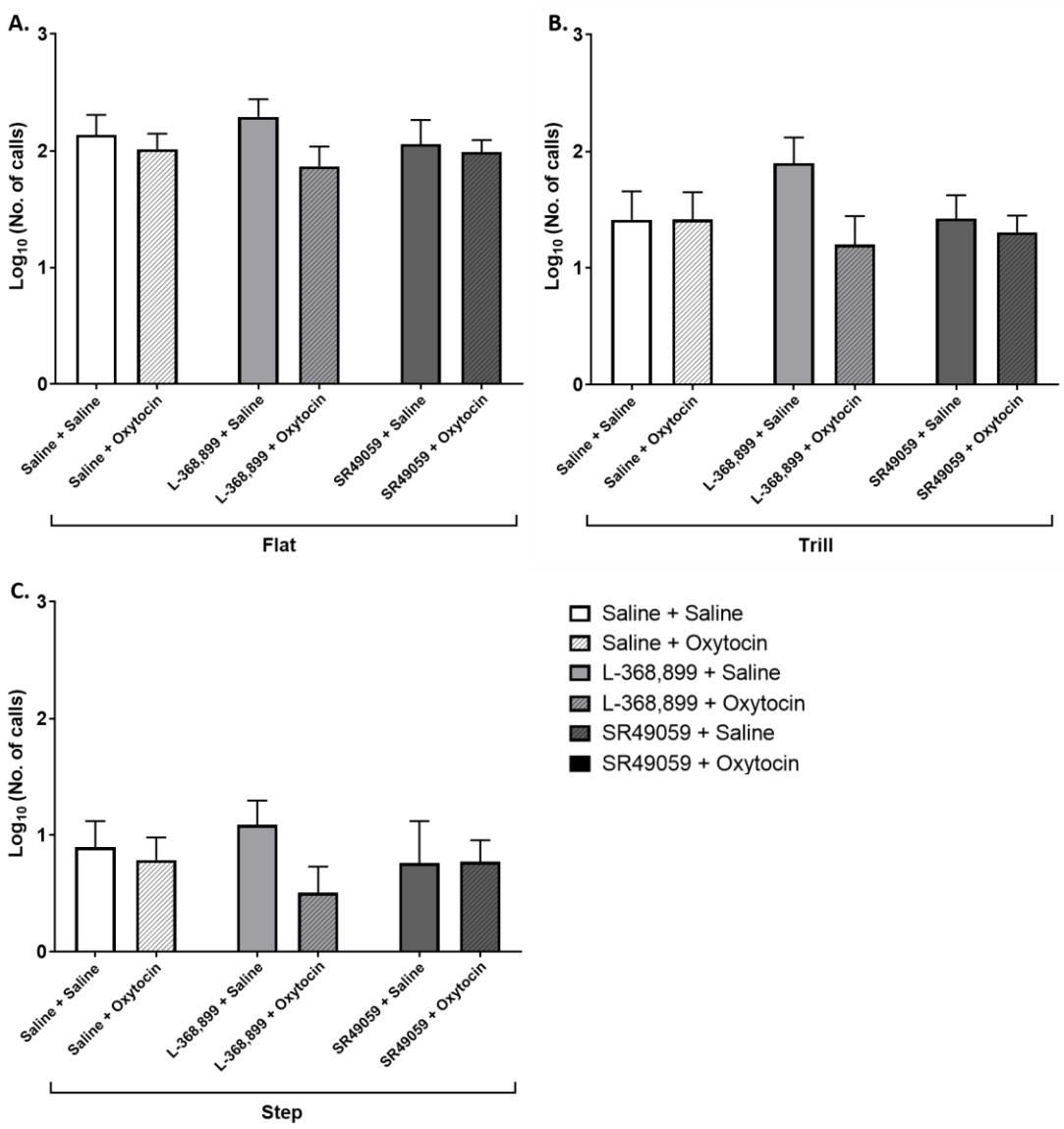

Figure 3.11: The effect of OXY, and OXTR and V1aR antagonism, on (A) flat, (B) trill, and (C) step prosocial 50 kHz calls (log_{10} mean + SEM, n= n=7 (saline + OXY and SR 49059 + OXY) or 8 (all other groups)). Data was log_{10} transformed to produce a normal distribution for analysis. Analysis with a two-way ANOVA revealed no effect of either OXY, or OXTR and V1aR antagonists, upon either trill, flat, or step calls.

FM prosocial 50 kHz calls (step and trill) are considered to be particularly reflective of a positive affective state and emitted during social interaction (Brudzynski, 2015), therefore the effect of the treatment upon the percentage of FM calls emitted was assessed (Figure 3.12). Two-way ANOVA revealed no significant effect of treatment on the percentage of FM prosocial calls emitted during social interaction ($F_{(2, 40)}$=2.432, p=0.10). In addition, the effect of treatment on the latency to first call was also analysed; reduced latency to first call has been observed in conjunction with

increased emission of 50 kHz calls following pharmacological manipulation (Mulvihill & Brudzynski, 2019). Two-way ANOVA revealed a significant main effect of OXY on the latency to first call ($F_{(1, 40)}$=4.614, p=0.037), such that OXY appeared to increase the duration to first call in the presence of both antagonists. However, no significant main effect of antagonist, nor an interaction between OXY and antagonist, was observed.

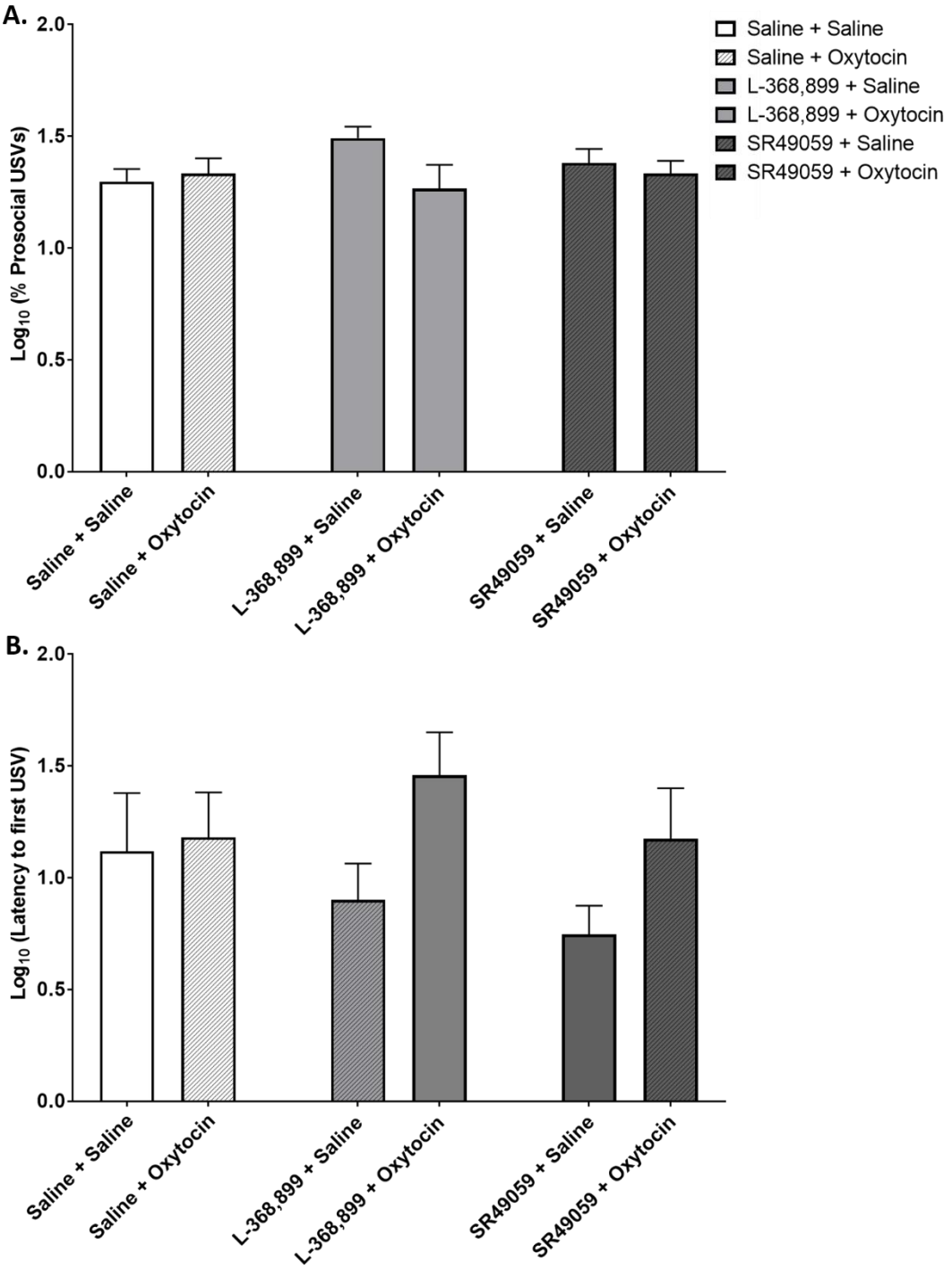

Figure 3.12 Effect of treatment on (A) the percentage of FM prosocial calls emitted and (B) the latency until the first USV produced during the social interaction task (log_{10} mean + SEM, n=7 (saline + OXY and SR 49059 + OXY) or 8 (all other groups)). Data was log_{10} transformed to produce a normal distribution for analysis. Two-way ANOVA revealed no significant effect of treatment upon the % prosocial calls emitted during social interaction ($F_{(2, 40)}$=2.432, p=0.10). However, a significant main effect of OXY was revealed on the latency to first call ($F_{(1, 40)}$=4.614, p=0.037), such that OXY appeared to increase the latency to first call in the presence of both SR 49059 and L-368,899. No significant main effect of antagonist, or an interaction between OXY and antagonist, was observed.

In addition to analysing the effect of treatment upon USV emission, the relationship between the production of FM prosocial USVs and the duration of prosocial behaviours during social interaction was also assessed (Figure 3.13A). A Pearson's correlation was run, however there was no significant correlation between the duration of prosocial behaviours and the percentage of FM prosocial USVs emitted during the social interaction task ($r(44) = 0.082$, $p=0.59$). In addition, a Pearson's correlation was also used to assess the relationship between the duration of prosocial behaviours and the latency to first call during social interaction (Figure 3.13B). Again, there was no statistically significant correlation between the duration of prosocial behaviours and the latency to first call during the social interaction task ($r(44) = -0.099$, $p=0.51$).

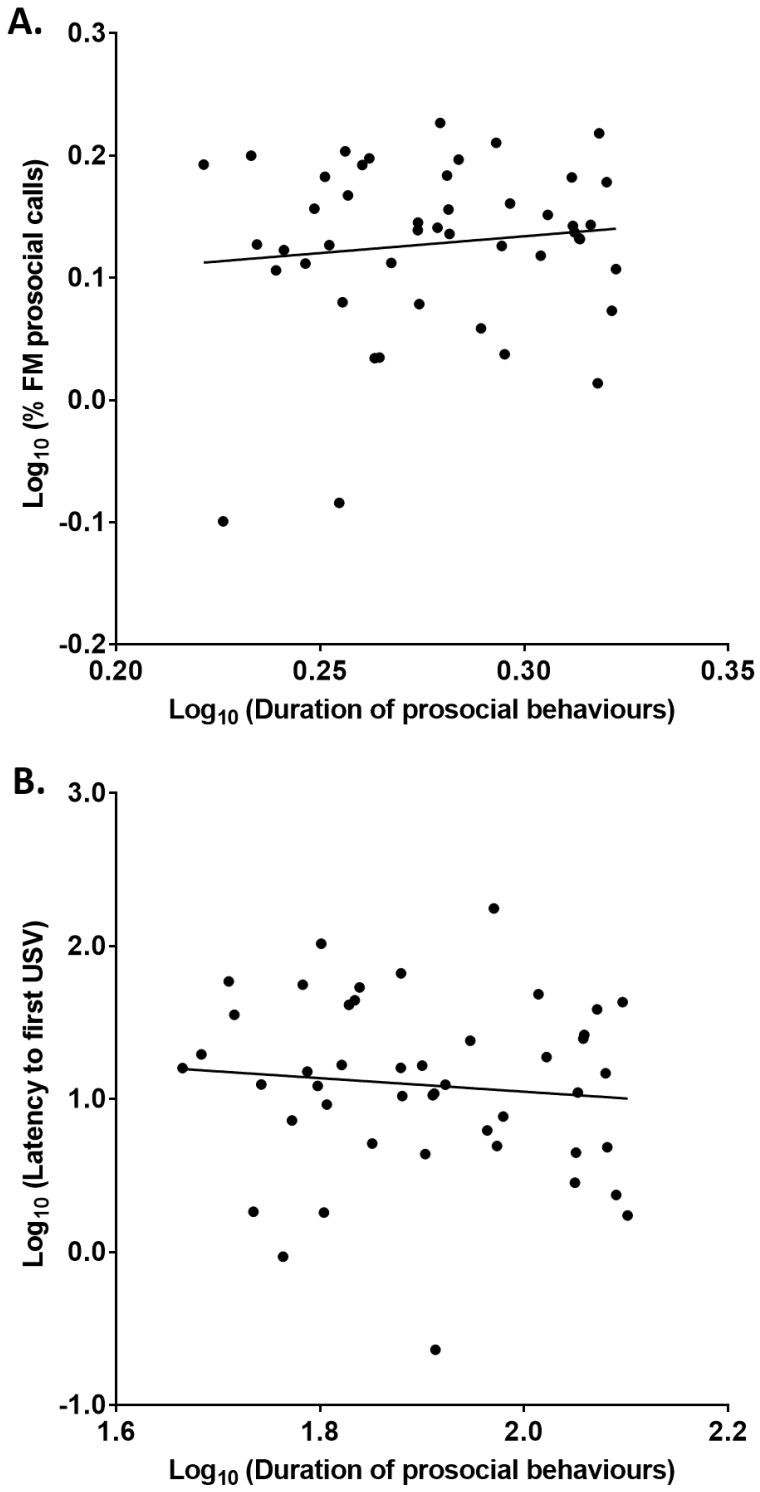

Figure 3.13: The correlation between the duration of prosocial behaviours in social interaction with (A) the percentage of prosocial calls emitted and (B) the latency to first USV (data was $\log_{10}$ transformed to produce a normal distribution for analysis). A Pearson's correlation found no statistically significant correlation between the duration of prosocial behaviours and % prosocial USVs emitted during the social interaction task ($r(44) = 0.082$, $p=0.59$), nor the latency to first call during social interaction ($r(44) = -0.099$, $p=0.51$).

3.5 Discussion

3.5.1 Effect of OXY, and OXTR and V1aR antagonism, on PCP-induced hyperactivity

In the LMA assessment we demonstrated OXY administration can reduce PCP-induced hyperactivity, consistent with previous work in this laboratory (Kohli *et al.*, 2019), and is suggestive of an OXY-induced antipsychotic-like effect. An antipsychotic-like effect of OXY has previously been noted in brown male Norway rats, with s.c. OXY facilitating latent inhibition in a dose dependent manner (Feifel *et al.*, 2015) and restoring prepulse inhibition in rats that had been disrupted by dizocilpine, a non-competitive NMDA-R antagonist (Feifel & Reza, 1999). Interestingly, as well as OXY reducing PCP-hyperactivity, administration of the OXTR antagonist L-368,899 also appeared to attenuate PCP-induced hyperactivity, although this did not reach significance. Administration of L-368,899 alongside OXY prevented OXY from significantly attenuating PCP-induced hyperactivity, suggesting a role for the OXTR in OXYs antipsychotic-like effect. However, L-368,899 did not fully reverse OXY-induced attenuation of PCP-hyperactivity back to the levels seen with PCP alone. In addition, PCP was able to significantly increase LMA in the presence of SR 49059 and OXY; suggesting the V1aR is also likely involved in OXY attenuation of PCP-hyperactivity. High doses of peripheral OXY (0.3mg/kg – 1.0mg/kg s.c.) can reduce LMA in the absence of any other drugs (Wolfe *et al.*, 2018). However, our low dose of OXY, nor SR 49059 and L-368,899, did not have any effect on LMA in the absence of PCP, suggesting the attenuation of PCP-induced hyperactivity was not due to the sedative effect of OXY often observed at higher doses (Angioni *et al.*, 2016; Wolfe *et al.*, 2018).

Other than this laboratory, no other group has studied the effect of OXY on NMDA-R antagonist-induced hyperactivity. However, OXY (2mg/kg i.p.) can decrease methamphetamine-induced hyperactivity in male Sprague Dawley rats (Carson *et al.*, 2010a), which was accompanied by reduced c-Fos expression in the NAc core and

subthalamic nucleus (Carson *et al.*, 2010b). Cocaine-induced hyperactivity in mice can also be attenuated following s.c. OXY administration, which occurred in conjunction with reduced NAc dopamine content (Kovacs *et al.*, 1990); these studies may implicate the NAc in the reversal of PCP-induced hyperactivity seen in this study. PCP administration dose dependently increases dialysate levels of both dopamine and 5-HT in the NAc, striatum, and frontal cortex in male Wistar rats (Millan *et al.*, 1999), and dopamine antagonism in the NAc can reduce NMDA antagonist-induced LMA (Del Arco *et al.*, 2008). However, given both PCP and our dose of OXY are known to increase NAc dopamine (Kohli *et al.*, 2019), it would seem unlikely reduced NAc dopamine could be responsible for OXY attenuation of PCP-induced hyperactivity. Indeed, NMDA-R antagonists are still able to cause hyperactivity following chemical lesions of NAc projecting dopaminergic neurons (Carlsson & Carlsson, 1989).

Several papers have implicated the PFC in NMDA-R antagonist-induced hyperactivity (Li *et al.*, 2010; Takahata & Moghaddam, 2003). PFC glutamatergic efferents project to the VTA, an area with dopaminergic outputs to motor areas, such as the supplementary motor area and premotor cortex (Hosp *et al.*, 2019; Tan *et al.*, 2019). It is hypothesised that PCP can cause hyperactivity through NMDA-R antagonism on PFC GABA neurons. This disinhibits cortico-cortical glutamatergic neurons, raising the concentration of extracellular glutamate in the PFC (Kehr *et al.*, 2018; Li *et al.*, 2010). Synaptic glutamate can then bind to non-NMDA receptors on glutamatergic PFC efferents, which project to areas such as the NAc and VTA (Qi *et al.*, 2012; Takahata & Moghaddam, 2003); increasing excitatory outputs to connected motor nuclei (Groenewegen & Uylings, 2000; Hosp *et al.*, 2019; Taha *et al.*, 2007). In support of this theory, AMPA antagonism in the PFC, NAc, and VTA can all inhibit PCP-induced hyperactivity, without modulating NAc dopamine (Takahata & Moghaddam, 2003). In the VTA OXTRs are located on excitatory dopaminergic and glutamatergic neurons (Peris *et al.*, 2017). Peris et al. also identified OXTRs present on neurons that were neither glutamatergic nor dopaminergic; hypothesising the other OXTRs may be present on GABAergic interneurons, known to be abundant in the VTA (Morales & Root, 2014). Therefore, OXY agonism on GABA interneurons in the VTA may be able to explain how OXTR agonism can attenuate PCP-induced hyperactivity, through

inhibiting VTA outputs to motor areas. OXTR expression on VTA glutamatergic neurons may also explain why we saw reduced PCP-hyperactivity following OXTR antagonism, albeit not significant, and why OXTR antagonism did not fully reinstate PCP-induced hyperactivity. These results may suggest there is a fine balance of OXTR activity needed to attenuate PCP-induced hyperactivity. V1aRs are also expressed in the VTA (Johnson *et al.*, 1993; Song *et al.*, 2016). Although the phenotype of the neurons they are expressed on is unknown, if V1aRs are also expressed on GABA interneurons, OXY agonism of V1aRs would reduce excitatory VTA outputs to motor areas, thus also reducing PCP-induced hyperactivity; explaining how PCP could still increase LMA in the presence of SR 49059 and OXY.

3.5.2 Effect of OXY, and OXTR and V1aR antagonism, on social interaction

This study demonstrated a significant increase in social interaction following s.c. OXY administration; predominantly due to increased body sniffing, consistent with previous work in our laboratory (Kohli *et al.*, 2019). Prior administration of the selective OXTR antagonist, L-368,899, prevented OXY enhancement of social interaction, while in contrast the V1aR antagonist had no effect. This suggests OXYs ability to enhance social behaviour in rats is likely modulated through activity at the OXTR, with no effect of the V1aR system in this paradigm. Interestingly, OXY administration also appeared to reduce the duration of aggressive behaviours (boxing and biting, and pinning), consistent with Calcagnoli *et al.* (2013).

The increase in social interaction seen in this study was mainly due to increased sniffing behaviours; unsurprising given olfactory cues play a crucial role in rodent interactions. Social recognition in rodents relies heavily upon pheromonal odour cues (Wacker & Ludwig, 2012), with the olfactory system being necessary for the processing of social stimuli (Marlin & Froemke, 2017). In fact olfactory stimuli are thought to the primary form of communication in rodents, and are evolutionarily older than USVs (Potasiewicz *et al.*, 2020). In this paradigm increased sniffing

following OXY administration may facilitate the social recognition process, allowing the conspecific to be recognised as 'novel' more rapidly, thus increasing social exploration and social interaction (Millan & Bales, 2013).

The precise brain regions involved in OXYs prosocial effects are unknown, with many OXTR expressing regions being implicated in different aspects of social behaviour. For example, the main olfactory bulb (OB) plays a key role in social recognition (Oettl *et al.*, 2016). OXY modulation of the olfactory system may explain the increase in sniffing duration seen in this study following s.c. OXY administration. The main OB is critical in processing olfactory information in rodents and lesions of the main OB impair conspecific recognition (Sanchez-Andrade & Kendrick, 2009). The processing of olfactory cues in the main OB are modulated by GABAergic granule cell interneurons, which receive glutamatergic inputs from the anterior olfactory nucleus (AON). The AON has high levels of OXTR expression and receives oxytocinergic inputs from the PVN (Knobloch *et al.*, 2012; Lee *et al.*, 2019b; Oettl & Kelsch, 2018). Activity of OXY in the AON can increase interneuron activity in the main OB, increasing the overall inhibitory tone. By decreasing the background activity within the OB, the peak response to odours is increased (Oettl & Kelsch, 2018). This improves the signal to noise ratio in the main OB, thereby facilitating social recognition (Oettl & Kelsch, 2018; Oettl *et al.*, 2016). Genetic deletion of AON OXTRs in adult mice impairs exploration of a conspecific, and optogenetically evoked OXY release in the AON increases the social investigation of same-sex conspecifics (Oettl *et al.*, 2016). In this study s.c. OXY increased exploration of conspecifics, and was prevented through OXTR antagonism, therefore OXY activity at AON OXTRs may explain this finding. OXTR expression in other brain regions may also enhance social recognition, such as those in the medial amygdala. The medial amygdala receives inputs from the accessory OB, which receives olfactory inputs (Ferguson *et al.*, 2000; Keverne, 1999; Scalia & Winans, 1975). In mice OXTR antagonism in the medial amygdala disrupts social recognition, while in OXY knockout mice direct OXY administration to the medial amygdala can rescue disrupted social recognition (Ferguson *et al.*, 2001).

However, other brain regions are also likely to be heavily implicated in OXY enhancement of social behaviour. The dose and route of OXY given in this study is

known to cause dopamine overflow in the NAc (Kohli *et al.*, 2019), suggesting that OXY-induced increases in social interaction may be, at least in part, mediated by NAc activity; a brain region that has been implicated in social approach (the time spent near an novel conspecific) and social reward (Dölen *et al.*, 2013; Williams *et al.*, 2020). Increases in extracellular NAc dopamine have also been demonstrated following OXY injection into the VTA and PVN of male Sprague Dawley rats, both of which were reversed by prior OXTR antagonist injection (Melis *et al.*, 2007). The NAc is part of the mesocorticolimbic system and contains mesolimbic dopaminergic nerve terminals (Floresco, 2015). The cell bodies of these neurons are located in the VTA and are understood to play a key role in reward; reinforcing behaviours such as social interaction (Melis *et al.*, 2007). Fiber photometry in mice has demonstrated the role of mesolimbic circuits in social behaviour; VTA projections to the NAc are able to encode and predict social interaction, and optogenetic modulation of these neurons can bidirectionally alter social behaviour (Gunaydin *et al.*, 2014). Furthermore, OXY in the VTA can alter mesolimbic neuronal activity and social behaviour through OXTR activity (Hung *et al.*, 2017; Xiao *et al.*, 2017); demonstrated through reduced social conditioned place preference following ablation of OXTRs expressed on VTA dopaminergic neurons (Hung *et al.*, 2017). Maternal behaviour is also regulated by OXY facilitation of mesolimbic dopamine (Insel, 1997; Love, 2014; Shahrokh *et al.*, 2010). During social interaction there is an increase in VTA-projecting PVN oxytocinergic neuronal activity (Hung *et al.*, 2017). Optogenetic activation of these neurons during social interaction can increase social behaviour, whilst inhibition decreases social behaviours (Hung *et al.*, 2017). Additionally, research has shown OXTRs in the NAc itself may also play a prominent role in social behaviour, from modulating partner preference (Young *et al.*, 2001) and pair bonding in female prairie voles (Keebaugh & Young, 2011), to social conditioned place preference in mice (Dolen *et al.*, 2013). The above provides good evidence that OXY modulation of mesocorticolimbic dopamine can alter social behaviour through OXTR activity; providing a possible explanation of the OXTR sensitive OXY enhancement of social behaviours observed in this chapter.

3.5.3 Effect of OXY, and OXTR and V1aR antagonism, on USVs

This study demonstrated no effect of OXY administration on the production of USVs during social interaction, nor a correlation between the number of FM 50 kHz calls and duration of social behaviours. These results suggest the OXY dose and route of administration used in this study, and the V1aR and OXTR, have no effect on the production of 50 kHz calls in male Lister-hooded rats. This is consistent with previous reports finding no OXY effect on USVs during a social interaction task in rats (Kohli *et al.*, 2019) and no effect of vasopressin administration on rat USV emission (Lukas & Wohr, 2015). Although previous research has shown USVs occur in unison with social behaviours, our research suggests they are likely to be modulated by a different neurocircuitry system.

Previous work has shown both peripheral and intracerebral NAc injections of dopamine agonists are able to reliably increase the emission of 50 kHz calls, in comparison to vehicle (Barker *et al.*, 2010; Burgdorf *et al.*, 2001; Burgdorf *et al.*, 2007; Thompson *et al.*, 2006; Williams & Undieh, 2010). Given our dose of OXY increases NAc dopamine it is surprising no effect on the production of prosocial 50 kHz USVs was observed (Kohli *et al.*, 2019). The lack of change in USV production observed during social interaction, despite the increase in social behaviours, suggests changes to social behaviour arising from OXY administration are not the result of increased social communication, but an alternative neural mechanism.

3.6 Summary and future work

During this chapter we demonstrated s.c. OXY can enhance prosocial behaviours during social interaction through OXTR activity, with no effect of the V1aR, in male Lister-hooded rats. However, we observed no effect of OXY or either antagonist upon the emission of prosocial 50 kHz calls during social interaction; suggesting changes to social behaviour arising from OXY administration are not the result of increased social communication, but of an alternative neurocircuitry mechanism. We also

demonstrated activity of OXY at both the OXTR and V1aR contributes to OXY attenuation of PCP-induced hyperactivity. OXYs ability to enhance non-reproductive social behaviours, such as social interaction, are thought to be brain region specific, with variations in OXTR expression in different regions controlling specific differences in social behaviours (Yu *et al.*, 2016). To further understand the mechanisms of this behavioural effect the precise brain regions involved need to be deciphered. The use of selective toxins, such as OXY-saporin, can be utilised to delineate the OXTR expressing regions involved and responsible for behavioural changes upon OXY administration, and will be explored in subsequent chapters.

Chapter 4: Oxytocin-saporin microinjection into the nucleus accumbens; effects on social behaviour and LMA activity.

4.1 Introduction

In Chapter 3 we demonstrated that OXTRs are responsible for enhancing social behaviour following systemic OXY administration and also play a role in the 'antipsychotic-like' effect of OXYs attenuation of PCP-induced hyperactivity. OXYs affects upon social behaviour are site specific, with differential activation of OXTRs in various regions of the brain leading to distinct forms of social behaviour (Newman, 1999; O'Connell & Hofmann, 2012; Smith *et al.*, 2019; Yu *et al.*, 2016). For example, NAc OXTR antagonist infusion can block partner preference in female prairie voles, whilst infusion into the adjacent caudate putamen has no effect (Young *et al.*, 2001). Furthermore, while i.c.v. OXY can enhance social preference in rats following social defeat, selective application of OXY and an OXTR antagonist into different sub-nuclei of the amygdala have no effect upon stress-induced social preference (Lukas *et al.*, 2011). In addition, in rats OXTR antagonist infusion (desGly-NH_2,d$(CH_2)_5$-[Tyr(Me)^2,Thr^4]OVT) reduced social novelty preference when injected into the NAc, but not when infused into the lateral septum or basolateral amygdala (Smith *et al.*, 2017b). Therefore, to fully understand the mechanisms through which OXY-induced behavioural changes occur, we need to decipher the brain regions involved.

The NAc is located within the ventral striatum, part of the of the mesocorticolimbic system and composed of two distinct subdivisions: the NAc shell and NAc core. The majority (>90%) of cells in the NAc are D_1 or D_2 dopamine receptor expressing GABAergic medium spiny neurons (MSNs) (Floresco, 2015; Li *et al.*, 2018) that exert balanced, opposing effects in the NAc (Lobo *et al.*, 2010; Lobo & Nestler, 2011). NAc MSNs receive excitatory glutamatergic inputs from numerous brain regions, including the PFC and hippocampus, and are connected by gap junctions and GABA-A containing synapses (Bamford & Wang, 2019; Zahm, 2000). The NAc also receives dopaminergic inputs from the VTA (Ikemoto, 2007), and abundant oxytocinergic

input from parvocellular neurons of the PVN, which release OXY into the NAc (Dolen & Malenka, 2014; Ferretti *et al.*, 2019; Knobloch *et al.*, 2012).

The NAc has high levels of OXTR expression in rats (Smith *et al.*, 2019; van Leeuwen *et al.*, 1985), and both central and peripheral OXY administration can enhance NAc dopamine release (Kohli *et al.*, 2019; Melis *et al.*, 2007; Melis *et al.*, 2009). In rats, OXTR expression in the NAc core reduces during development from juvenile to adulthood, but remains high (Smith *et al.*, 2017a). In contrast, OXTR expression in the NAc shell remains constant across age (Nardou *et al.*, 2019). Interestingly, in contrast to other brain regions, NAc OXTR shell expression is consistent between males and females (Smith *et al.*, 2017a). High NAc OXTR expression across multiple species, and between males and females, suggests NAc OXTRs have a translational, key role in social behaviour (Dolen *et al.*, 2013; Insel & Shapiro, 1992; Lee *et al.*, 2017; Ross *et al.*, 2009b; Schorscher-Petcu *et al.*, 2009; Smith *et al.*, 2017a). In fact, the NAc is considered a key node in the social decision-making network (O'Connell & Hofmann, 2011) and plays an important role in social reward (Dolen *et al.*, 2013; Resendez *et al.*, 2013), social novelty preference (Smith *et al.*, 2017b), pair bond formation (Keebaugh *et al.*, 2015; Ross *et al.*, 2009a), and alloparental care (Keebaugh *et al.*, 2015). Preclinical evidence has also suggested natural variations in NAc OXTR expression may contribute to individual variations in social behaviour seen within species (Keebaugh *et al.*, 2015; Olazábal & Sandberg, 2020; Ross *et al.*, 2009b).

While there is an abundance of research to suggest NAc OXTRs play an important role in reproductive social behaviours, such as pair bonding, maternal bonding, and alloparental care (Keebaugh & Young, 2011; Liu & Wang, 2003; Olazabal & Young, 2006; Ross *et al.*, 2009b; Young *et al.*, 2001), there is much less research investigating the role of NAc OXTRs in non-reproductive social behaviours. However, research has shown bilateral infusion of OXY into the NAc can increase social preference between same sex prairie voles, which is dose dependently reduced by NAc OXTR antagonist infusion ([d$(CH_2)_5$,Tyr(Me)^2,Thr^4,Tyr-NH_2^{9}]-OVT) (Yu *et al.*, 2016). NAc OXTRs can also drive social recognition in male rats; increasing the time spent interacting with a novel conspecific, as opposed to a familiar cage mate, demonstrated by NAc microinjections of the selective OXTR antagonist desGly-NH_2,d$(CH_2)^5$-

[Tyr(Me)^2,Thr^4]OVT (Smith *et al.*, 2017b). In mice, NAc OXTR expression influences social hierarchies; socially dominant males have higher NAc core OXTR densities than subordinate males (Lee *et al.*, 2019b). Similarly, following chronical social defeat voles have reduced levels of both OXY and OXTRs in the NAc, and social withdrawal arising from chronic social defeat can be reversed by NAc OXY administration, causing OXTR activation (Hou *et al.*, 2020; Wang *et al.*, 2018).

The rewarding nature of social interactions is what drives social behaviour in both rodents and humans, and is essential for the formation of hierarchical, reproductive, and non-reproductive social relationships, and if impaired can result in profound psychiatric disorders (Dölen *et al.*, 2013; Fone & Porkess, 2008; Hany *et al.*, 2021; Nardou *et al.*, 2019; Song *et al.*, 2016). Social reward likely evolved to motivate social behaviours, as they are essential for survival, reproduction, and neural development. Mesolimbic dopamine has long been associated with reward; therefore it is likely that the oxytocinergic system interacts with the mesolimbic system to influence social behaviour (Dölen *et al.*, 2013; Lobo & Nestler, 2011). Consistent with this hypothesis, the dose of s.c. OXY used in this thesis to reliably increase social interaction through OXTR activity also increased dopamine overflow in the NAc of male Lister-hooded rats (Kohli *et al.*, 2019). Voltammetry studies in rats have demonstrated significant increases in NAc dopamine during conspecific interaction (Robinson *et al.*, 2002), and optogenetic activation of mesolimbic VTA-NAc projections increased social interaction in mice (Gunaydin *et al.*, 2014). In addition, electrophysiological recordings in mice show increased NAc activity during social interaction, but not novel objection exploration (Gunaydin *et al.*, 2014). Interestingly, the prosocial effect observed following optogenetic activation of VTA-NAc dopaminergic projections in mice is attenuated by a NAc micro-infusion of the D_1 receptor antagonist SCH23390 (Gunaydin *et al.*, 2014). Furthermore, optogenetic activation of NAc D_1 expressing cells significantly increased social interaction; indicating dopamine signalling through the NAc D_1 receptor is involved in non-reproductive social behaviours (Gunaydin *et al.*, 2014). Activation of D_1 MSNs has long been associated with increasing the effects of rewarding stimuli (reviewed by Lobo and Nestler (2011)), therefore demonstrating a role for NAc dopamine and reward circuitry in the enhancement of social behaviour.

OXTRs are expressed on MSNs within the NAc (Williams *et al.*, 2020), making it likely NAc OXTRs contribute to social reward. As such, NAc OXTR blockade inhibits social conditioned place preference but not cocaine conditioned place preference in mice, and selective ablation of presynaptic NAc OXTRs inhibits social conditioned place preference (Dolen *et al.*, 2013).

The mesolimbic dopamine system may also be involved in the communication of rats during social interaction. The NAc, in particular, appears to be critically involved in the production of 50 kHz prosocial USVs. Emission of prosocial USVs are increased following injection of dopamine agonists both systemically and directly into the NAc (Brudzynski, 2015; Burgdorf *et al.*, 2001; Mulvihill & Brudzynski, 2019; Simola *et al.*, 2012; Thompson *et al.*, 2006), and synaptic release of dopamine in the NAc from VTA mesolimbic dopaminergic neurons can initiate emission of 50 kHz USVs (Scardochio *et al.*, 2015). Furthermore, playback of 50 kHz calls to freely moving rats initiates both social approach towards the speaker and phasic NAc dopamine release (Willuhn *et al.*, 2014), and 50 kHz call emission induced by tickling in adolescent rats is blocked through NAc administration of both D_1 and $D_{2/3}$ receptor antagonists (SCH23390 and raclopride, respectively) (Hori *et al.*, 2013). However, in Chapter 3 we saw no effect of s.c. OXY on prosocial USV emission during social interaction, consistent with Kohli *et al.* (2019), despite the observation that the dose of OXY used increases NAc dopamine efflux (Kohli *et al.*, 2019).

Interestingly, the NAc OXTRs involvement in social behaviours appear to be translational. Human fMRI studies have shown carriers of an OXTR SNP (rs237915) have aberrant ventral striatum activation in response to viewing angry emotions, and report increased emotional and peer problems in low stress environments (Loth *et al.*, 2014). Although this polymorphism is not currently associated with ASD or schizophrenia, people with ASD have demonstrated reduced NAc neural activity during social play (Assaf *et al.*, 2013).

The OXTR is also involved in the attenuation of PCP-induced hyperactivity. As discussed in Chapter 3, PCP administration dose dependently increased dialysate levels of both dopamine and 5-HT in the NAc (Millan *et al.*, 1999), and dopamine antagonism in the NAc can reduce NMDA-R antagonist induced LMA (Del Arco *et al.*,

2008). However, NMDA-R antagonists are still able to cause hyperactivity following chemical lesions of NAc projecting dopaminergic neurons (Carlsson & Carlsson, 1989). We previously demonstrated OXY administration was able to significantly attenuate PCP-induced hyperactivity, and pre-treatment with an OXTR and a V1aR antagonist could prevent the OXY attenuation of PCP-induced hyperactivity, although not returning the response fully back to that of PCP alone (Chapter 3). With both PCP and OXY increasing NAc dopamine it would seem paradoxical that attenuation of PCP-hyperactivity could be achieved through changes in NAc dopamine activity. However, within the NAc OXTRs are primarily expressed upon MSNs (Williams *et al.*, 2020), with a population of D_1 MSNs directly projecting to the VTA (Baimel *et al.*, 2019). The VTA has dopaminergic outputs to motor areas, such as the supplementary motor area and premotor cortex (Hosp *et al.*, 2019; Nguyen *et al.*, 2018), making it plausible OXTR activity within the NAc could in some way be contributing to OXY attenuation of PCP-hyperactivity.

Saporin is a cytotoxic compound derived from the soapwort plant that causes cell death through ribosome inhibition, preventing protein synthesis (Baskin *et al.*, 2010). Saporin has been conjugated to a number of ligands to take advantage of this mechanism to selectively destroy neurons expressing the ligand binding site, such as 192 IgG-saporin to selectively destroy cholinergic neurons (Book *et al.*, 1994). In an analogous manner, conjugation of saporin to OXY confers specific binding of the conjugate to OXTR expressing cells, selectively destroying OXTR expressing neurons (Angioni *et al.*, 2016; Baskin *et al.*, 2010).

Saporin is a ribosome inactivating protein (RIP) which inhibits protein synthesis and causes cell death (Bagga *et al.*, 2003; Baskin *et al.*, 2010). Saporin achieves this by cleaving the *N*-glycosidic bond, which connects ribonucleic acid (RNA) bases to the sugar phosphate backbone of a specific adenine in 28 S ribosomal RNA, a structural component of the ribosome large subunit. This cleavage prevents the binding of elongation factors to ribosomes, preventing protein synthesis (Bagga *et al.*, 2003; Walsh *et al.*, 2013). There are two types of RIPs: type 1 and type 2, with saporin falling into the type 1 category. Type 1 RIPS are monomeric proteins, which have RNA *N*-glycosidase enzymatic activity. In contrast, type 2 RIPSs consist of two disulphide-

linked polypeptides; an A-chain which has RNA *N*-glycosidase activity and one or more lectin-like B-chains. While the *N*-glycosidase activity promotes the cytotoxic effects of RIPs, the B-chains of type 2 RIPs promote cell entry, through interacting sugars expressed on cell surfaces, promoting cell entry (Bagga *et al.*, 2003; Walsh *et al.*, 2013). As saporin is a type 1 RIP, and therefore lacks a B-chain, it is unable to enter cells on its own. However, when conjugated to a cell receptor ligand penetration of the cell membranes expressing the ligand receptor can selectively occur (Walsh *et al.*, 2013). In this case conjugation to OXY, forming OXY-saporin, results in internalisation into OXTR expressing neurons, selectively destroying OXTR expressing neurons whilst minimising unwanted side-effects (Baskin *et al.*, 2010).

OXY-saporin has previously been used to determine the specific brain regions involved in OXY modulated behaviours, such as LMA (Angioni *et al.*, 2016) and satiety (Baskin *et al.*, 2010). Work in the next two chapters takes advantage of the selective destruction of OXTR expressing neurons that occurs following OXY-saporin micro-injection to discrete brain nuclei, to decipher the role of specific OXTR expressing neuronal populations on behaviours, including social interaction and attenuation of PCP-hyperactivity.

As well as high OXTR expression in the NAc, OXTRs are expressed within the VTA on glutamatergic and dopaminergic neurons that project to the NAc (Peris *et al.*, 2017). The NAc also receives innervation from oxytocinergic PVN neurons, making the NAc highly susceptible to modulation by OXY (Dölen *et al.*, 2013; Gunaydin *et al.*, 2014; Melis *et al.*, 2007; Song *et al.*, 2016). In addition, the NAc also receives inputs from other brain regions associated with social behaviour, including the hypothalamus and PFC (Gunaydin & Deisseroth, 2014). It is therefore likely that OXY activity in the NAc contributes to some of the behavioural effects seen following s.c. OXY in rodents, such as increased social interaction and attenuation of PCP-induced hyperactivity, which are reported herein.

4.2 Aims

No studies to date have examined the role of NAc OXTRs in rats during non-reproductive social behaviours. To decipher the role of NAc OXTRs this study will use a bilateral micro-injection of the OXTR targeting toxin, OXY-saporin, to destroy OXTR expressing neurons in the NAc. The aim of this study is to determine the role of NAc OXTRs in OXY enhancement of social interaction and OXY attenuation of PCP-induced hyperactivity in male Lister-hooded rats.

4.3 Materials and Methods

4.3.1 Animals

80 male Lister-hooded rats (42-48 days; Charles River UK) were housed in groups of four in individually ventilated cages with their litter mates. Upon ordering rats were specifically requested to be caged and transported with their litter mates. Rats were gently handled for seven days prior to beginning behavioural studies to reduce anxiety enhance animal welfare (LaFollette *et al.*, 2017). Housing details and ethical requirements are as described in section 3.3.1. At the end of the study all rats were killed by the S1 method of concussion, and death was confirmed by exsanguination.

4.3.2 Experimental Design

As detailed in Chapter 3.3.2, group sizes of n=10 was aimed for. This gives the statistical test >80% power to detect an effect size of η^2=0.14 at a significance threshold of p=0.05, when using a when using a repeated measures, between factors ANOVA. Power analysis was conducted with GPower 3.1 (Faul *et al.*, 2007), ensuring sufficient power to allow statistically significant findings to be obtained, while still adhering to the 3R's principle.

All treatments were randomly allocated so that all litters received all treatment groups. During LMA assessment all treatment groups were randomly allocated across multiple runs to all 10 LMA arenas, to prevent any extraneous bias, and the experimenter was blinded to treatment.

4.3.3 Drugs

OXY was obtained from Bachem (St. Helens, UK) and PCP from Sigma Aldrich (Poole, UK). The toxins OXY-saporin and blank-saporin (Kit-46) were obtained from Advanced Targeting Systems (Amsterdam, The Netherlands). All drugs were dissolved in sterile saline (0.154M) to the desired concentration.

4.3.4 NAc saporin injection

Rats were anaesthetised with isoflurane (AnimalCare, UK) delivered in oxygen and nitrous oxide (induced at 5% and maintained at 1.5-3%; flow rate of 500cc/min oxygen and 1L/min nitrous oxide), before being transferred to a stereotaxic frame. The local anaesthetic EMLA (AstraZeneca, UK) was applied to the ear bars to minimise discomfort, and Lacri-Lube eye gel (Lubrithal; Dechra, UK) was applied to prevent the eyes drying out during surgery.

A craniotomy was performed, and two Burr holes drilled at predetermined co-ordinates relative to bregma. Using a 25 gauge, flat tipped, 1µL Hamilton syringe, 0.5µL of 0.06µg/µL OXY-saporin or blank saporin was injected into the NAc (AP: +1.6, L: -1.0, V: -7.0, Figure 4.1) according to the rat brain (Paxinos & Watson, 2007). Blank-saporin is comprised of the toxin saporin conjugated to a missense protein with no known biological targets, thus providing a suitable control (Baskin *et al.*, 2010). Dosing concentrations and volumes were based upon previous published literature using OXY-saporin (Angioni *et al.*, 2016; Baskin *et al.*, 2010). A volume of 0.5µL (thought to diffuse less than 1mm) was used to prevent off target effects of saporin

in the brain, although spread is dependent on diffusion properties (Bast & Feldon, 2003; Myers, 1966). Saporin was injected slowly over 3 minutes and the syringe was left in place for 3 minutes following injection to prevent loss of injected saporin-conjugate during removal of the injector (Angioni *et al.*, 2016). The wound was sutured using braided silk W502 suture (Mersilk, UK), followed by topical administration of 2% lignocaine (AnimalCare, UK) and administration of 1mL s.c. saline (0.154M) to prevent dehydration. Carprofen (4mg/kg, AnimalCare, UK) was administered s.c. pre-surgery, and for 4 days post-surgery. No further analgesia was administered for the duration of the study. Following surgery rats were monitored daily and received a highly palatable diet. OXY-saporin neurotoxicity is reported to reach a maximal effect 14 days post-surgery, which is maintained for at least 28 days (Angioni *et al.*, 2016). Therefore, no behavioural testing was conducted until a minimum of 15 days post-surgery which also allowed for full recovery from surgery, including regaining their pre-surgical weight and complete analgesic drug washout.

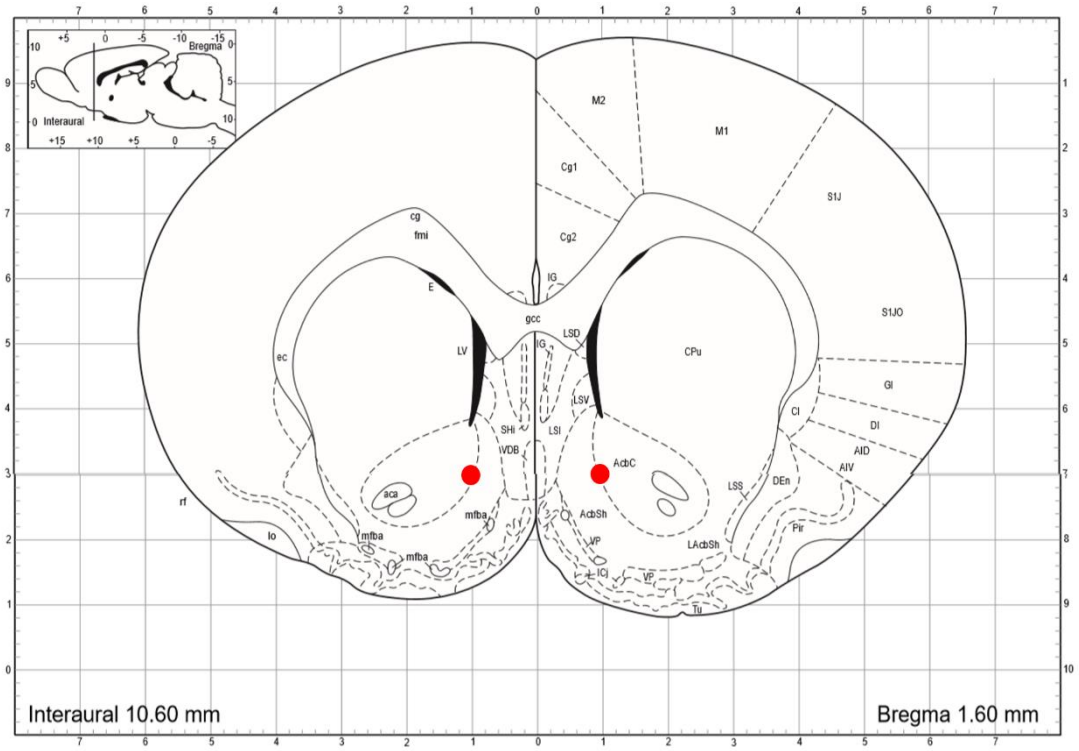

Figure 4.1: Coronal slice showing the saporin injection site at AP: +1.6, L: -1.0, V: -7.0, in the NAc on the boundary of the NAc shell and core (Image adapted from Paxinos and Watson (2007).

4.3.5 LMA

LMA was undertaken 15-17 days post-surgery as previously described in chapter 3.3.4 (Figure 3.1), excluding the administration of any antagonist. Recording was taken for 120 minutes; 30 minutes habituation and 90 minutes after OXY administration. After testing animals were returned to their home cage. Rats received OXY (0.1mg/kg s.c.) 30 minutes into testing, followed by PCP (5.2mg/kg i.p.) 30 minutes later. Cumulative activity data counts were calculated as mean + SEM for each 5-minute epoch.

4.3.6 Social interaction

Social interaction was undertaken 22-25 days post-surgery, as described in chapter 3.3.5 (Figure 3.2), without antagonist administration. 24 hours prior to undertaking the social interaction task rats were singly housed to enhance their motivation to interact during the task (Niesink & van Ree, 1982). Rats were treatment and weight matched with a novel rat from a different litter, so that they had never met their conspecific before. Each rat was sprayed with either red (Superdrug UK) or green (Claire's Accessories, UK) hairspray on the nape to enable identification during tracking and behavioural scoring. 0.1mg/kg of OXY was administered 45 minutes before undertaking the task. After the trial rats were removed from the arena and returned to their cage.

4.3.7 USVs

During the social interaction task USVs were recorded and analysed as described in chapter 3.3.6. After analysis calls were characterised into either flat, trill, or step calls (further detailed in chapter 3.3.6).

4.3.8 Tissue collection

Rats were killed by the S1 method of concussion, accompanied by immediate exsanguination to confirm death following social interaction. Rat brains were immediately removed from the skull, placed on a cool tray, and divided down the coronal midline using anatomical structures into left and right hemispheres.

The left hemisphere was stored in 4% paraformaldehyde (PFA) and left at 4°C for 24 hours, before being transferred into sucrose for an additional 24 hours, to remove water from the brain. Following 24 hours in sucrose, brains were blotted dry, and submersed in 2-methylbutane (Isopentane Solution, Sigma Aldrich, UK) cooled to -20°C, and left for 2 minutes. Once frozen, brains were placed into labelled bags to allow identification, and stored at -80°C until slicing.

At the time of dissection, the right hemisphere was dissected into the NAc, hypothalamus, and medial amygdala for future *in vitro* analysis using tissue punctures. All brain regions were immediately placed into an Eppendorf, and placed into liquid nitrogen, before being transferred into a -80°C freezer.

4.3.9 Immunohistochemistry

4.2.9.1 Solution preparation

0.1M Phosphate buffered saline (PBS) was prepared by adding 1 Dulbecco 'A' Oxoid PBS tablet (ThermoFisher Scientific, UK) to 100mL of distilled water and stirred until dissolved and stored at 4°C until use.

To prepare Day 1 (D1) buffer for immunohistochemistry, 1.25g Bovine Serum Albumin (BSA) (Sigma Aldrich, UK) and 750µL Triton-X100 (Sigma Aldrich, UK) were dissolved in 250mL 0.1M PBS, and stored at 4°C. To prepare Day 2 (D2) buffer for immunohistochemistry 375mg BSA (Sigma Aldrich, UK) and 250µL Triton-X100 (Sigma Aldrich, UK) were dissolved in 250mL 0.1M PBS, and stored at 4°C.

Anti-freeze solution was prepared to store brain slices in prior to immunohistochemistry. 600mL 1,2-Ethanediol (Ethylene glycol; Sigma Aldrich, UK), 600mL 1,2,3-Propanetriol (Glycerol; Sigma Aldrich, UK) and 800mL 0.1M PBS were added to a glass beaker and stirred until dissolved, and then stored at 4°C.

4.3.10.2 Tissue Preparation

For immunohistochemistry 60µm coronal brain slices were made on a freezing microtome (Anglia Scientific, Cambridge, UK), rinsed in PBS, and stored in antifreeze solution (-20°C) until use.

4.3.10.3 Immunohistochemistry protocol

To confirm the OXY-saporin injection site and neuronal loss immunohistochemistry was performed on brain slices using an anti-NeuN primary antibody. It was initially planned the loss of OXTR neurons would be confirmed using an anti-OXTR primary antibody. Initial trials obtained successful staining with the ThermoFisher anti-OXTR antibody, however this was discontinued before staining could be completed on tissue from this experiment. Subsequent pilot studies with two different anti-OXTR primary antibodies, and numerous optimisations, failed to obtain any specific staining so it was not possible to confirm specific OXTR neuronal loss. Optimisations tried included antigen retrieval, using biotinylated secondary antibodies, varying concentrations of blocking serum and Triton-X100, as well as varying incubation times and temperatures at all stages. Optimisations were also performed on uterine control tissue due to extremely high expression of the OXTR. However, no specific staining was found (further detailed in Appendix 1). Therefore, NeuN was selected as it is a neuron specific nuclear protein expressed in the majority of neuronal cell types in the CNS, with the immunohistochemical staining localised to the neuronal nucleus (Mullen *et al.*, 1992).

Slices to be stained were identified using Paxinos and Watson's stereotaxic rat brain atlas (Paxinos & Watson, 2007). Well volumes were kept at 1mL, with five slices per well. Slices were washed in 0.1M PBS and non-specific background blocked using 2% donkey serum (Sigma Aldrich, UK) diluted in D1 buffer (1 hour, room temperature). Slices were then incubated with the primary anti-NeuN antibody (twenty-four hours at 4°C, 1:1000 dilution in D1 buffer, MAB377, Merck Millipore, UK).

After twenty-four hours slices were washed in D2 buffer, before incubation with the secondary antibody, Donkey Anti-Mouse IgG H&L Alexa Fluor® 555 (A-31570 (ThermoFisher Scientific, UK), one hour at room temperature). Slices were kept covered during incubation to avoid bleaching of the light sensitive secondary antibody. Following incubation slices were then washed in D2 buffer twice, followed by two washes in 0.1M PBS, before being mounted onto gelatinised slides. Once dry slides were rinsed with PBS, counterstained with 4',6-diamidino-2-phenylindole (DAPI dilactate, 1:1000 in distilled H_2O, Sigma Aldrich, UK) and rinsed with distilled water. Slides were then cover slipped with 1,4-diazabicyclo[2.2.2]octane (DABCO) fluorescent mounting medium. During immunohistochemistry no primary and no secondary controls were performed alongside staining, to ensure all staining was specific for the NeuN nuclear protein.

4.3.10.4 Cell Quantification

During imaging the NAc location was confirmed according to Paxinos and Watson's stereotaxic atlas (Paxinos & Watson, 2007). Images (Nikon E200 microscope x20 and saved as .Tiff files) were taken within the NAc at the location of the micro-injection. Exposure and gain settings were selected for each filter and kept constant throughout imaging, ensuring all images were comparable. All photos were taken within 24 hours of staining with the secondary antibody. SPOT Advanced™ imaging software (Diagnostic Instruments Inc., Michigan, USA) was used to take, merge, and add scale bars to photos.

ImageJ software was used to count NeuN positive cells. Images were converted to 16-bit greyscale, with the threshold adjusted to highlight all NeuN positive cells. Highlighted cells were then counted automatically with the ImageJ software, with the minimum size adjusted to 120 pixels (Schneider *et al.*, 2012).

4.3.10 Data analysis

Data were analysed using GraphPad Prism (Version 7.04 for Windows) and JASP (Version 0.12.1). To analyse the LMA data a 4-way repeated measures ANOVA was selected. This enabled the examination of the group differences over time, when groups are formed by a combination of the between-subject factors. Between subject factors were OXY, PCP, and saporin, with time as the within-subjects variable. Before conducting the four-way ANOVA it was ensured that all the assumptions required for a 4-way repeated measures ANOVA were satisfied: one dependent variable that is measured at the continuous level, three within-subjects factors that consists of two or more categorical levels, no significant outliers, homogeneity of variance (using Levene's test), the dependent variable is normally distributed for each group of the independent variable (using Shapiro-Wilk's test), and sphericity of the data (using Mauchly's sphericity test). To determine which specific groups significantly differed from each other, if applicable, a Tukey's post-hoc was run. Tukey's post-hoc was selected as it compares all groups to each other and corrects for multiple comparisons; reducing the risk of type 1 errors (Lee & Lee, 2018; Moore *et al.*, 2012; Whitlock & Schluter, 2015).

To analyse the social interaction data a two-way ANOVA was selected. A two-way ANOVA was used to determine whether there was an interaction effect between two independent variables (OXY and saporin) on a continuous dependent variable (social interaction duration). Similarly, a Tukey's post-hoc was selected to determine which specific groups significantly differed from each other, if applicable.

NeuN positive cells were counted by ImageJ and were analysed using an unpaired Student's t-test. A student's t-test was used to determine whether a difference exists

between the means of two the independent groups, OXY-saporin and blank-saporin, on the continuous dependent variable (NeuN positive cell counts). All assumptions for the student's t-test were satisfied: independence of observations, one dependent variable measured at the continuous level, one independent variable that consists of two categorical, independent groups, homogeneity of variance and normally distributed data.

4.4 Results

4.4.1 LMA

To determine the role of NAc OXTRs on OXY-attenuation of PCP-induced hyperactivity, widely used to determine potential antipsychotic efficacy (Jentsch & Roth, 1999), a LMA task was performed following NAc OXY-saporin injection and OXY administration. During stereotaxic surgery one rodent died under anaesthesia, and during LMA one rodent received the incorrect dose, so both were excluded from analysis. For analysis data was divided into three sections and analysed separately: the habituation phase (0-30min, data not shown), the 30 min following OXY administration (30-60 min) and then the post-PCP phase (60-120 min). Mauchly's test of sphericity indicated the assumption of sphericity had been violated for all interactions (p<0.05), therefore a Greenhouse-Geisser correction was used. There was no significant difference between treatment groups in the habituation phase ($F_{(4.299,\ 301)}$=0.124, p=0.300; four-way repeated measures ANOVA), nor any effect of OXY on LMA prior to PCP administration ($F_{(3.806,\ 266)}$=0.555, p=0.687; four-way repeated measures ANOVA), therefore all treatment groups had the same level of basal activity before PCP administration. Data analysis subsequently focussed on the data accrued following PCP administration (60 min onwards).

A four-way repeated measures ANOVA on the 60 minutes post PCP administration revealed a significant three-way interaction between time * PCP * OXY ($F_{(4.793,\ 335)}$=5.69, p<0.001), as well as between time * PCP ($F_{(4.793,\ 335)}$=28.97, p<0.001) and time * OXY ($F_{(4.793,\ 335)}$=7.141, p<0.001) (all details in Appendix 2). There was also a

significant main effect of time upon LMA ($F_{(4.793, 335)}$=78.37, p<0.001). Tukey's post-hoc on the 3-way time * PCP * OXY interaction confirmed PCP significantly increased activity for 25 minutes following administration (P<0.001 for 65-80 min, p<0.01 at 85 min) when compared to control groups, regardless of NAc saporin administration. In addition, OXY significantly attenuated PCP-induced hyperactivity for 15 minutes following PCP administration (p<0.01) in both blank-saporin and OXY-saporin groups, such that there was no alteration by the NAc OXY-saporin injection (Figure 4.2). The effect of treatment on rears was also assessed, however a four-way repeated measures ANOVA revealed no significant effect of treatment on the number of rears ($F_{(4.243, 301)}$=0.782, p=0.544, data not shown).

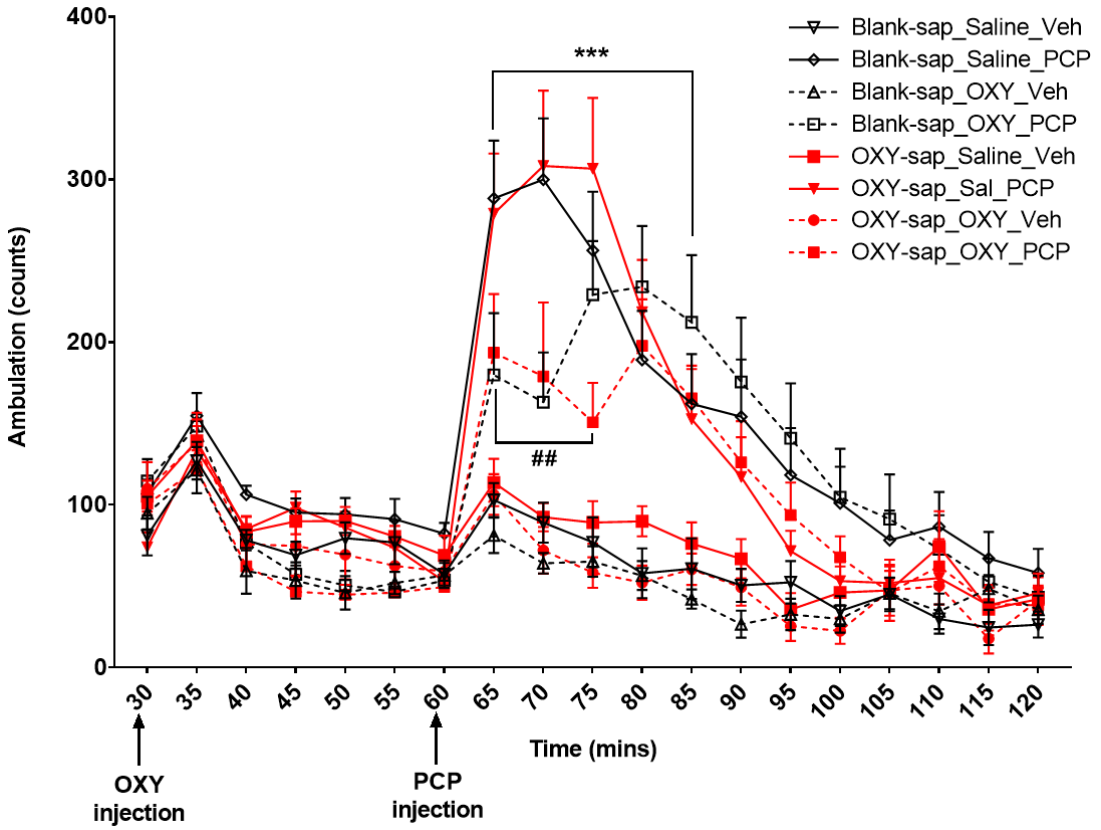

Figure 4.2: Time course of PCP-induced hyperactivity in the presence and absence of OXY in rats that had received NAc injections of either OXY-saporin or blank-saporin (mean ± SEM, n=9 (blank-sap_OXY_veh, and blank-sap_saline_PCP) or 10 (all other groups)). Four-way ANOVA (on the post-PCP data from 60 min post-test) revealed a significant three-way interaction between time * PCP * OXY ($F_{(4.793,\ 335)}$=5.69, p<0.001), as well as time * PCP ($F_{(4.793,\ 335)}$=28.97, p<0.001) and time * OXY ($F_{(4.793,\ 335)}$=7.141, p<0.001), and a significant main effect of time ($F_{(4.793,\ 335)}$=78.37, p<0.001). PCP significantly increased activity for 25 minutes following administration (p<0.001 for 65-80 min, p<0.01 at 85 min, Tukey's post-hoc), and was significantly attenuated by OXY administration for 15 minutes following PCP administration (p<0.01, Tukey's post-hoc), with no effect of the NAc saporin injection. *** p<0.001 compared to saline_veh, and ## p<0.01 compared to saline_PCP, Tukey's post-hoc.

In this experiment the peak effect of PCP only lasted 15 minutes, similar to but 5 minutes shorter than in Chapter 3; likely attributed to batch differences in PCP and a different batch of rats. Therefore, the effect of OXY on the total ambulation in the 15 minutes post-PCP was assessed (Figure 4.3). Three-way ANOVA analysis revealed a significant two-way interaction between OXY * PCP ($F_{(1,\ 70)}$=9.349, p=0.003), and a significant main effect of both OXY ($F_{(1,\ 70)}$=18.39, p<0.001) and PCP ($F_{(1,\ 70)}$=97.52, p<0.001). Regardless of whether rats had received the NAc saporin injection or

control, PCP significantly increased LMA (p<0.001, Tukey's post-hoc), and was significantly attenuated by OXY administration (p<0.001, Tukey's post-hoc) (Figure 4.3).

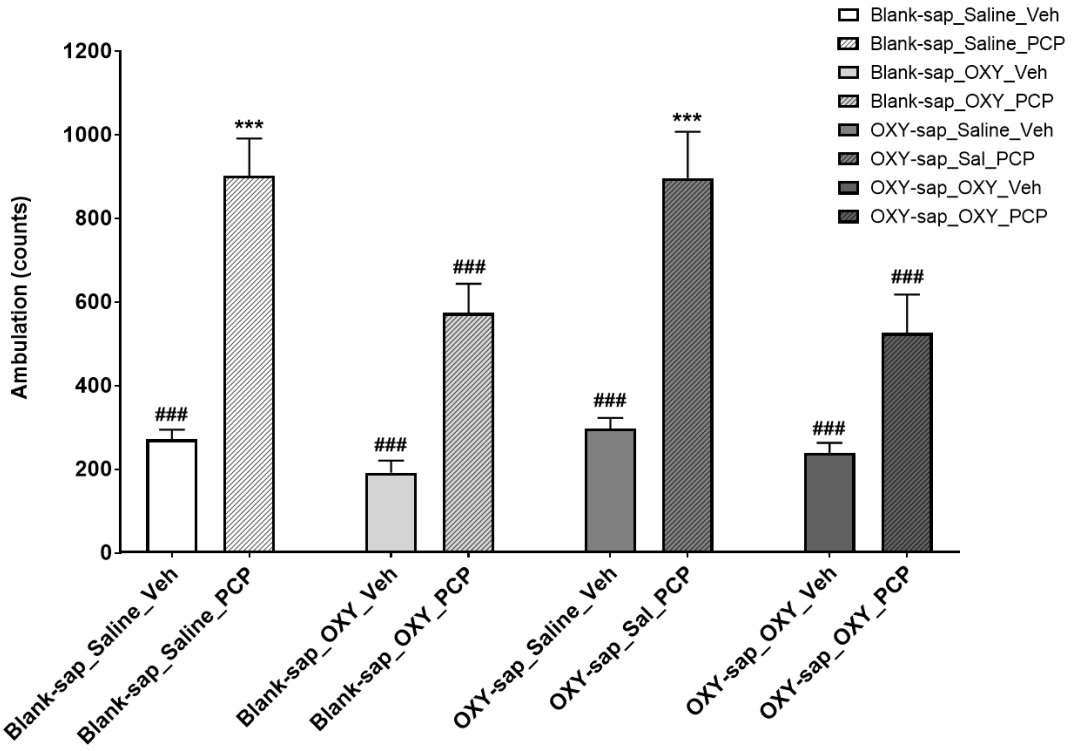

Figure 4.3: Total ambulation 15 minutes post-PCP administration, following NAc injection of either blank-saporin (control) or OXY-saporin (mean + SEM, n=9 (blank-sap_OXY_veh, and blank-sap_saline_PCP) or 10 (all other groups) per group). Three-way ANOVA revealed a significant interaction between OXY * PCP ($F_{(1, 70)}$=9.349, p=0.003), and a significant main effect of both OXY ($F_{(1, 70)}$=18.39, p<0.001) and PCP ($F_{(1, 70)}$=97.52, p<0.001). PCP significantly increased LMA (p<0.001, Tukey's post-hoc), which was significantly attenuated by OXY administration (p<0.001, Tukey's post-hoc). NAc saporin injection had no effect on the ability of OXY to attenuate PCP-hyperactivity. *** p<0.001 compared to saline_veh, and ### p<0.001 compared to saline_PCP, Tukey's post-hoc.

4.4.2 Social interaction

To determine the effect of NAc OXY-saporin micro-injection on OXY changes in social behaviour, social interaction was performed in an open field arena. During recording one video was not recorded due human error, thus resulting in an n=8 for one

treatment group (Blank-saporin and OXY). A two-way ANOVA of the total duration of prosocial behaviours (body sniffing, anogenital sniffing, following, crawling over and under and lying side-by-side) was performed. This revealed a significant interaction between OXY * saporin ($F_{(1, 34)}$=8.923, p=0.005), and a significant main effect of OXY ($F_{(1, 34)}$=4.381, p=0.044). OXY significantly increased the total duration of prosocial behaviours (p<0.01, Tukey's post-hoc), which was not seen following injection of OXY-saporin into the NAc (Figure 4.4).

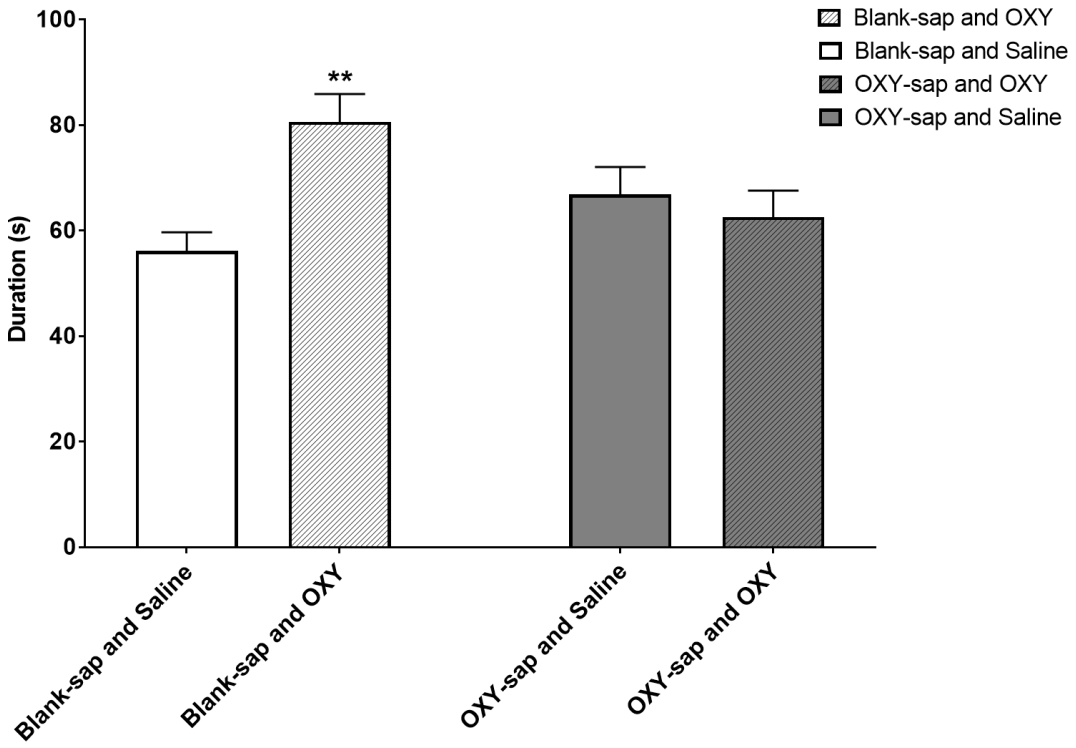

Figure 4.4: Total duration of prosocial behaviours in a social interaction task with a treatment and weight matched conspecific, following NAc injection of either blank-saporin or OXY-saporin and acute OXY administration (mean + SEM, n=8 (blank-sap and OXY) or 10 (all other groups) per group). Two-way ANOVA showed a significant interaction between OXY and saporin ($F_{(1, 34)}$=8.923, p=0.005), and a main effect OXY ($F_{(1, 34)}$=4.381, p=0.044). OXY significantly increased the total duration of prosocial behaviours (p<0.01, Tukey's post-hoc) in the blank-sap controls but this was not observed in rats given NAc injection of OXY-saporin. ** p<0.01 compared to blank-sap and saline, Tukey's post-hoc.

A more comprehensive breakdown of the individual behavioural components during social interaction showed that the majority of prosocial behaviours were anogenital

and body sniffing; used by rodents to determine familiarity through odour cues (Marlin & Froemke, 2017; Wacker & Ludwig, 2012). Two-way ANOVA analysis of body sniffing showed a significant OXY * saporin interaction ($F_{(1, 34)}$=7.18, p=0.011). OXY administration significantly increased the duration of body sniffing (p<0.05, Tukey's post-hoc) in control rats, and this did not occur following injection of OXY-saporin into the NAc. No significant effect of treatment was seen upon anogenital sniffing duration ($F_{(1, 34)}$=1.25, p=0.27). (Figure 4.5).

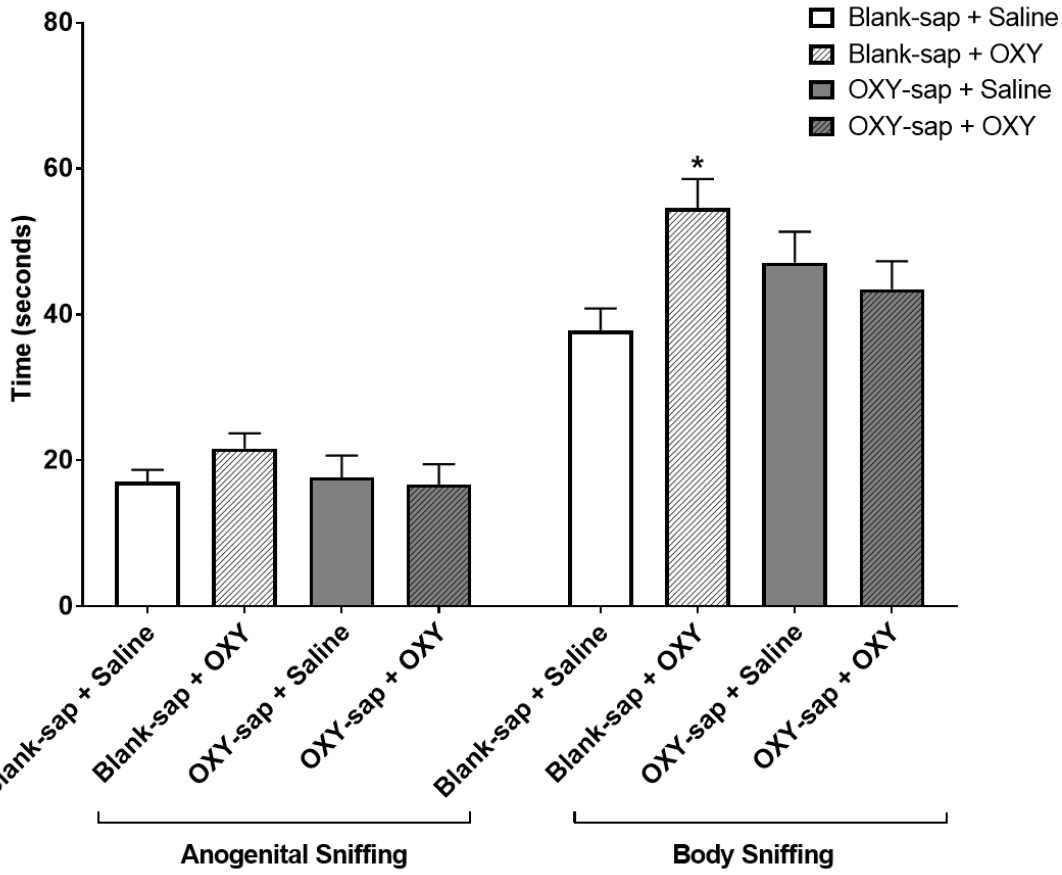

Figure 4.5: Total duration of anogenital and body sniffing during a social interaction task following NAc injection of either blank-saporin or OXY-saporin, and acute OXY administration (mean + SEM, n=8 (blank-sap and OXY) or 10 (all other groups) per group). Two-way ANOVA revealed no significant effect of OXY or OXY-saporin on anogenital sniffing duration ($F_{(1, 34)}$=1.25, p=0.27). However, analysis of body sniffing showed a significant interaction between OXY and saporin ($F_{(1, 34)}$=7.18, p=0.011), with OXY significantly increasing the duration of body sniffing (p<0.05, Tukey's post-hoc). OXY enhancement of body sniffing was not seen following NAc OXY-saporin, and was comparable in time to that in the saline control. *p<0.05 compared to blank-sap and saline, Tukey's post-hoc.

All other prosocial behaviours (crawling over and under, lying side by side and following) were also analysed individually. There was no effect of treatment upon either the duration or frequency of crawling over and under (data not shown), with less than 10% of pairs exhibiting this behaviour. However, when analysing the durations of lying side-by-side, two-way ANOVA revealed no significant interaction between OXY and saporin, but a significant main effect of OXY ($F_{(1, 34)}$=6.23, p=0.012), with OXY appearing to increase lying side-by-side duration (Figure 4.6A). Two-way ANOVA of following duration also showed a significant interaction of OXY * saporin ($F_{(1, 34)}$=4.62, p=0.039) but no main effect of OXY or saporin (Figure 4.6B). Tukey's post-hoc revealed no significant differences between groups.

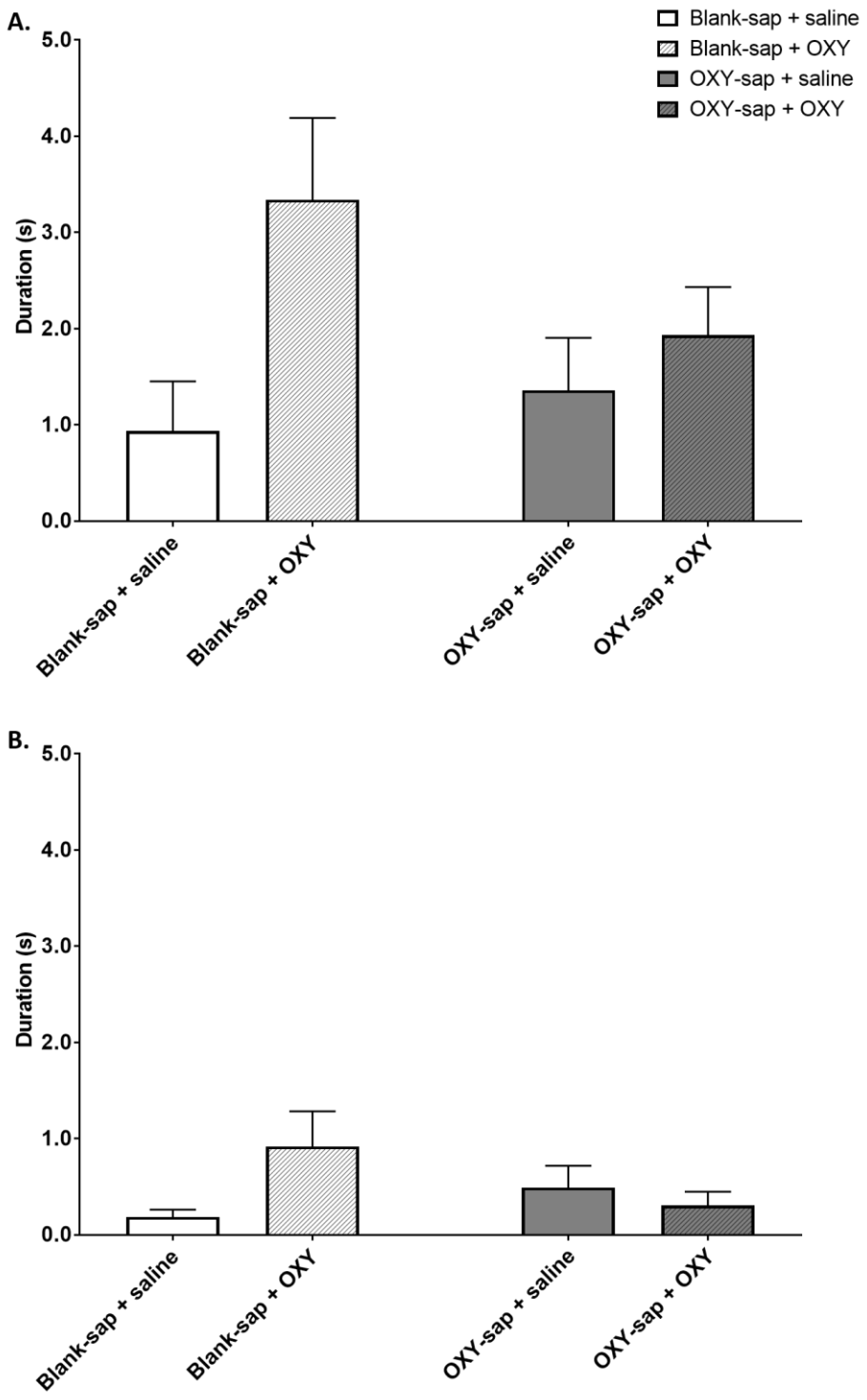

Figure 4.6: The effect of NAc injection OXY-saporin and acute OXY administration on the durations of lying side by side (A) and following (B) during social interaction (mean + SEM, n=8 (blank-sap and OXY) or 10 (all other groups) per group). (A) Two-way ANOVA of lying side by side durations revealed a significant main effect of OXY ($F_{(1, 34)}$=6.231, p=0.012); OXY appearing to increase the duration of lying side by side in control conditions (B) Two-way ANOVA of following duration showed a significant interaction between OXY * saporin ($F_{(1, 34)}$=4.62, p=0.039) but no significant main effect of either OXY or saporin.

Aggressive behaviours were also assessed; the summation of boxing and biting and pinning durations. Two-way ANOVA indicated no significant interaction between OXY and saporin ($F_{(1, 34)}$=0.5638, p=0.45). However, there was a significant main effect of OXY ($F_{(1, 34)}$=4.235, p=0.047), such that OXY decreased the duration of aggressive behaviours (Figure 4.7).

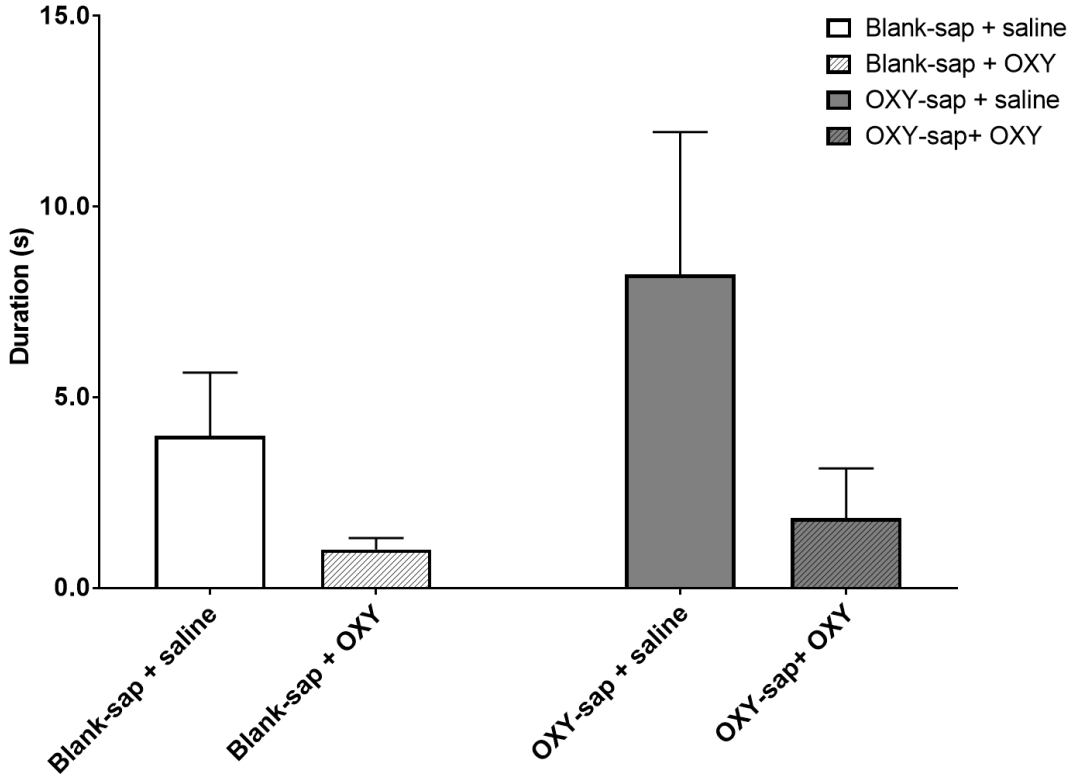

Figure 4.7: The effect of OXY, and NAc OXY-saporin injection, on the duration of total aggressive behaviours in the social interaction task (mean + SEM, n=8 (blank-sap and OXY) or 10 (all other groups) per group). Two-way ANOVA showed there was no significant interaction between OXY and saporin ($F_{(1, 34)}$=0.5638, p=0.45). However, there was a significant main effect of OXY ($F_{(1, 34)}$=4.235, p=0.047), such that OXY appeared to decrease the duration of aggressive behaviours.

4.4.3 USVs

To determine the role of NAc OXTRs on communication during social behaviours, USVs emitted by the rats were recorded during the social interaction task. As with

social interaction data mean values were calculated for each pair as it is not possible to identify which rat produced each USV. In addition, the behaviour and USVs of each rat is also, at least in part, dependent upon its pair.

Following identification of each call, every 50 kHz call was further classified into flat, step and trill subtypes (see Chapter 1.3, Figure 1.10). All USV data were $\log_{10}$ transformed to satisfy Shapiro-Wilks test for normality. A two-way ANOVA was performed to determine the effect of treatment upon the production of each call subtype (Figure 4.8), however no significant effect of treatment was found on any call subtype ($F_{(1, 35)}$=0.87, p=0.35, $F_{(1, 35)}$=2.62, p=0.11 and $F_{(1, 35)}$=0.55, p=0.46 for flat, step, and trill calls respectively).

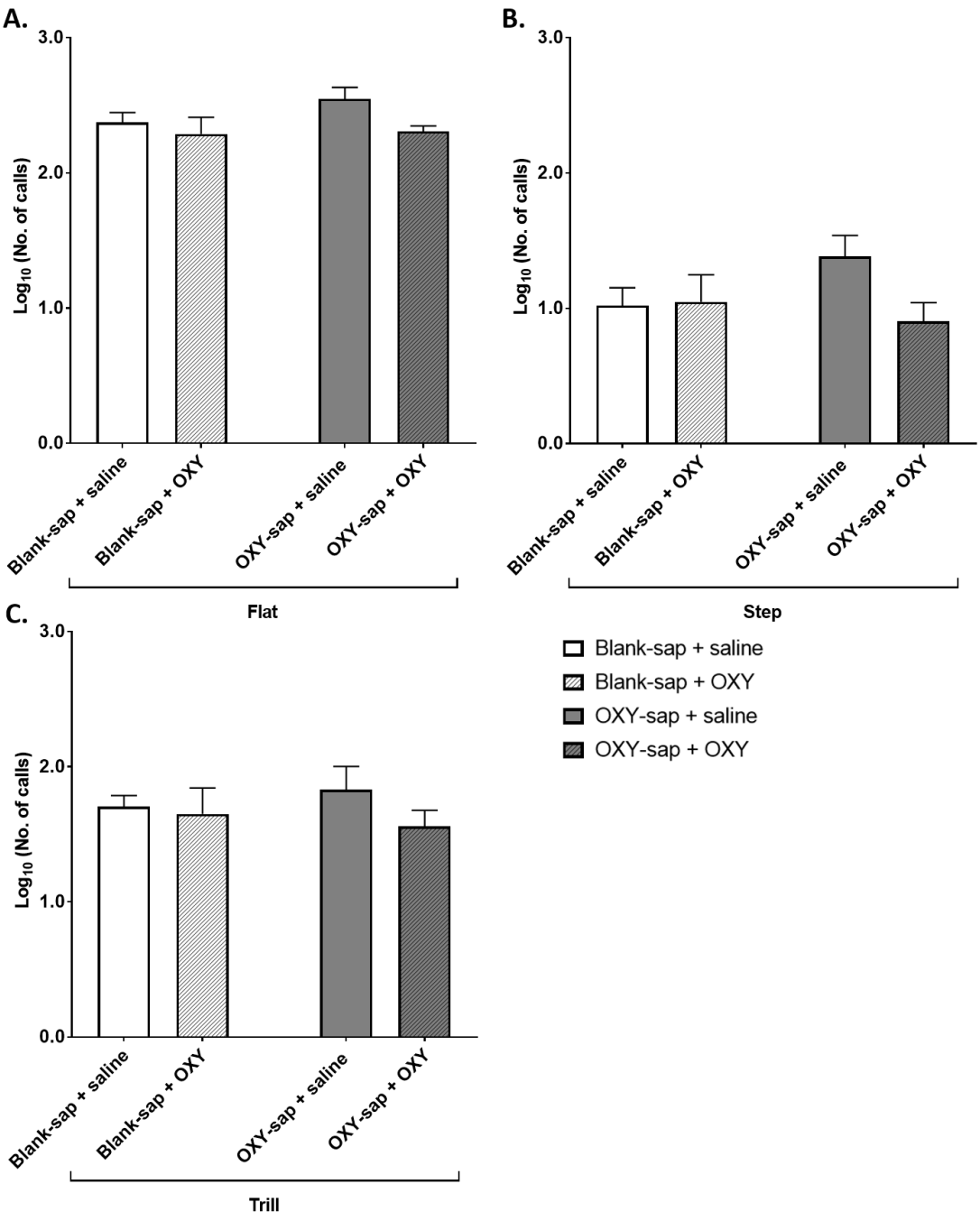

Figure 4.8: The effect of NAc OXY-saporin injection and OXY administration on the production of (A) flat, (B) step, and (C) trill 50 kHz calls (log_{10} mean + SEM, n=9 (blank-sap and OXY) or 10 (all other groups) per group). Data was log_{10} transformed to produce a normal distribution for analysis. Two-way ANOVA revealed no significant effect of treatment on the production of either flat, step, or trill calls ($F_{(1, 35)}$=0.87, p=0.35, $F_{(1, 35)}$=2.62, p=0.11 and $F_{(1, 35)}$=0.55, p=0.46 for flat, step, and trill calls respectively).

Frequency modulated (FM) 50 kHz calls are considered to be especially prosocial and strongly indicative of a positive affect, therefore the effect of the treatment upon only FM calls emitted was assessed (Figure 4.9A) (Brudzynski, 2015). In addition, the effect of treatment on the latency to first call was also analysed, as reduced latency to first call has been observed in combination with increased emission of 50 kHz calls following drug administration (Mulvihill & Brudzynski, 2019) (Figure 4.9B). However, two-way ANOVA revealed no significant effect of treatment upon either the percentage of prosocial calls emitted ($F_{(1, 35)}$=0.95, p=0.33), or the latency to the first call ($F_{(1, 35)}$=0.18, p=0.67).

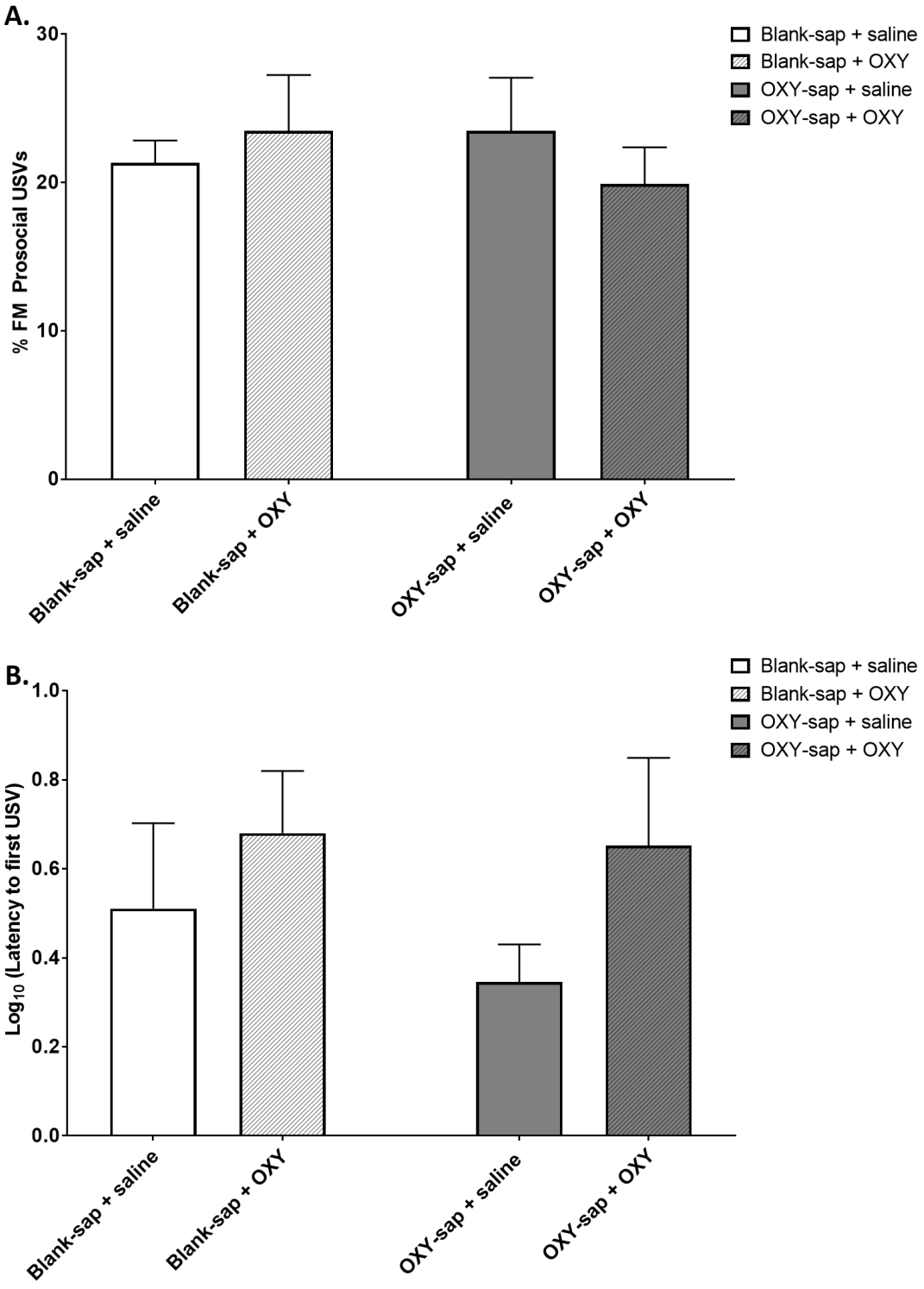

Figure 4.9: The effect of NAc OXY-saporin injection and OXY administration on (A) the percentage of FM prosocial calls emitted and (B) the latency to first call (mean + SEM, n=9 (blank-sap and OXY) or 10 (all other groups) per group). Two-way ANOVA revealed no significant effect of treatment upon either the percentage prosocial calls ($F_{(1, 35)}$=0.95, p=0.33), or the latency to first call ($F_{(1, 35)}$=0.18, p=0.67, data was $\log_{10}$ transformed to produce a normal distribution for analysis).

In addition to determining the effect of treatment upon USV emission, the relationship between duration of prosocial behaviours and USVs was also assessed. A Pearson's correlation was run to assess the relationship between the duration of prosocial behaviours during social interaction, and the percentage of prosocial calls emitted during the social interaction task (Figure 4.10A). However, there was no statistically significant correlation between the duration of prosocial behaviours and % prosocial FM USVs emitted during the social interaction task ($r(36)=0.06$, $p=0.73$). In addition, a Pearson's correlation was also used to assess the relationship between the duration of prosocial behaviours and the latency to first call during social interaction (Figure 4.10B). Again, there was no statistically significant correlation between the duration of prosocial behaviours and the latency to first call during the social interaction task ($r(36)=0.05$, $p=0.74$).

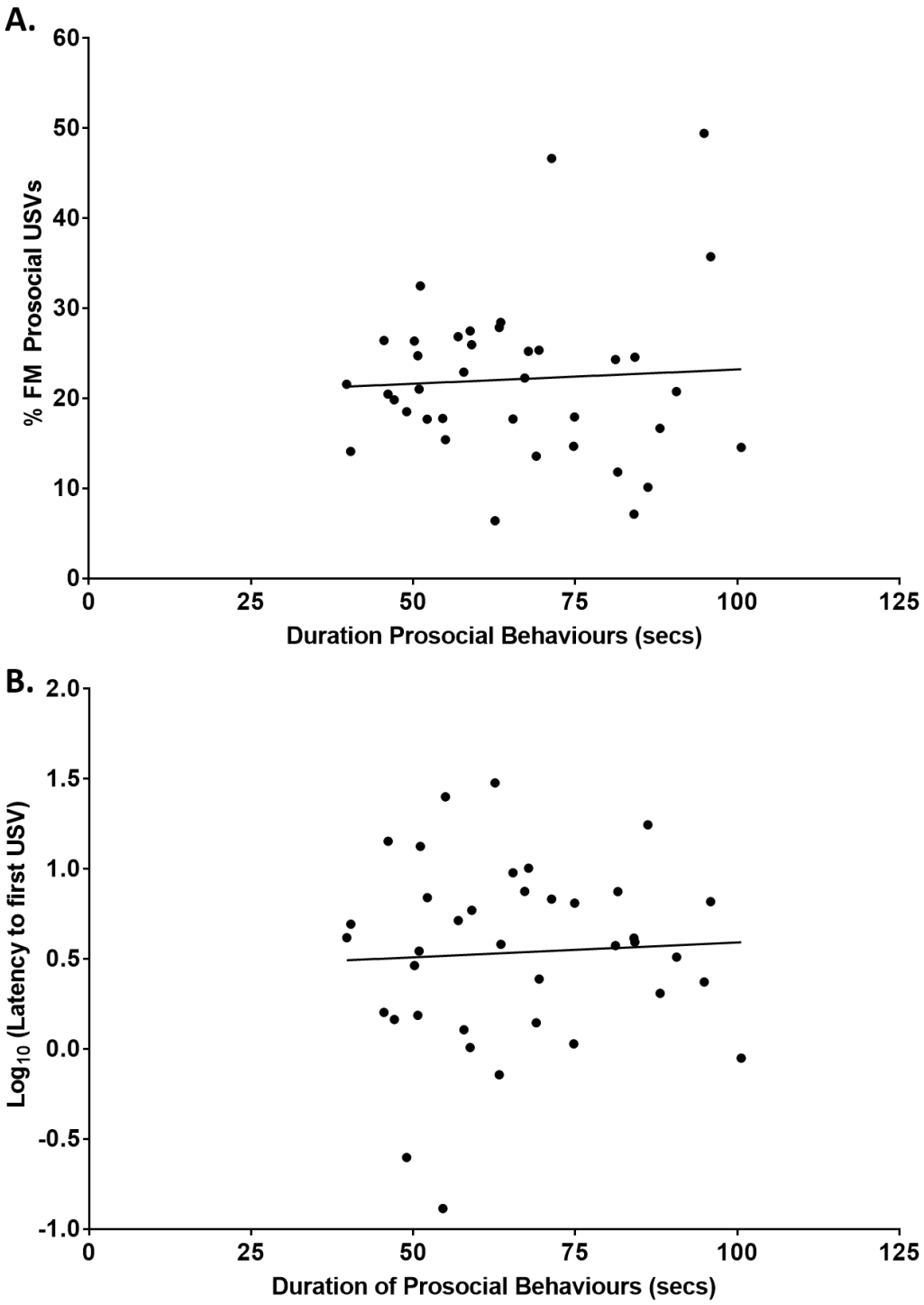

Figure 4.10: The correlation between the duration of prosocial behaviours in social interaction with (A) the percentage of prosocial calls emitted and (B) the latency to first USV. A Pearson's correlation found no significant correlation between the duration of prosocial behaviours and the percentage of prosocial FM USVs emitted during the social interaction task ($r(36)=0.06$, $p=0.73$), nor between the duration of prosocial behaviours with the latency to first call during social interaction ($r(36)=0.05$, $p=0.74$) (latency to first call data was $\log_{10}$ transformed to produce a normal distribution for analysis).

4.4.4. Immunohistochemistry

In order to confirm the saporin injection placement, and neuronal loss, immunohistochemistry was performed using an anti-NeuN primary antibody; a neuronal nuclear antigen that can be used as a neuronal biomarker (Mullen *et al.*, 1992). Immunohistochemistry was performed on NAc slices around the planned injection site (Figure 4.11). Counting NeuN positive cells from images taken at x20 magnification (Figure 4.11B) revealed a significant reduction of neurons in the NAc of rats receiving OXY-sap injection compared to blank-sap control (105.4 ± 4.96 and 182.3 ± 3.26; mean ± SEM respectively, $p<0.001$; Student's unpaired *t*-test (Figure 4.12)). Three brains were excluded due to damage upon removal.

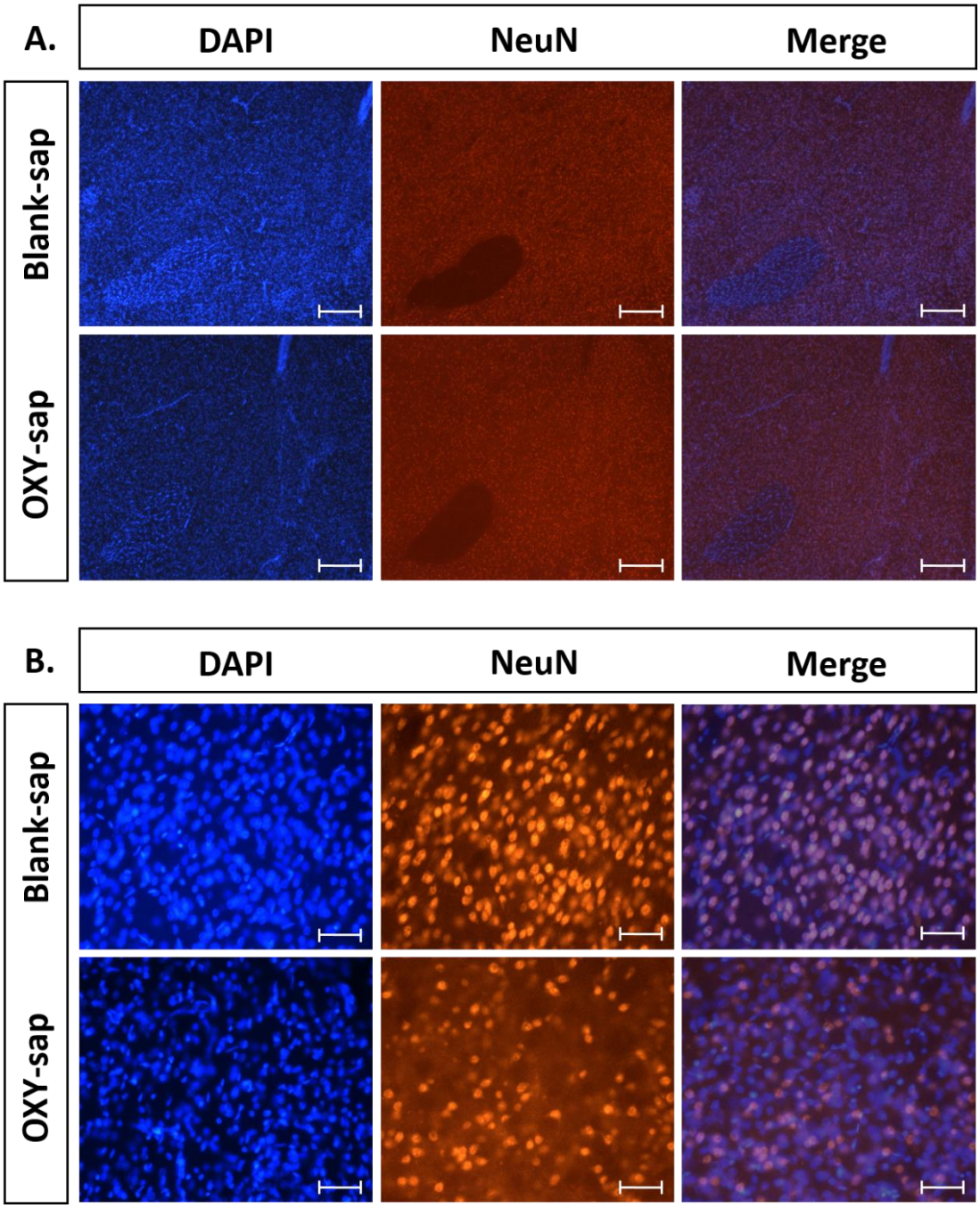

Figure 4.11: Representative images (showing approximately the group mean) of the NAc taken at magnification x4 (A) and x20 (B) following immunohistochemical staining of cell bodies (DAPI; blue) and neuronal nuclei (NeuN; red). Images were taken on 60µm coronal sections in the location of NAc saporin injection (AP: +1.6, L: -1.0, V: -7.0), and show a visible loss of neurons in the OXY-sap condition. Scale bar represents 250µm in x4 images and 50µm in x20 images.

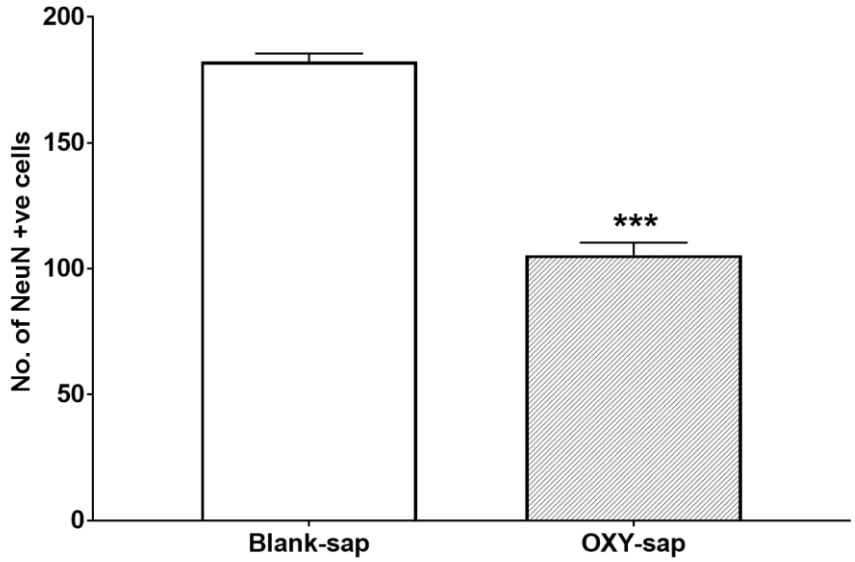

Figure 4.12: The effect of NAc OXY-sap injection on the number of NeuN positive cells in the NAc (mean + SEM, n=37 (OXY-sap)/39 (blank-sap). NAc OXY-sap injection caused a significant reduction in the number of neurons in the NAc (mean = 105.4 ± 4.96), compared to the blank-sap control injection (mean = 182.3 ± 3.26; $t_{(74)}$=13.08, p<0.001; Student's unpaired *t*-test).

4.5 Discussion

4.5.1 Effect of NAc OXY-saporin on OXY attenuation PCP-induced hyperactivity

As well as enhancing social interaction, OXY can attenuate PCP-induced hyperactivity; thought to be indicative of an antipsychotic-like effect. As discussed in chapter 3, PCP administration dose dependently increases NAc dopamine in male Wistar rats (Millan *et al.*, 1999). NAc dopamine release was initially thought to be responsible for NMDA-R antagonist induced hyperactivity, since it can be prevented through dopamine antagonism in the NAc (Del Arco *et al.*, 2008). However, NMDA-R antagonists are still able to increase LMA following lesions of NAc projecting dopaminergic neurons (Carlsson & Carlsson, 1989). In this chapter we demonstrated no effect of NAc OXY-saporin injection upon either PCP-induced hyperactivity, nor OXY attenuation of PCP-induced hyperactivity. As previously stated, 58% of NAc OXTRs are thought to be

expressed upon D_1 or D_2 expressing MSNs (Williams *et al.*, 2020), therefore OXY-saporin injection would cause destruction of these neurons. The loss of dopamine receptors in the NAc in this experiment suggests it is unlikely that PCP-induced hyperactivity, and OXY attenuation of this locomotor effect, is directly caused by NAc dopamine or NAc OXTR activity.

As well as modulating NAc dopamine, PCP is also associated with increased PFC glutamatergic activity (Takahata & Moghaddam, 2003), so the effect of PCP on NAc dopamine may be secondary to increased glutamatergic neurotransmission. AMPA antagonism in multiple brain regions including the PFC, NAc, and VTA can inhibit PCP-induced hyperactivity (Takahata & Moghaddam, 2003). In addition, the antipsychotics cariprazine and aripiprazole both attenuate PCP-induced hyperactivity, as well as also simultaneously attenuating PCP-induced increases in mPFC glutamate, dopamine, and 5-HT (Kehr *et al.*, 2018). As discussed in chapter 3, PCP NMDA-R antagonism in the PFC can disinhibit cortico-cortical glutamatergic neurons (Kehr *et al.*, 2018; Li *et al.*, 2010). This increases PFC extracellular glutamate (Li *et al.*, 2010), which consequently binds to non-NMDA receptors on glutamatergic PFC efferents (Qi *et al.*, 2012; Takahata & Moghaddam, 2003) and increases excitatory outputs to the VTA and connected motor sites (Groenewegen & Uylings, 2000; Hosp *et al.*, 2019; Tan *et al.*, 2019). Therefore, it is possible OXY may be altering glutamatergic neurotransmission within the PFC to attenuate PCP-induced hyperactivity. Within the PFC OXTRs are expressed on both GABAergic interneurons and glutamatergic neurons that project to mesolimbic areas such as the NAc and VTA (Li *et al.*, 2016; Marlin *et al.*, 2015; Nakajima *et al.*, 2014; Tan *et al.*, 2019). PFC OXY microinjections and optogenetic activation of OXTR expressing GABAergic interneurons can increase the concentration of synaptic GABA in the PFC (Qi *et al.*, 2012; Tan *et al.*, 2019). Therefore, it seems plausible that OXY-induced increases in mPFC GABA transmission could reduce PFC glutamatergic efferent activity to the VTA and connected motor sites, reducing PCP-induced hyperactivity. However, OXTRs in the mPFC are also expressed on glutamatergic neurons, which project to areas such as the VTA and NAc (Tan *et al.*, 2019). Therefore, excitatory OXY activity on

glutamatergic PFC neurons may counteract the inhibitory effects of OXTR expressing GABAergic neurons.

OXTRs are also expressed on glutamatergic terminals in the substantia nigra, an area heavily involved in the regulation of movement (Angioni *et al.*, 2016). Using OXY-saporin to identify key regions involved in OXY control of LMA, Angioni *et al.* (2016) hypothesised OXY can influence movement through activity on presynaptic OXTRs expressed on nigral glutamatergic nerve terminals. OXTR agonism on these neurons would lead to increased glutamate release, synapsing onto GABAergic efferents projecting to the brain stem, thus decreasing LMA through increased inhibitory outputs. This could provide an explanation as to how s.c. OXY can attenuate PCP-induced hyperactivity.

4.5.2 Effect of NAc OXY-saporin on OXY enhancement of social interaction

Consistent with previous work in this laboratory this chapter demonstrated s.c. OXY significantly increases social interaction, predominantly due to increased duration of body sniffing, and reduces aggressive behaviours (Kohli *et al.*, 2019). We also demonstrated that following NAc injection of OXY-saporin s.c. OXY can no longer enhance social interaction; suggesting NAc OXTR expressing neurons are required for s.c. OXY enhancement of social behaviour. However, NAc saporin injection had no effect upon OXY attenuation of aggressive behaviours. Consistent with our findings, NAc OXTR blockade reduced social novelty preference in juvenile rats (Smith *et al.*, 2017b) and social approach in voles (Yu *et al.*, 2016). In addition, NAc OXTRs are required for social conditioned place preference in mice (Dölen *et al.*, 2013), and NAc OXTR activation can reverse social withdrawal arising from chronic social defeat in voles (Hou *et al.*, 2020; Wang *et al.*, 2018).

Fluorescent *in situ* hybridization has shown that in adult mice the majority (58%) of NAc OXTRs are expressed on MSNs, and OXY is able to modulate their activity (Moaddab *et al.*, 2015; Peris *et al.*, 2020; Williams *et al.*, 2020). Despite high

dopaminergic input from the VTA, NAc OXTRs are not thought to be expressed on dopaminergic neurons; lesions of VTA dopaminergic neurons projecting to the NAc have no effect on NAc OXTR expression (Young *et al.*, 2001). NAc dopamine release and activity of D_1 dopamine receptors on MSNs are strongly associated with feelings of reward (reviewed by Lobo and Nestler (2011)). Mesolimbic dopamine is well known to regulate motivational behaviour, and changes in NAc dopamine transmission can alter behavioural responses to multiple stimuli associated with reward, such as self-administration of drugs of abuse (Willuhn *et al.*, 2010). The rewarding properties of social interaction has been demonstrated through its ability to be used as motivation for place conditioning (Calcagnetti & Schechter, 1992; Dölen *et al.*, 2013) and learning in the T maze paradigm (Ikemoto & Panksepp, 1992). Indeed, consociate interactions in adolescent and adult male rats lead to increased NAc dopamine activity, as measured by fast scan cyclic voltammetry (Robinson *et al.*, 2002; Robinson *et al.*, 2011). In addition, the Fischer 344 strain of rat, known to be significantly less playful than other inbred strains, show less NAc dopamine release when compared to Sprague Dawley rats during social play (Siviy *et al.*, 2011). Furthermore, during brief consociate interactions fast-scan cyclic voltammetry has shown increased frequency and amplitude of NAc core dopamine transients in male Long-Evans rats (Robinson *et al.*, 2011). Increased NAc activity is also seen when mice preferentially chose to explore a chamber with a novel conspecific, in contrast to a neutral chamber; again, suggesting increased NAc neural activity is associated with enhancement of prosocial behaviour (Gunaydin & Deisseroth, 2014; Gunaydin *et al.*, 2014). Additionally, NAc dopamine release is seen in Lister-hooded rats following s.c. OXY, alongside increased social interaction (Kohli *et al.*, 2019), and NAc infusions of both the dopamine agonist apomorphine and the dopamine reuptake inhibitor amphetamine increase social play in male Wistar rats (Manduca *et al.*, 2016). Therefore, s.c. OXY enhancement of social behaviour is likely to involve its ability to modulate NAc dopamine; increasing the rewarding properties of social interaction, thus enhancing the motivation to engage in this behaviour (Kohli *et al.*, 2019).

Dopamine transmission in the NAc can be classified into two distinct modes: phasic and tonic. Phasic dopamine release is rapid (<1 second) and arises from synchronised

burst firing of dopaminergic neurons, resulting in a large increase in NAc synaptic dopamine (µM to mM levels) (Floresco, 2015; Grace, 1991; Soares-Cunha *et al.*, 2016b). In contrast, tonic release maintains low steady-state levels of synaptic dopamine in the NAc (10-20 nM), which is tightly regulated (Keefe *et al.*, 1993; Soares-Cunha *et al.*, 2016b). Following NAc dopamine release, dopamine acts at both D_1 and D_2 receptors in the NAc; expressed on GABAergic MSNs (Gerfen & Surmeier, 2011; Manduca *et al.*, 2016). The D_1 receptor has a lower affinity for dopamine in comparison to the D_2 receptor (µM vs. nM) (Richfield *et al.*, 1989). Therefore, phasic dopamine release primarily activates D_1 receptors, while tonic release primarily activates D_2 receptors (Goto & Grace, 2005; Hikida *et al.*, 2016). As our dose of OXY causes phasic NAc dopamine release it is likely the behavioural effects are due to D_1 receptor modulation of MSN activity (Kohli *et al.*, 2019). NAc D_1 receptors are understood to be responsible for feelings of reward in drugs of abuse (Smith *et al.*, 2013b), and are also critical for the expression of social behaviours in mice (Gunaydin *et al.*, 2014; Kopec *et al.*, 2018). Agonism of $G\alpha_q$ coupled OXTRs on D_1 expressing MSNs would further increase excitability of D_1 MSNs, through PLC activation and subsequent release of Ca^{2+} from intracellular stores, thus driving the rewarding nature of social interactions; potentially explaining how s.c. OXY can signal through NAc OXTRs to increase the duration of social interaction observed in this study. However, expression of OXTRs upon D_1 MSNs in the NAc means the NAc injection of OXY-saporin would be expected to cause destruction of D_1 MSNs, removing D_1 receptors as well as OXTRs from the NAc. Removal of MSNs in the NAc would have great effects upon the mesolimbic neural circuity, therefore, it is possible that the inability of OXY to increase social interaction in OXY-saporin rats could be attributed to loss of D_1 expressing MSNs, and not the loss of NAc OXTRs.

It is also possible OXY is acting elsewhere in the mesolimbic system to increase social behaviour, such as the VTA; an area which contains abundant dopaminergic neurons projecting to the NAc (Gunaydin *et al.*, 2014; Hung *et al.*, 2017). As well as expression within the NAc, OXTRs are expressed upon the cell bodies of NAc-projecting dopaminergic neurons within the VTA (Hung *et al.*, 2017). Following s.c. OXY administration and RAGE dependent transportation across the BBB, OXY levels in the

CSF and PVN are increased (Yamamoto *et al.*, 2019). As well as direct projections to the NAc, the PVN has an abundance of oxytocinergic projections to the VTA, releasing OXY onto VTA cell bodies. Immunohistochemical studies have demonstrated these oxytocinergic fibres in the VTA are in close contact to and surround mesolimbic dopaminergic fibres that project to the NAc (Melis *et al.*, 2007). Both D_1 and D_2 expressing MSNs in the NAc receive direct dopaminergic inputs from the VTA, thus OXY activity in the VTA can initiate dopamine release in the NAc (Fallon & Moore, 1978; Melis *et al.*, 2007; Nauta *et al.*, 1978). Detailed studies in mice by Hung *et al.* (2017) revealed OXY release in the VTA from PVN oxytocinergic neurons is necessary for social reward, and these neurons have increased activity during social interaction. Furthermore, activation of both PVN oxytocinergic neurons and their terminals in the VTA are sufficient to increase prosocial behaviours, while inhibiting oxytocinergic PVN terminals in the VTA decreases prosocial behaviours (Hung *et al.*, 2017). Consistent with this, Song *et al.* (2016) demonstrated VTA OXTRs are critical for the rewarding properties of social interaction in male hamsters. Therefore, loss of NAc D_1 receptors through NAc OXY-saporin injection could also explain how s.c. OXY was unable to increase social interaction following NAc OXY-saporin. It is of interest that Peris *et al.* (2017) found that fibres of VTA OXTR expressing neurons were predominantly found on the border of the NAc shell and core; the location at which we targeted our OXY-saporin injection. Although it is unknown whether these specific neurons terminated in the NAc, if they were to release dopamine in this location, where there is likely to have been a loss of D_1 MSNs, the ability of OXY to enhance to rewarding nature of social interaction would have been lost. This may explain how s.c. OXY could no longer increase social interaction following administration. It is very likely that OXTR activation in multiple mesolimbic sites is involved in OXY enhancement of social behaviour, including the NAc and VTA, and may interact with other neural circuits to enhance social interaction by as yet unknown mechanisms. For example, Melis *et al.* (2007) demonstrated OXY in the VTA can not only increase NAc dopamine, but also dopamine in the PVN, suggesting VTA OXY activity can also influence hypothalamic circuits.

It is also worth noting OXTRs are likely also expressed on D_2 expressing MSNs. Although D_1 and D_2 expressing MSNs were originally thought to have opposing effects on behaviour and neuronal activity, brief stimulation of both D_1 and D_2 MSNs can drive reward and cocaine induced conditioned place preference, suggesting both D_1 and D_2 activity can increase motivation for natural rewards, such as social interaction (Soares-Cunha *et al.*, 2016a; Soares-Cunha *et al.*, 2020). In addition, Manduca *et al.* (2016) demonstrated blockade of both NAc D_1 and D_2 receptors can reduce social play in male Wistar rats, and in mice Dölen *et al.* (2013) demonstrated OXY can generate long-term depression on both D_1 and D_2 expressing MSNs, both of which are needed for social reward. It is also worth noting D_2 receptors are also expressed on NAc cholinergic interneurons, with receptor activation leading to phasic dopamine release in the NAc (Aubry *et al.*, 1993; Cachope *et al.*, 2012). Therefore, it is possible activity of these cholinergic interneurons may also enhance social interaction.

There are a few other important aspects to consider when discussing the role of NAc OXTRs in social behaviour. Firstly, the role of NAc OXTRs appears to change across development, thus the effects of the OXTR demonstrated here may not be consistent with what would be observed in pups. In young male mice Dölen *et al.* (2013) found OXTRs expressed on axon terminals of dorsal raphe neurons in the NAc core, with social reward requiring coordinated activity of both OXY and 5-HT to generate long term depression of excitatory synapses onto D_1 and D_2 MSNs in the NAc. However, this is not the case in adult mice (Nardou *et al.*, 2019; Williams *et al.*, 2020). Secondly, the NAc shell and core may have differing roles in social behaviour. Yang *et al.* (2018) demonstrated different sub regions of the NAc shell innervate different VTA neurons. The medial NAc shell primarily synapses to VTA GABA receptors on dopaminergic neurons, through $GABA_B$ receptors (Edwards *et al.*, 2017; Yang *et al.*, 2018). In contrast, the NAc lateral shell projects to VTA GABAergic neurons, synapsing with $GABA_A$ receptors (Edwards *et al.*, 2017; Yang *et al.*, 2018). Therefore, to fully understand the role of NAc OXTRs in social behaviour role of OXTRs in different regions of the NAc need to be considered. Finally, although NAc saporin prevented OXY enhancement of social behaviour, it had no effect on OXYs attenuation of aggressive behaviours during social interaction. Therefore, OXY is likely to be exerting

its effects elsewhere to reduce aggression. For example, OXY may be acting in the central amygdala to reduce aggression, as shown by Calcagnoli *et al.* (2015b).

Due to the lack of a commercially available anti-OXTR antibody and other constraints it was not possible to identify the phenotype of the neurons destroyed by OXY-saporin injection. Identifying the phenotype of the OXTR expressing neurons involved would shed further light on the mechanisms through which s.c. OXY can increase social interaction. However, as NeuN is a nuclear stain it enabled us to count the cells; enabling us to quantify the neuronal loss following OXY-saporin. Following NAc OXY-saporin we saw a 42% reduction of neurons in the NAc. Baskin *et al.* (2010) found a greater than 50% reduction in OXTR mRNA in the NTS following injection of OXY-saporin at the same volume and dose used in this chapter. In addition, Angioni *et al.* (2016) found a significant reduction in substantia nigra neurons immunoreactive for tyrosine hydroxylase (50% and 90% for the pars compacta and pars reticulata respectively) and the VGlut1 glutamate transporter (73% and 75% for the pars compacta and pars reticulata respectively) following bilateral OXY-saporin at the same dose and volume used in this chapter. However, it is difficult to compare draw comparisons between these figures as the extent of neuronal loss is dependent on OXTR expression, thus widely varies between brain regions.

4.5.3 Effect of NAc OXY-saporin on production of USVs during social interaction

Consistent with Kohli *et al.* (2019) and findings from chapter 3, we saw no effect of OXY, nor NAc OXY-saporin injection, upon the production of USVs during the social interaction task. Although there is an abundance of research suggesting NAc dopamine is heavily involved in the USV production, as detailed in the introduction (Chapter 4.1), contrasting results have been observed following peripheral administration of some selective dopamine agonists. For example, peripheral administration of D_1 (A68930), D_2 (quinpirole) and D_3 (PD 1289072) agonists inhibited 50 kHz emission when given alone (Scardochio & Clarke, 2013), and while systemic

amphetamine (which binds to monoamine transporters, increasing dopamine and noradrenaline transmission) increases the rate of 50 kHz USVs, systemic administration of dopamine and noradrenaline reuptake inhibitors (GBR 12909 and nisoxetine, respectively) have no effect upon 50 kHz calls (Wright *et al.*, 2013). As the current dose of s.c. OXY increases NAc dopamine, and OXY-saporin injection likely removed OXTR expressing D_1 and D_2 MSNs, it is unlikely NAc OXTR activity is involved in the production of 50 kHz USVs. It is possible glutamatergic signalling is involved in the production of USVs. Injection of glutamate to the into the anterior hypothalamic-preoptic area in rats can dose-dependently initiate 50 kHz emission, which is antagonised by the NMDA-R antagonist, MK-801 (Fu & Brudzynski, 1994). In addition, PCP administration can reduce the emission of 50 kHz USVs in rats during tickling and the presentation of a visual stimulus (Boulay *et al.*, 2013; Swalve *et al.*, 2016). Therefore, future work should look at the role of the NMDA-R in the production of 50 kHz calls during social interaction.

4.5.3 Summary and future work

To summarise, the work in this chapter confirmed that OXTR expressing neurons in the NAc are critical for OXY enhancement of social behaviour following s.c. administration, consistent with work in mice showing NAc OXTRs are needed for social reward and social approach (Dolen *et al.*, 2013; Williams *et al.*, 2020). In addition, this study confirmed that NAc OXTR expressing neurons are not critical for OXYs 'antipsychotic-like effect', having no effect on OXYs ability to attenuate PCP-induced hyperactivity. However, the question still remains, does OXY increase social behaviours through activity at OXTRs on D_1 and D_2 expressing MSNs, enhancing NAc MSN activity and driving the rewarding aspects of social interaction, or, does OXY act elsewhere in the mesolimbic circuit, such as the VTA, to increase NAc dopamine, thereby enhancing social interaction. Therefore, the next chapter aims to gain more of an insight into the role of OXTR expressing neurons in the mesolimbic circuit through OXY-saporin injection into the VTA, and the effects this has upon social behaviour and reversal of PCP-induced hyperactivity.

Chapter 5: Oxytocin-saporin microinjection into the ventral tegmental area; effects on social behaviour and LMA activity.

5.1 Introduction

In Chapter 4 it was established NAc OXY-saporin prevents OXY enhancement of social interaction but does not alter OXY attenuation of PCP-induced hyperactivity. Within the NAc OXTRs are expressed upon MSNs (Williams *et al.*, 2020), therefore the behavioural effects following NAc OXY-saporin injection may have been due to either depletion of D_1 MSNs, or depletion of NAc OXTRs, or a combination of both. Thus, this chapter seeks to further understand the role of OXTRs in OXYs enhancement of social interaction, and OXY attenuation of PCP-induced hyperactivity.

The VTA is located towards the floor of the midbrain and is comprised of predominantly dopaminergic neurons (Xiao *et al.*, 2017). The VTA is a key component of the mesolimbic system and has been implicated in a variety of behaviours, such as cognition, motivation, and addiction (Lammel *et al.*, 2014; Morales & Margolis, 2017). The primary projections of the VTA are to the ventral striatum, primarily serving a role in reward learning (Howe & Dombeck, 2016; Xiao *et al.*, 2017). It is well documented that the VTA is highly susceptible to modulation by OXY. Within the VTA OXTRs are expressed on dopaminergic, GABAergic, and glutamatergic neurons (Peris *et al.*, 2017; Xiao *et al.*, 2017). Axonal projections of magnocellular and parvocellular PVN OXY neurons project to the VTA where they release OXY, with their terminals in close proximity to OXTR expressing dopaminergic neurons (Beier *et al.*, 2015; Hung *et al.*, 2017; Melis *et al.*, 2007; Tang *et al.*, 2014; Xiao *et al.*, 2017).

Similar to the NAc, early work indicating a role for the VTA in social behaviour came from studies of maternal and reproductive behaviours. Lesions of NAc projecting VTA dopaminergic neurons and VTA OXTR antagonism both impair maternal behaviour (Hansen *et al.*, 1993; Pedersen & Prange, 1979). Furthermore, injection of OXY into the VTA and subsequent OXTR activation causes penile erection in male Sprague

Dawley rats through activation of mesolimbic dopaminergic neurons and NAc dopamine release (Melis *et al.*, 2007; Succu *et al.*, 2008). As well as effects on reproductive social behaviours OXY can also influence non-reproductive social behaviours through VTA activity. Both OXY and vasopressin VTA administration in hamsters can enhance social conditioned place preference through OXTR activation, suggesting a role for VTA OXTRs in social reward (Song *et al.*, 2016).

Complex experiments by Hung *et al.* (2017) showed OXY release in the VTA from PVN neurons can enhance the activity of NAc projecting dopaminergic neurons that control social behaviours and social reward. Inhibiting OXY activity in the VTA, through either inhibition of VTA-projecting PVN neurons or selective ablation of OXTRs from VTA dopamine neurons, prevents social conditioned place preference but not cocaine conditioned place preference in mice (Hung *et al.*, 2017). Furthermore, PVN oxytocinergic neuron activity markedly increases during social interaction, and optogenetic activation of these neurons is sufficient to increase social behaviours (Hung *et al.*, 2017). Consistent with this work, Xiao *et al.* (2017) also demonstrated parvocellular PVN oxytocinergic neurons project to the VTA in mice, with OXTRs expressed upon both VTA dopaminergic and GABAergic neurons. Furthermore, OXY can increase the firing rates of VTA dopaminergic neurons through OXTR activity, with no effect of numerous GABAergic and glutamatergic antagonists; suggesting a monosynaptic effect of OXY release onto VTA dopaminergic neurons (Xiao *et al.*, 2017). This is likely to be consistent in rats as well as mice, since Melis *et al.* (2007) demonstrated OXY activity in the VTA in rats can increase NAc dopamine. Further evidence in support of VTA dopaminergic neurons and their role in social behaviours comes from Gunaydin *et al.* (2014). During social interaction fiber photometry has revealed marked increases in VTA dopaminergic neuronal activity which coincide with the start of social interaction. Peak calcium activity in these neurons can encode and predict the onset of social interaction, and optogenetic activation of these neurons can significantly increase interaction with a novel same sex mouse during a social interaction task in both males and females (Gunaydin *et al.*, 2014). Furthermore, inhibition of these neurons significantly decreases social interaction, suggesting activation of VTA dopaminergic neurons is sufficient to drive

social interaction, and disruption of this signalling pathway can disrupt normal social interaction (Gunaydin *et al.*, 2014). In addition, Gunaydin *et al.* (2014) et al. also demonstrated D_1 receptors within the NAc mediate the effects of VTA stimulation. Infusion of SCH23390, a D_1 receptor selective antagonist, into the NAc before social interaction prevents the prosocial effect of VTA dopaminergic neuron stimulation; suggesting NAc D_1 receptor activity is critical for the prosocial effects of VTA-NAc dopaminergic projections. As such, optogenetic activation of NAc D_1 expressing cells is sufficient to increase social interaction in mice (Gunaydin *et al.*, 2014). Numerous other groups have also shown both OXY and optogenetic activation of VTA dopaminergic neurons can evoke NAc dopamine release (Jing *et al.*, 2019; Melis & Argiolas, 2011; Melis *et al.*, 2007; Shahrokh *et al.*, 2010; Succu *et al.*, 2008). In addition to the wealth of preclinical research implicating VTA OXTR activity in the control of social behaviour, fMRI studies have shown intranasal OXY administration in humans can enhance blood oxygen level–dependent activity in the VTA, suggestive of a common role for VTA OXTRs across species (Groppe *et al.*, 2013; Paloyelis *et al.*, 2016).

While there is conflicting evidence surrounding the expression and role of V1aRs in the VTA in rodents (Caldwell *et al.*, 2008; Dubois-Dauphin *et al.*, 1996; Insel *et al.*, 1994; Johnson *et al.*, 1993; Ostrowski *et al.*, 1994; Tribollet *et al.*, 1988; Xiao *et al.*, 2017), recent work has suggested that the V1aR is also expressed on VTA dopaminergic and GABAergic neurons (Xiao *et al.*, 2017). OXY can signal through the V1aR to modulate behaviour (Smith *et al.*, 2019). However, due to its low affinity at the V1aR it is estimated that OXY activation of V1aRs only results in 1% of its full activity (Manning *et al.*, 2008; Tribollet *et al.*, 1988; Xiao *et al.*, 2017). We previously showed a selective V1aR antagonist had no effect on OXY enhancement of social interaction (Chapter 3), and Song *et al.* (2016) demonstrated social reward is modulated through VTA OXTR activity, and not V1aR activity, through administration of selective V1aR and OXTR antagonists to the VTA (desGly-NH^2-$d(CH_2)^5$[D-Tyr^2,Thr^4]OVT and $d(CH_2)_5$[$Tyr(Me)^2$]AVP, respectively), therefore it is unlikely the V1aR is involved in OXYs prosocial effects.

As shown in chapters 3 and 4, a low dose of s.c. OXY increases social interaction between two male Lister-hooded rats. S.c. OXY is transported into the brain across the BBB via the RAGE protein and is able to elevate OXY levels in the PVN (Yamamoto *et al.*, 2019). We hypothesised that OXY may be increasing PVN oxytocinergic drive onto VTA dopaminergic neurons, increasing social interaction through increased mesolimbic dopaminergic activity. Thus, using OXY-saporin injected into the VTA, we sought to determine the role of VTA OXTRs in social interaction.

The production of USVs may also be influenced by VTA activity, although we are yet to see any evidence for OXY modulation of USVs during social interaction. Studies have shown NAc dopamine from neurons originating the in VTA can initiate 50 kHz call emission (Hori *et al.*, 2013; Scardochio *et al.*, 2015; Willuhn *et al.*, 2014). Interestingly, the synthetic μ-opioid receptor agonist DAMGO can initiate 50 kHz call emission during conditioned place preference when injected directly into the VTA, while both 6-hydroxydopamine and electrolytic lesions of the VTA reduce 50 kHz call emission during tickling (Burgdorf *et al.*, 2007). It is therefore likely dopaminergic activity within the VTA may contribute, at least in part, to the emission of prosocial calls during social interaction.

OXTRs in the VTA may also be involved in OXY attenuation of PCP-induced hyperactivity. Retigabine, a KCNQ channel opener, can reduce PCP-induced hyperactivity in rats following acute administration into the VTA and substantia nigra (Hansen *et al.*, 2007; Sotty *et al.*, 2009). When administered in the VTA retigabine dose dependently reduces basal dopaminergic activity and PCP-induced c-Fos expression in the primary motor cortex (Hansen *et al.*, 2007; Sotty *et al.*, 2009), thus reductions in dopamine activity in the VTA can attenuate PCP-induced hyperactivity. In addition, Jing *et al.* (2019) found selective activation and inhibition of VTA dopaminergic neurons can increase or decrease LMA in mice respectively; indicating VTA dopaminergic neurons have a direct influence on LMA. OXY can indirectly inhibit VTA dopaminergic activity through OXTR activation of VTA GABAergic interneurons (Xiao *et al.*, 2017), thereby possibly attenuating PCP-induced hyperactivity through reduced excitatory VTA projections to motor areas.

5.2 Aims

Using VTA micro-injections of the selective OXTR toxin OXY-saporin, this chapter aimed to determine the role of VTA OXTRs on OXY enhancement of social interaction and OXY attenuation of PCP-induced hyperactivity.

5.3 Methods

5.3.1 Animals

80 male Lister-hooded rats (175-200 grams; Charles River UK) were housed in groups of three or four in individually ventilated cages with their litter mates and were specifically requested to be caged and transported with their litter mates. Cages were kept at a 12-hour light-dark cycle with all cages maintained at identical parameters, as detailed in Chapter 3.3.1. All rats were handled for seven days prior to beginning behavioural studies to reduce anxiety enhance animal welfare (LaFollette *et al.*, 2017). Housing details and ethical requirements are as described in section 3.3.1. At the end of the study all rats were killed by the S1 method of concussion, and death was confirmed by exsanguination.

5.3.2 Experimental Design

As detailed in Chapter 3.3.2, group sizes of n=10 was aimed for. This gives the statistical test >80% power to detect an effect size of η^2=0.14 at a significance threshold of p=0.05, when using a when using a repeated measures, between factors ANOVA. Power analysis was conducted with GPower 3.1 (Faul *et al.*, 2007), ensuring sufficient power to allow statistically significant findings to be obtained, while still adhering to the 3R's principle.

All treatments were randomly allocated so that all litters received all treatment groups. During LMA assessment all treatment groups were randomly allocated across multiple runs to all 10 LMA arenas, to prevent any extraneous bias, and the experimenter was blinded to treatment.

5.3.3 Drugs

OXY was obtained from Bachem (St. Helens, UK) and PCP from Sigma Aldrich (Poole, UK). The toxins OXY-saporin and blank-saporin (Kit-46) were obtained from Advanced Targeting Systems (Amsterdam, The Netherlands). OXY, PCP and OXY-saporin were prepared as described in Chapter 4.3.3.

5.3.4 Saporin injection

The saporin injection was performed as described in Chapter 4.3.4, with the additional application of Gluture (Zoetis, Leatherhead, UK) to the incision following surgery, aiming to reduce the likelihood of having to re-suture animals during the recovery window. OXY-saporin was targeted to the VTA instead of the NAc, specifically co-ordinates AP: -5.8, L: ±0.5, V: -8.2, according to the rat brain atlas (Figure 5.1) (Paxinos & Watson, 2007). A volume of 0.5µL was used to target to the whole VTA, consistent with other groups performing VTA stereotaxic injections in rats (Maccioni *et al.*, 2018; Tabbara & Fletcher, 2019). Following surgery rats were monitored daily and healthcare checks were performed. To allow OXY-saporin to reach its maximal effect no behavioural testing was conducted until a minimum of 15 days post-surgery (Angioni *et al.*, 2016), which also allowed for full recovery from surgery, including regaining their pre-surgical weight and analgesic drug washout.

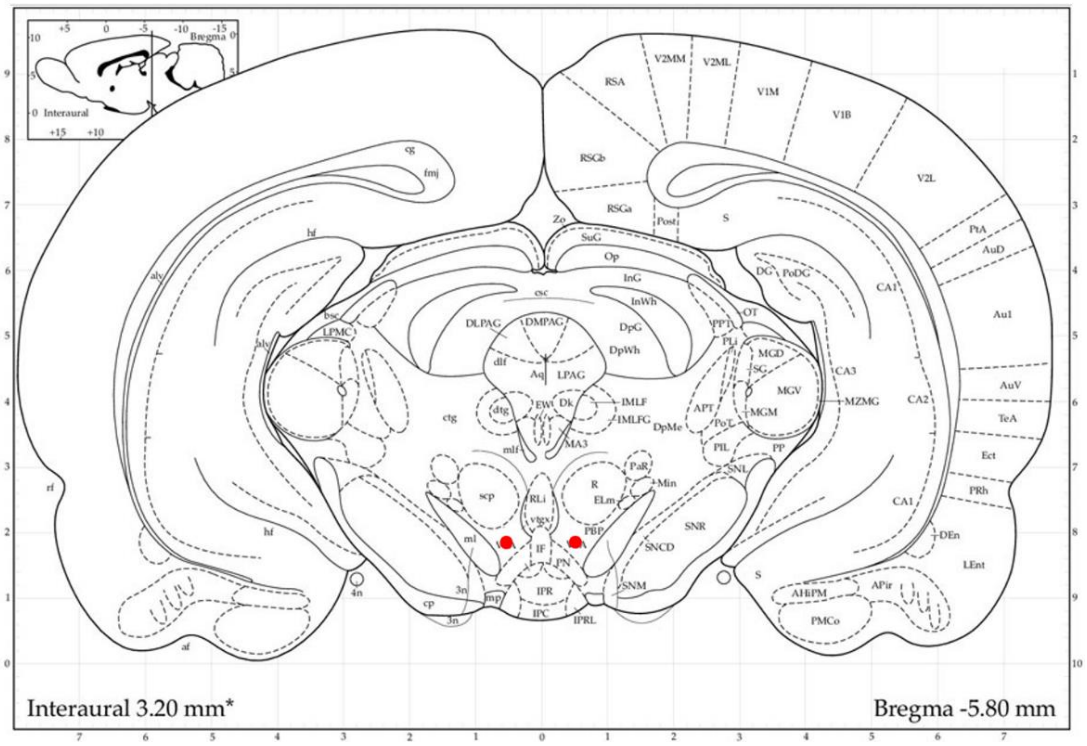

Figure 5.1: Coronal slice showing the saporin injection site located centrally within the VTA (AP: -5.8, L: ±0.5, V: -8.2), according to the rat brain atlas (Paxinos & Watson, 2007).

5.3.5 LMA

LMA was undertaken 15-17 days post-surgery, and performed as previously described in chapter 3.3.4, excluding the administration of selective antagonists. Recording was taken for 120 minutes; comprising of 30 minutes habituation and 90 minutes behavioural testing. After testing animals were returned to their home cage. Rats received a single dose of OXY (0.1mg/kg s.c.) 45 minutes into testing, followed a single dose of PCP (5.2mg/kg i.p.) 15 minutes later. Data were calculated as mean with SEM for each five-minute epoch.

5.3.6 Social interaction

Social interaction was undertaken as described in chapter 3.3.5, excluding the administration of selective antagonists, and performed 7 days after LMA allowing for drug washout. 0.1mg/kg of OXY was administered 45 minutes before undertaking the task, and rats were sprayed with either red or green hairspray (Claire's Accessories, UK) on the nape to enable identification during tracking and behavioural scoring.

5.3.7 USVs

USVs were recorded during social interaction and analysed as described in chapter 3.3.6. After analysis, calls were further characterised into either flat, trill or step calls (further detailed in chapter 3.3.6).

5.3.8 Tissue Collection

Rats were S1 killed by concussion following social interaction, and death was immediately confirmed with exsanguination. Rat brains were then removed from the skull. Using a razor blade, brains were sliced down the midline into left and right hemispheres. Brains were fixed and frozen as described in chapter 4.3.8.

5.3.9 Data analysis

Data were analysed using GraphPad Prism (Version 7.04 for Windows) and JASP (Version 0.12.1). To analyse the LMA data a 4-way repeated measures ANOVA was selected. This enabled the examination of the group differences over time, when groups are formed by a combination of the between-subject factors. Between subject factors were OXY, PCP, and saporin, with time as the within-subjects variable. Before

conducting the four-way ANOVA it was ensured that all the assumptions required for a 4-way repeated measures ANOVA were satisfied: one dependent variable that is measured at the continuous level, three within-subjects factors that consists of two or more categorical levels, no significant outliers, homogeneity of variance (using Levene's test), the dependent variable is normally distributed for each group of the independent variable (using Shapiro-Wilk's test), and sphericity of the data (using Mauchly's sphericity test). To determine which specific groups significantly differed from each other, if applicable, a Tukey's post-hoc was run. Tukey's post-hoc was selected as it compares all groups to each other and corrects for multiple comparisons; reducing the risk of type 1 errors (Lee & Lee, 2018; Moore *et al.*, 2012; Whitlock & Schluter, 2015).

To analyse the social interaction data a two-way ANOVA was selected. A two-way ANOVA was used to determine whether there was an interaction effect between two independent variables (OXY and saporin) on a continuous dependent variable (social interaction duration). Similarly, a Tukey's post-hoc was selected to determine which specific groups significantly differed from each other, if applicable.

NeuN positive cells were counted by ImageJ and were analysed using an unpaired Student's t-test. A student's t-test was used to determine whether a difference exists between the means of two the independent groups, OXY-saporin and blank-saporin, on the continuous dependent variable (NeuN positive cell counts). All assumptions for the student's t-test were satisfied: independence of observations, one dependent variable measured at the continuous level, one independent variable that consists of two categorical, independent groups, homogeneity of variance and normally distributed data.

5.3.10 Immunohistochemistry

Immunohistochemistry using the primary anti-NeuN antibody (MAB377, Merck Millipore, UK) and all subsequent image analysis was performed as described in

Chapter 4.3.9, with all immunohistochemistry being performed on the left hemisphere.

5.4 Results

5.4.1 LMA

To determine whether VTA OXTRs are involved in OXY attenuation of PCP-hyperactivity, a model commonly used to assess antipsychotic efficacy (Jentsch & Roth, 1999; Jones *et al.*, 2011), a LMA task was performed 15-16 days following administration of OXY-saporin in the VTA. LMA was recorded in computerised, Perspex arenas areas for two hours; allowing habituation to the novel environment and the assessment of LMA following drug administration. One rodent was S1 killed following surgery due to adverse effects (possible stroke during surgery) and 5 rats were not included in the analysis due to human error during drug administration. For LMA analysis data was divided into three sections and analysed separately: the habituation phase (0-30min, data not shown), the 30 min following OXY administration (30-60 min), and then the post-PCP phase (60-120 min). Mauchly's test of sphericity indicated the assumption of sphericity had been violated for all interactions (p<0.05), therefore a Greenhouse-Geisser correction was used. There was no significant difference between the treatment groups in the habituation phase ($F_{(4.326, 285)}$=0.411, p=0.815; four-way repeated measures ANOVA), nor any effect of OXY on LMA prior to PCP administration ($F_{(5, 330)}$=0.716, p=0.612; four-way repeated measures ANOVA), therefore all treatment groups had the same level of basal activity before PCP administration. Data analysis subsequently focussed on the data accrued following PCP administration (60 min onwards).

A four-way repeated measures ANOVA of the 60 minutes following PCP administration was performed and revealed a significant three-way interaction between time * OXY * PCP ($F_{(4.141, 273)}$=2.45, p=0.045), as well as significant two-way interactions between time * OXY ($F_{(4.141, 273)}$=2.99, p=0.018) and time * PCP ($F_{(4.141,}$

$_{273)}$=23.11, p<0.001). There was also a significant main effect of time ($F_{(4.141, 273)}$=49.27, p<0.001) (all details in Appendix 2). Regardless of OXY-saporin administration, PCP was able to significantly increase LMA for 25 minutes following administration (Tukey's post-hoc; 65-80mins P<0.001, 85min p<0.01). Furthermore, OXY administration attenuated PCP-hyperactivity regardless of black-saporin control or OXY-saporin administration, although this only reached significance at the 70min time point (Tukey's post-hoc; p<0.05) (Figure 5.2).

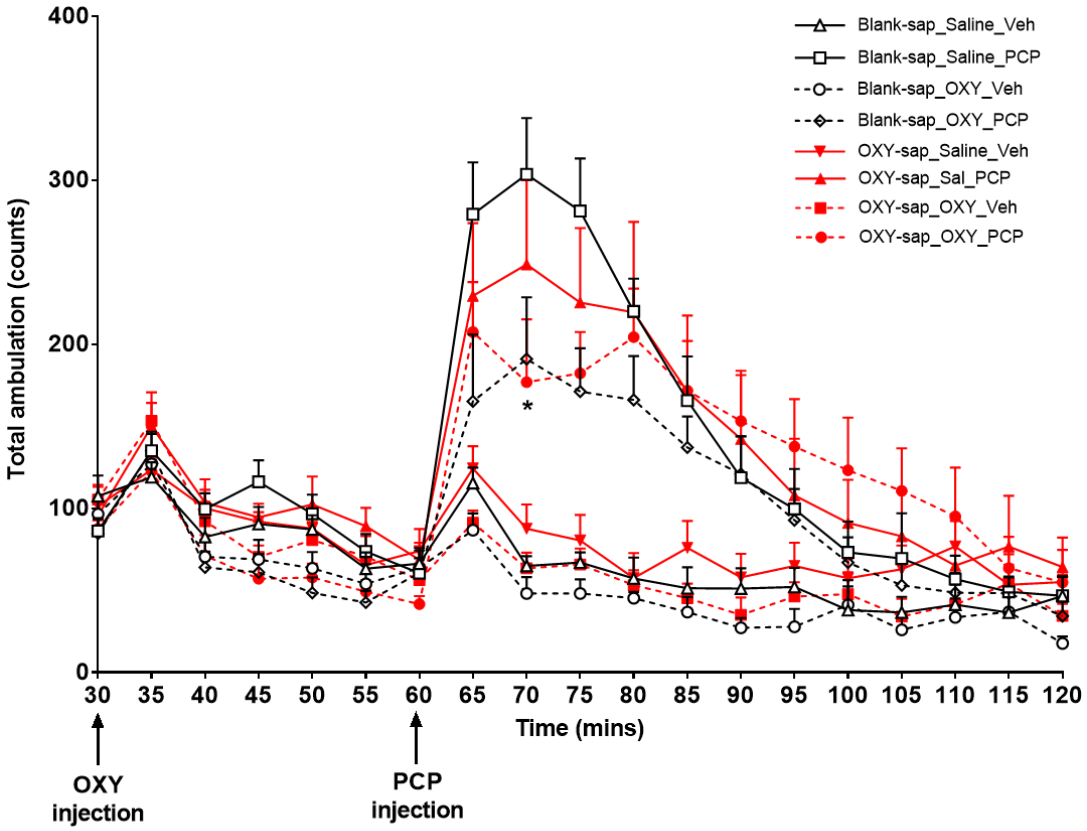

Figure 5.2: Time course of PCP-induced hyperactivity in the presence and absence of OXY, in rats that had received bilateral VTA injections of either OXY-saporin or blank-saporin (mean + SEM, n=9 (blank_sap_saline_veh, blank-sap_OXY_veh, blank-sap_saline_PCP, OXY-sap_saline_veh, OXY-sap_saline_PCP and OXY-sap_OXY_veh) or 10 (all other groups)). Four-way ANOVA revealed significant interactions between time * OXY * PCP ($F_{(4.141, 273)}$=2.45, p=0.045), time * OXY ($F_{(4.141, 273)}$=2.99, p=0.018) and time * PCP ($F_{(4.141, 273)}$=23.11, p<0.001). There was also a significant main effect of time ($F_{(4.141, 273)}$=49.27, p<0.001). Tukey's post-hoc on the three-way interaction showed PCP was able to significantly increase LMA from 65-85 minutes, regardless of saporin administration (Time point 65-80mins p<0.001, time point 85min p<0.01). Administration of OXY alongside PCP was able to attenuate PCP-hyperactivity regardless of saporin administration, although this only reached significance at time point 70min (p<0.05). * p<0.05 compared to saline_PCP, Tukey's post-hoc.

Consistent with LMA experiments in chapter 4, the peak effect of PCP administration on LMA lasted for 15 minutes, therefore further analysis focussed specifically on this window. Analysis with a three-way ANOVA revealed no significant interactions; only a significant main effect of PCP ($F_{(1, 66)}$=76.71, p<0.001) and OXY ($F_{(1, 66)}$=9.66, p=0.003). PCP administration increased LMA counts (864 ± 67.2 and 703.6 ± 127, for blank-sap and OXY-saporin respectively) compared to the control group (247 ± 10.9), while administration of OXY seemed to attenuate this, regardless of VTA OXY-saporin (527 ± 98.7 and 566 ± 65.8, for blank-sap and OXY-saporin respectively) (Figure 5.3).

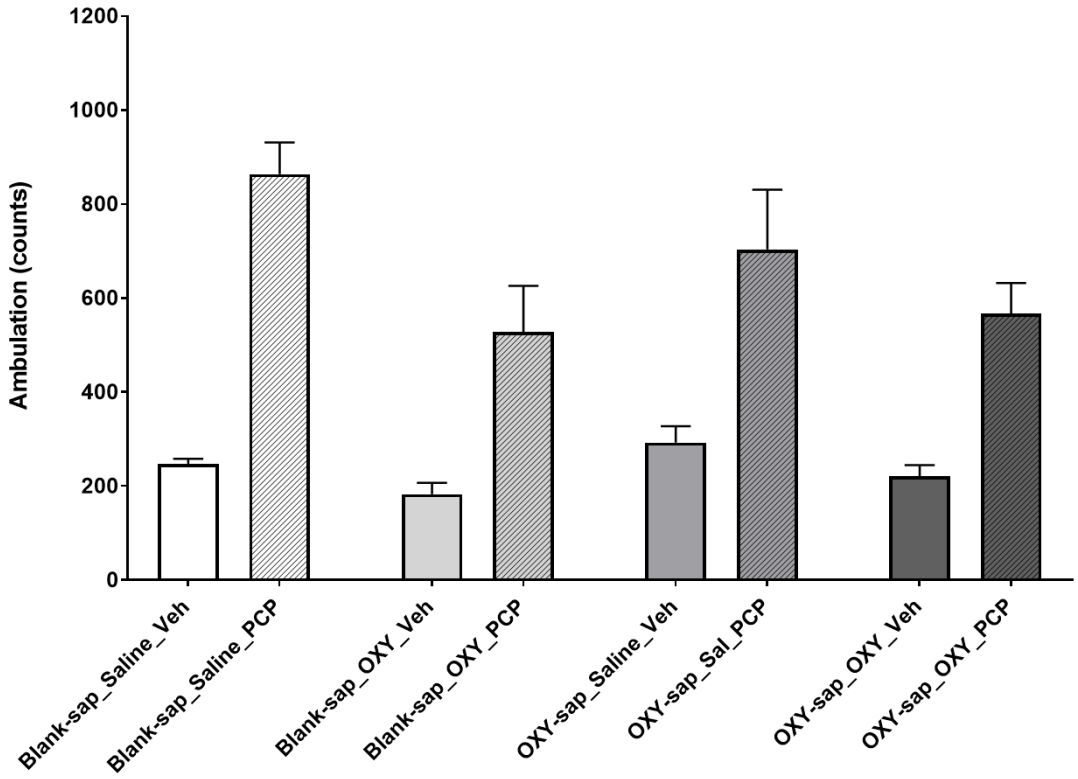

Figure 5.3: Total ambulation for the first 15 minutes following PCP administration in rats that had received bilateral VTA injections of either OXY-saporin or blank-saporin (mean + SEM, n=9 (blank_sap_saline_veh, blank-sap_OXY_veh, blank-sap_saline_PCP, OXY-sap_saline_veh, OXY-sap_saline_PCP and OXY-sap_OXY_veh) or 10 (all other groups)). Analysis with a three-way ANOVA revealed no significant interactions; only a significant main effect of PCP ($F_{(1, 66)}$=76.71, p<0.001) and OXY ($F_{(1, 66)}$=9.66, p=0.003). PCP appeared to increase LMA, with administration of OXY seeming to attenuate this.

5.4.2 Social Interaction

To determine whether OXTRs in the VTA play a role in OXY enhancement of social interaction, we performed a social interaction task 22-24 days following surgery. Social interaction was performed in an open field arena with a novel weight- and treatment-matched rat. Behaviours were analysed on video following recording. The total duration of prosocial behaviours (body sniffing, anogenital sniffing, following, crawling over and under, and lying side-by-side) were analysed using a two-way ANOVA. Analysis revealed a significant interaction between OXY and saporin ($F_{(1, 35)}$=12.66, p=0.001), and a significant main effect saporin ($F_{(1, 35)}$=23.01, p<0.001). OXY significantly increased the total duration of prosocial behaviours in blank-saporin controls (p<0.01, Tukey's post-hoc), but this not seen following VTA injection of OXY-saporin (Figure 5.4).

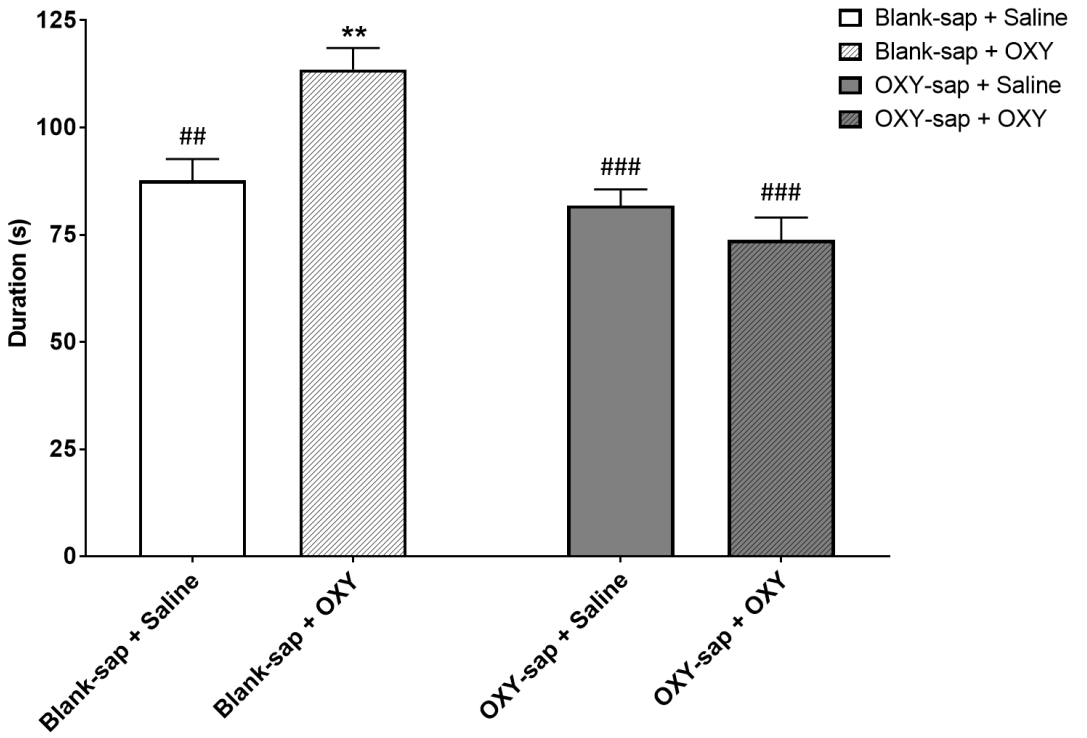

Figure 5.4: Total duration of prosocial behaviours during a social interaction task following VTA OXY-saporin injection and acute OXY (mean + SEM, n=9 (blank-sap and OXY) or 10 (all other groups) per group). Two-way ANOVA showed a statistically significant interaction between OXY and saporin ($F_{(1, 35)}$=12.66, p=0.001), and a significant main effect saporin ($F_{(1, 35)}$=23.01, p<0.001). OXY significantly increased the total duration of prosocial behaviours (p<0.01, Tukey's post-hoc), which was not seen following VTA injection of OXY-saporin. ** p<0.01 compared to blank-sap and saline, ### p<0.001 and ## p<0.01 compared to blank-sap and OXY, Tukey's post-hoc.

Sniffing behaviours made up the biggest proportion of prosocial behaviours observed. Therefore, the effect of treatment upon both body sniffing and anogenital sniffing was further examined. Two-way ANOVA of body sniffing duration showed a significant interaction between OXY and saporin ($F_{(1, 35)}$=5.42, p=0.026), as well a significant main effect of saporin ($F_{(1, 35)}$=11.79, p=0.002), such that OXY significantly increased body sniffing in blank-saporin rats (p<0.05) but in contrast failed to increase the duration of body sniffing following VTA OXY-saporin. Similarly, two-way ANOVA of anogenital sniffing revealed a significant interaction between OXY and saporin ($F_{1, 35)}$=11.35, p<0.01), as well as a significant main effect saporin ($F_{(1, 35)}$ =13.79, p<0.001). As with body sniffing, OXY significantly increased the total duration of anogenital

sniffing (p<0.05), but this was not observed following VTA injection of OXY-saporin (Figure 5.5).

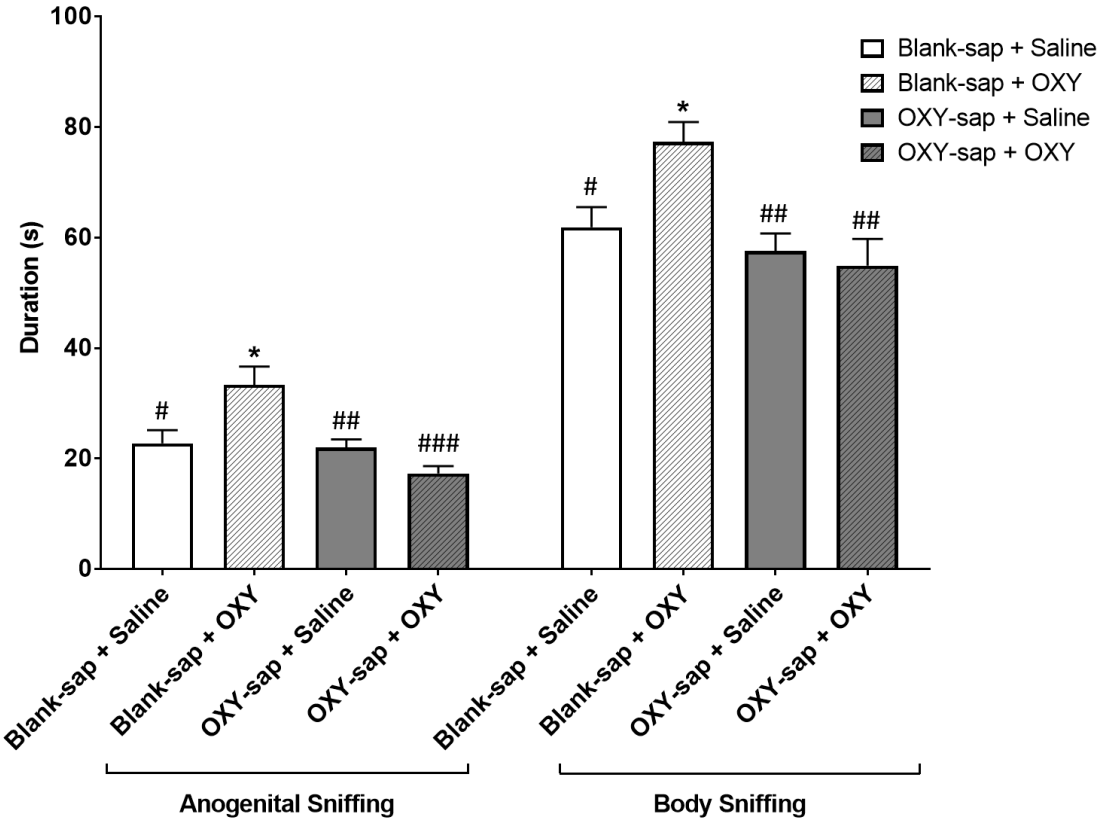

Figure 5.5: Total duration of anogenital and body sniffing behaviours during social interaction, following VTA OXY-saporin injection and acute OXY (mean + SEM, n=9 (blank-sap and OXY) or 10 (all other groups) per group). Two-way ANOVA of anogenital sniffing duration showed a significant interaction between OXY and saporin ($F_{(1, 35)}$=11.35, p<0.001), and a significant main effect saporin ($F_{(1, 35)}$ =13.79, p<0.001). OXY significantly increased the duration of anogenital sniffing (p<0.05, Tukey's post-hoc), which was not observed following VTA injection of OXY-saporin. Similarly, two-way ANOVA of body sniffing revealed a significant interaction between OXY and saporin ($F_{(1, 35)}$=5.42, p=0.026), as well a significant main effect of saporin ($F_{(1, 35)}$=11.79, p=0.002), such that OXY could no longer significantly increase the duration of body sniffing following VTA OXY-saporin (p<0.05, Tukey's post-hoc). * p<0.05 compared to blank-sap and saline, # p<0.05, ## p<0.01, and ### p<0.001 compared to blank-sap and OXY.

The other prosocial behaviours analysed during social interaction included crawling over and under, lying side by side, and following. There was no significant effect of treatment upon the duration of crawling over and under, with less than 50% of pairs

exhibiting this behaviour (Two-way ANOVA, $F_{(1, 35)}$=0.88, p=0.35, data not shown). However, two-way ANOVA of lying side by side durations revealed a significant interaction between OXY and VTA saporin ($F_{(1, 35)}$=4.85, p=0.034), though Tukey's post-hoc indicated no significant differences between groups. Analysis of following duration also showed a significant interaction between OXY and VTA saporin (Two-way ANOVA; $F_{(1, 35)}$=4.39, p=0.044), with OXY-saporin and saline showing significantly less following behaviours than blank-saporin and saline (p<0.05, Tukey's post-hoc) (Figure 5.6).

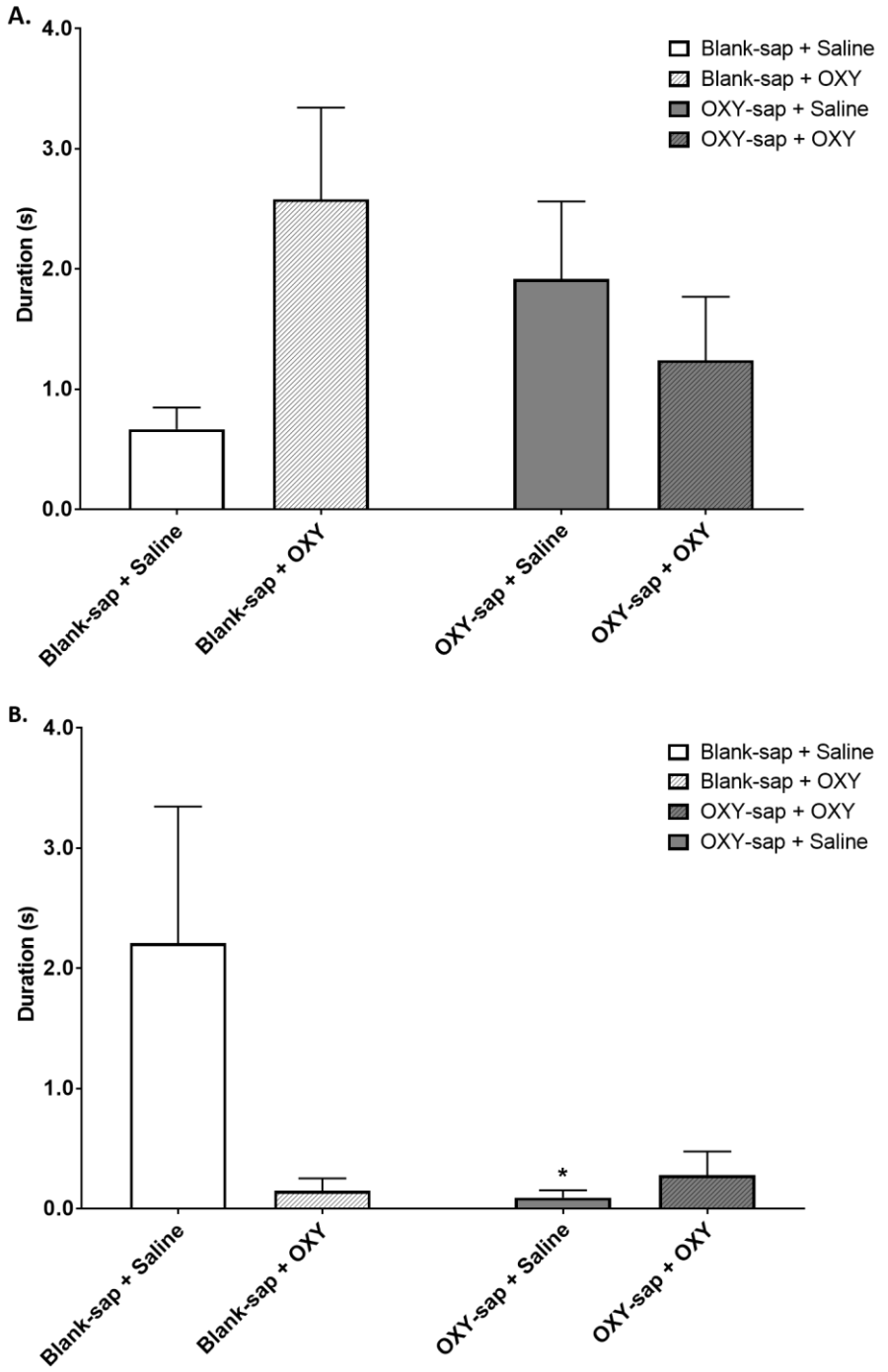

Figure 5.6: The effect of VTA OXY-saporin injection and acute OXY administration on the durations of lying side by side (A) and following (B) during social interaction (mean + SEM, n=9 (blank-sap and OXY) or 10 (all other groups) per group). (A) Two-way ANOVA of lying side by side durations revealed a significant interaction OXY and VTA saporin ($F_{(1,\ 35)}$=4.85, p=0.034), however Tukey's post-hoc showed no significant differences between groups. (B) Analysis of following duration also showed a significant interaction between OXY and VTA saporin (Two-way ANOVA; ($F_{(1,\ 35)}$=4.39, p=0.044), with OXY-saporin + saline showing significantly less following behaviours than blank-saporin + saline (p<0.05, Tukey's post-hoc). * p<0.05 compared to blank-sap and saline.

Two of the behaviours scored during social interaction are considered aggressive behaviours (boxing and biting and pinning), therefore the effect of treatment on the duration of aggressive behaviours was examined. However, analysis with a two-way ANOVA indicated no significant effect of treatment upon the duration of aggressive behaviours ($F_{(1, 35)}$ =0.10, p=0.75).

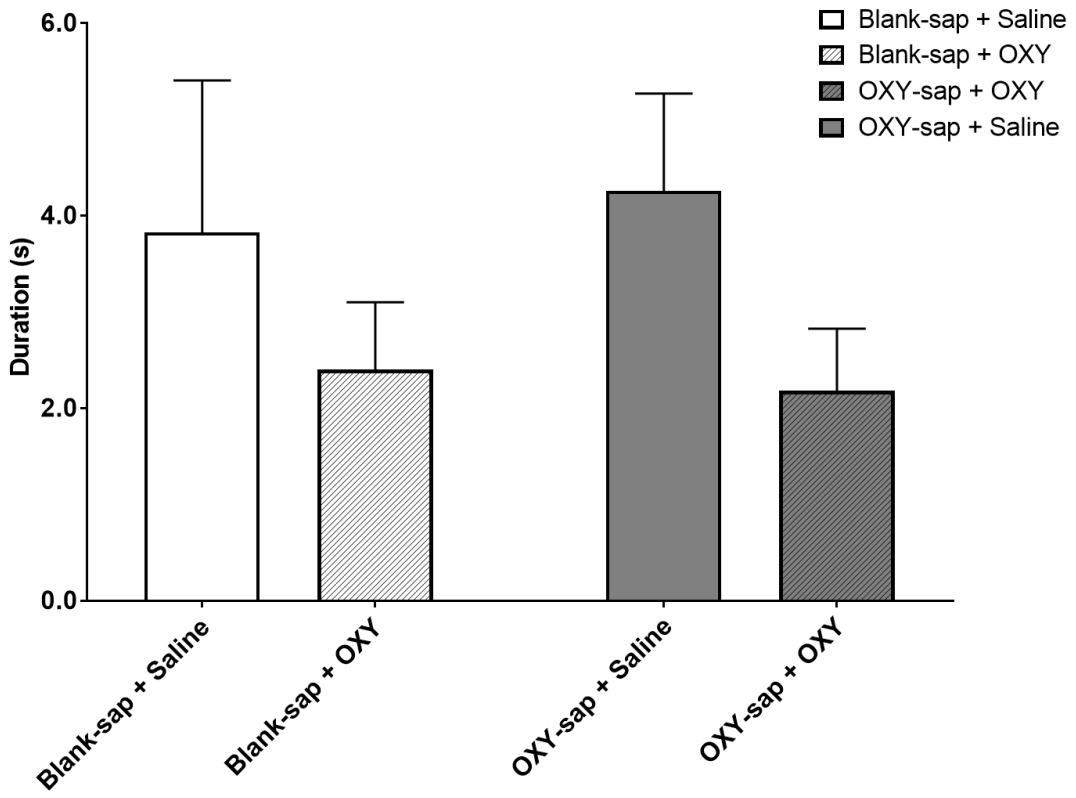

Figure 5.7: Total duration of aggressive behaviours during a social interaction task following VTA OXY-saporin injection and acute OXY (mean + SEM, n=9 (blank-sap and OXY) or 10 (all other groups) per group). Two-way ANOVA showed no significant effect of treatment upon the duration of aggressive behaviours ($F_{(1, 35)}$ =0.10, p=0.75).

5.4.3 USVs

During social interaction rats emit USVs as a means of communication, with 50 kHz prosocial calls being indicative of a positive affect (Brudzynski, 2015). Following identification 50 kHz calls were classified into three different types of prosocial call:

flat, trill, and step, and the effect of treatment upon each call type was examined. One day of USV recordings were lost due to a technical fault with the recording equipment, therefore n=6 (blank-sap and saline), 7 (OXY-saporin and saline) or 8 (all other groups) per group. As with previous USV data, call counts were $\log_{10}$ transformed to produce a normal distribution for analysis. A two-way ANOVA for each call type revealed no significant main effects of treatment on any type of call emitted during social interaction ($F_{(1, 25)}$ =1.61, p=0.21, $F_{(1, 25)}$ =4.16, p=0.06, $F_{(1, 25)}$ =0.97, p=0.33 for flat, step, and trill calls respectively) (Figure 5.8).

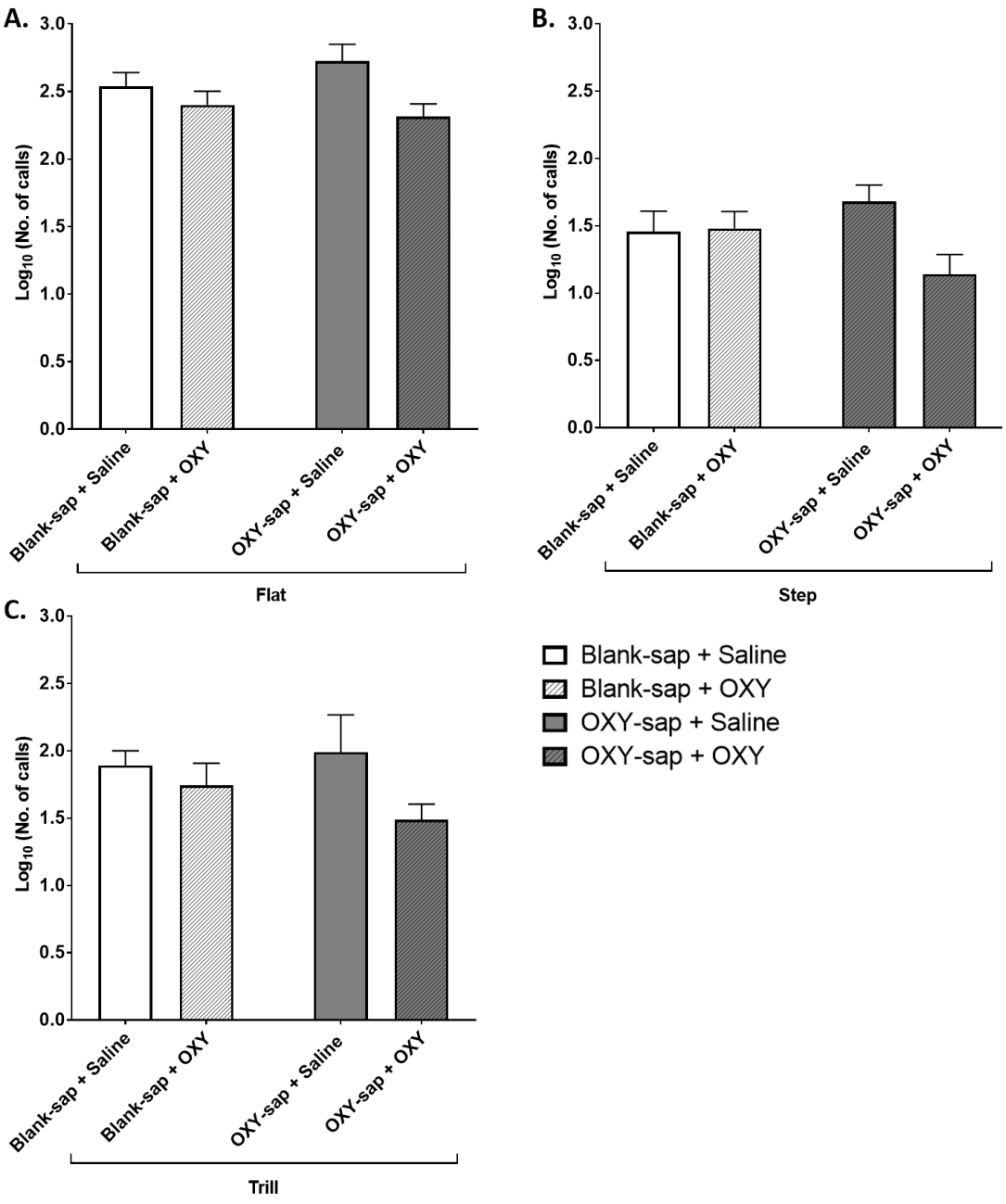

Figure 5.8: The effect of VTA OXY-saporin on the production of (A) flat, (B) step, and (C) trill 50 kHz calls (log_{10} mean ± SEM, n=6 (blank-sap and saline), 7 (OXY-saporin and saline)/ or 8 (all other groups) per group). Data was log_{10} transformed to produce a normal distribution for analysis. Two-way ANOVA revealed there was no significant effect of treatment upon either flat ($F_{(1, 25)}$ =1.61, p=0.21), step ($F_{(1, 25)}$ =4.16, p=0.06), or trill ($F_{(1, 25)}$ =0.97, p=0.33) call production.

Further analysis specifically focussed on the percentage of FM 50 kHz calls emitted, as these are strongly associated with a positive affect and rewarding situations, such as social interaction (Brudzynski, 2015). Two-way ANOVA revealed no significant

effect of treatment upon the percentage of FM 50 kHz calls emitted during social interaction ($F_{(1, 25)}$=3.10, p=0.091) (Figure 5.9A). The latency to first USV was also analysed, as Mulvihill and Brudzynski (2019) demonstrated reduced latency to first call occured in conjunction with increased emission of 50 kHz calls following pharmacological manipulation. However, no effect of treatment was observed upon the latency to first USV (Two-way ANOVA; $F_{(1, 25)}$=1.14, p=0.30) (Figure 5.9B).

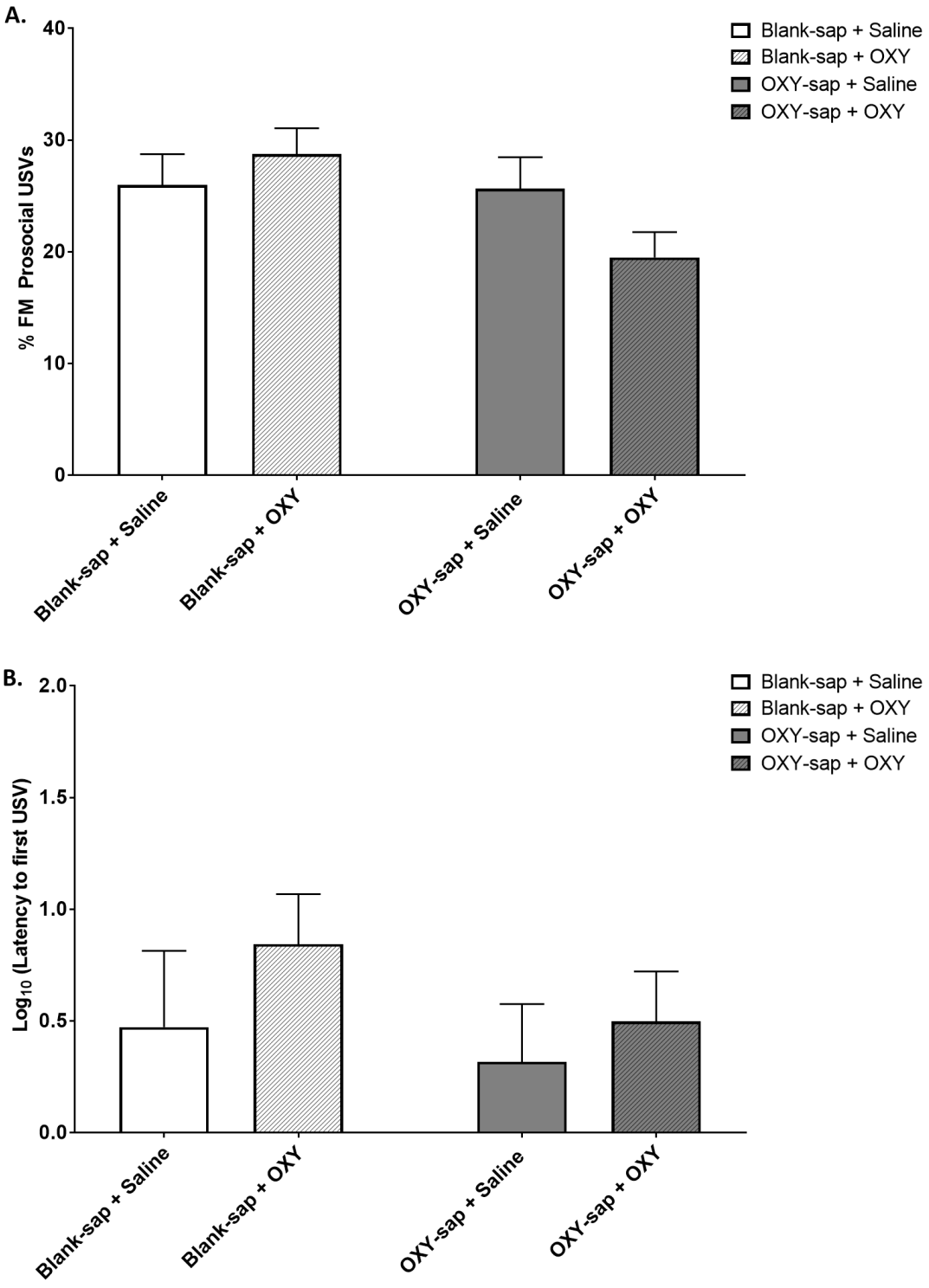

Figure 5.9: The effect of VTA saporin and acute OXY on (A) the percentage of FM 50 kHz calls emitted during social interaction and (B) the latency to first USV (mean + SEM, n=6 (blank-sap and saline), 7 (OXY-saporin and saline)/ or 8 (all other groups) per group). (A) Two-way ANOVA of the percentage FM 50 kHz calls emitted during social interaction revealed no significant effect of treatment ($F_{(1, 25)}$=3.10, p=0.091) (B) Two-way ANOVA of the latency to first call also revealed no significant effect of treatment ($F_{(1, 25)}$=1.14, p=0.30, data was $\log_{10}$ transformed to produce a normal distribution for analysis).

Finally, to determine whether there was any relationship between the duration of prosocial behaviours and percentage of FM prosocial calls emitted during social interaction, a Pearson's correlation was conducted (Figure 5.10A). A Pearson's correlation was also used to determine whether there was a significant correlation between the duration of prosocial behaviours and the latency to first USV (Figure 5.10B). However, Pearson's correlation found no significant correlation between either the duration of prosocial behaviours and the percentage of prosocial FM USVs emitted during the social interaction task ($r(27)=0.18$, $p=0.35$), nor the duration of prosocial behaviours with the latency to first call ($r(27)=-0.06$, $p=0.75$).

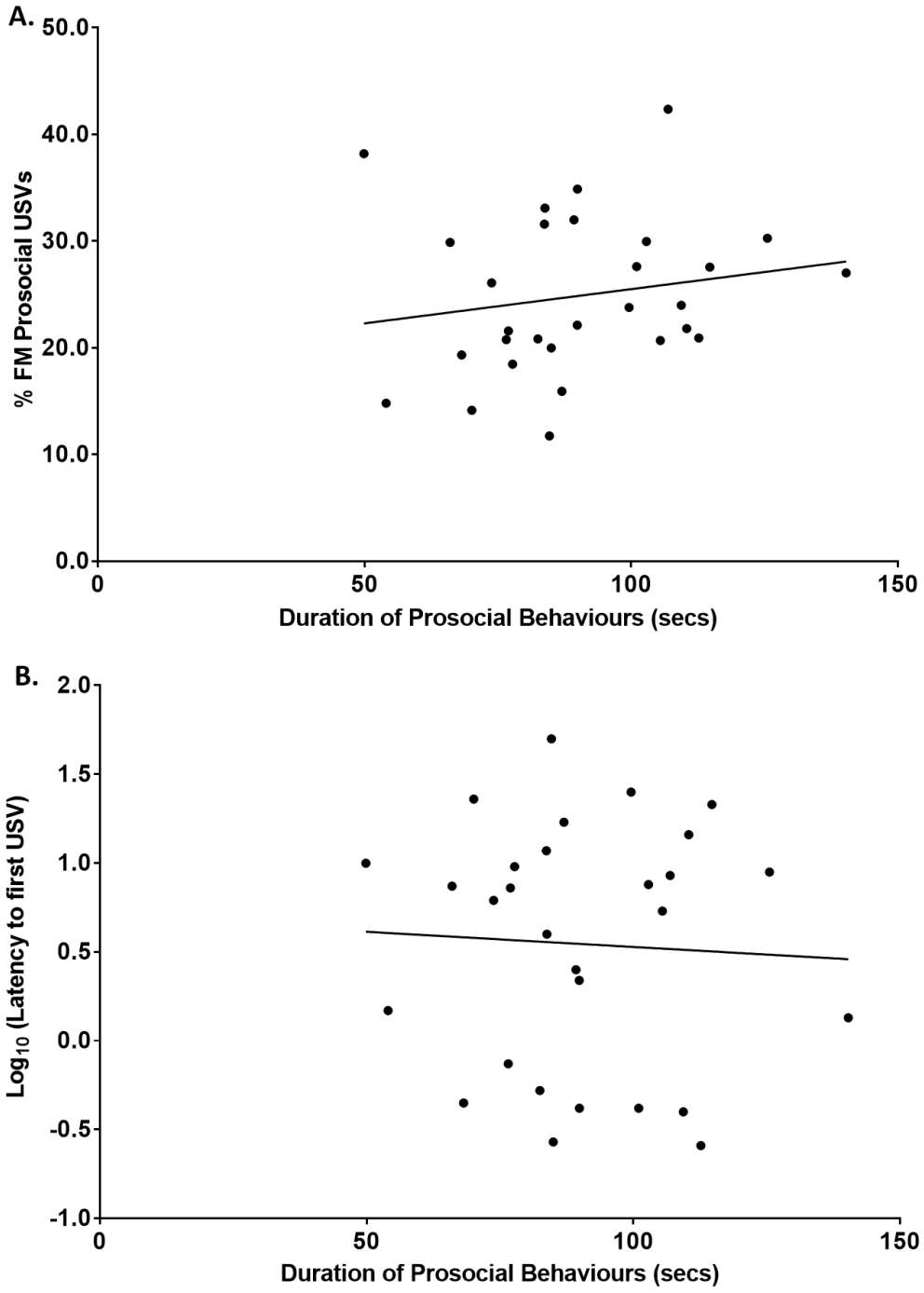

Figure 5.10: The correlation between the duration of prosocial behaviours in social interaction with (A) the percentage of FM prosocial calls emitted and (B) the latency to first USV. A Pearson's correlation found no statistically significant correlation between either the duration of prosocial behaviours and percentage prosocial FM USVs emitted during the social interaction task ($r(27)=0.18$, $p=0.35$), nor the duration of prosocial behaviours with the latency to first call during social interaction ($r(27)=-0.05$, $p=0.79$, latency to first call data was $\log_{10}$ transformed to produce a normal distribution).

5.4.4 Immunohistochemistry

In order to confirm the saporin injection placement was within the VTA and to determine whether there had been any loss of neurons following the OXY-saporin micro-injection, immunohistochemistry was performed using an anti-NeuN primary antibody. Slices containing the planned VTA injection site, as determined using the Rat Brain Atlas (Paxinos & Watson, 2007), were used for immunohistochemistry. One brain was excluded due to damage upon removal from the skull. Images revealed a visual loss of neurons within the VTA (Figure 5.11). Counting VTA NeuN positive cells in images taken at x20 magnification revealed there was a significant reduction in neurons following OXY-saporin injection to the VTA (mean = 20.95 ± 1.78), when compared to blank-saporin control injection (mean = 60.2 ± 2.56; $t_{(76)}$=12.61, p<0.001; Student's unpaired *t*-test) (Figure 5.12).

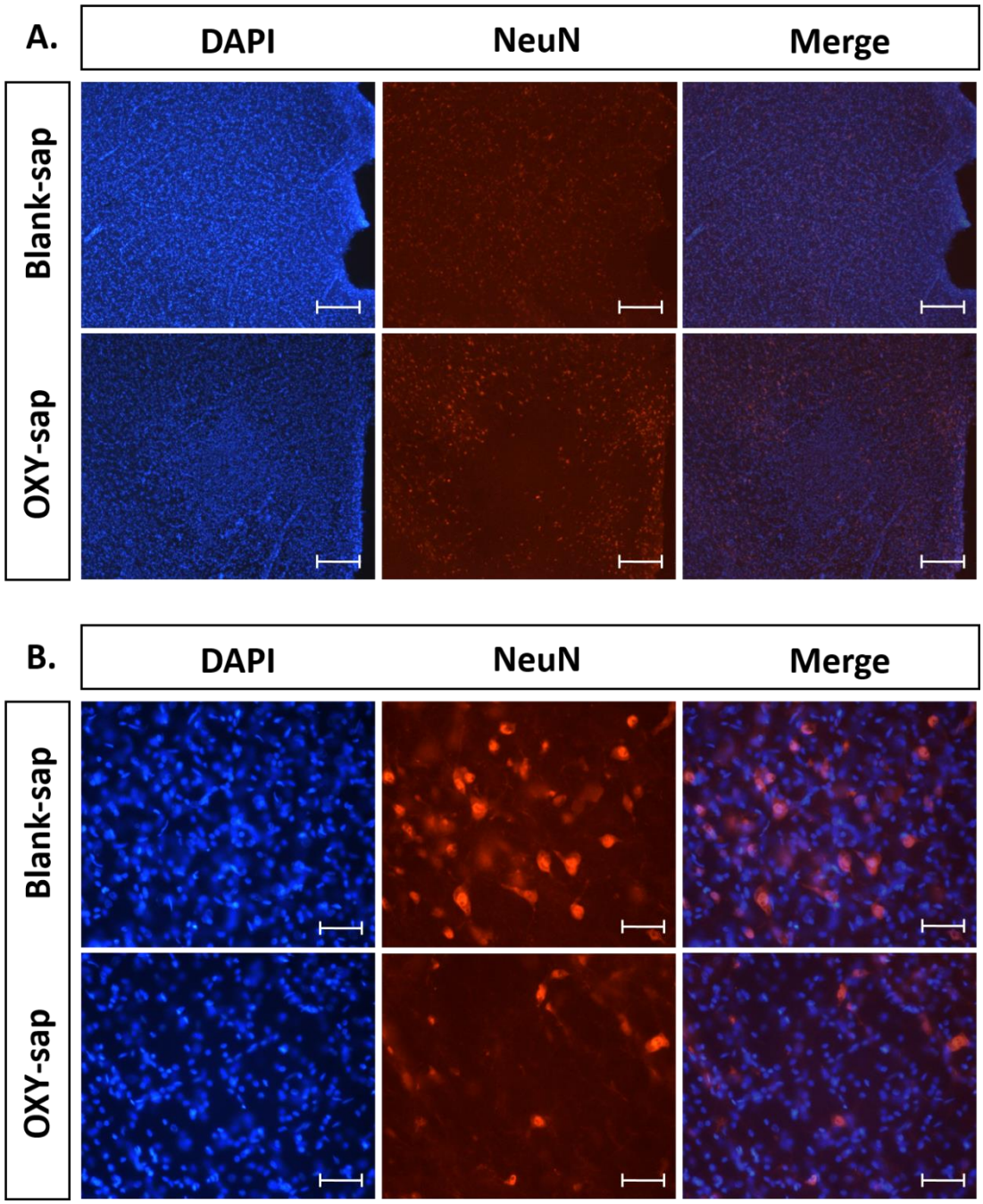

Figure 5.11: Representative images (showing the group mean) taken of the VTA after VTA OXY-saporin injection, taken at magnification x4 (A) and x20 (B) following immunohistochemical staining of cell bodies (DAPI; blue), neuronal nuclei (NeuN; red), and both images merged (Merge; blue and red). Images were taken on 60µm coronal sections in the location of VTA saporin injection (AP: -5.8, L: ±0.5, V: -8.2) and show a visible loss of neurons in the OXY-saporin condition. Scale bar represents 250µm in x4 images and 50µm in x20 images.

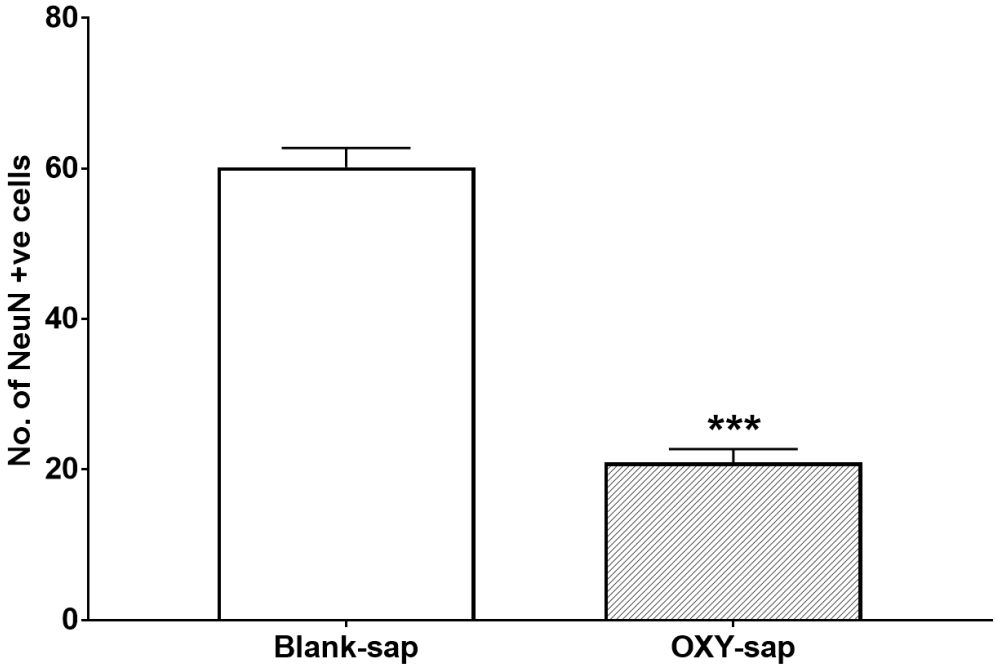

Figure 5.12: The effect of a VTA OXY-sap injection on the number of NeuN positive cells in the VTA (mean ± SEM, n=39/group). VTA OXY-sap injection caused a significant reduction in the number of neurons in the VTA (mean = 20.95 ± 1.78), compared to the blank-sap control injection (mean = 60.2 ± 2.56; $t_{(76)}$=12.61, p<0.001; Student's unpaired *t*-test).

5.5 Discussion

5.5.1 Effect of VTA OXY-saporin on OXY attenuation of PCP-induced hyperactivity

During this study we saw no effect of VTA saporin on OXY attenuation of PCP-hyperactivity. Despite the fact previous work has suggested reducing VTA dopaminergic activity can prevent PCP-induced hyperactivity (Hansen *et al.*, 2007; Sotty *et al.*, 2009), it is very unlikely this is the mechanism through which OXY achieves this. Although OXY can indirectly inhibit VTA dopaminergic activity through OXTR activation of VTA GABAergic interneurons, this is likely counteracted by direct OXY action on dopamine neurons. This would result in an overall increase in dopaminergic activity, the proposed mechanism through which OXY enhances social

interaction (Peris *et al.*, 2017; Xiao *et al.*, 2017). Furthermore, OXTRs are also expressed on glutamatergic neurons in the VTA (Peris *et al.*, 2017). As no effect of VTA OXY-saporin was observed upon PCP-hyperactivity, or on OXY attenuation of PCP-hyperactivity, it is unlikely VTA glutamatergic neurotransmission is involved. However, although there was a 66% reduction in the number of VTA neurons following OXY-saporin administration, due to the absence of any commercially available selective OXTR antibody we were unable to determine whether there were still OXTR expressing neurons remaining following OXY-saporin micro-injection. Therefore, it is possible there may be sufficient expression of OXTR-neurons remaining to enable OXY to reduce PCP-hyperactivity through modulation of VTA activity, despite preceding toxin administration.

It is of note that as well as the PVN sending axonal oxytocinergic projections to the VTA, it also sends monosynaptic projections to the substantia nigra (SN) (Watabe-Uchida *et al.*, 2012). The SN is part of the nigrostriatal dopamine pathway; connecting the SN with the dorsal striatum through dopaminergic connections (Björklund & Dunnett, 2007). The nigrostriatal pathway is critical in the modification of movement, with reduced dopaminergic activity associated with impairments in normal movement, as seen in Parkinson's disease (Hodge & Butcher, 1980). SN dopaminergic projections to the dorsal striatum synapse onto two separate populations of neurons; D_1 and D_2 expressing MSNs (Gerfen & Surmeier, 2011; Lobo, 2009). D_1 neurons form the 'direct pathway' of the basal ganglia, sending inhibitory projections to the internal globus pallidus (GPi), while the D_2 pathway forms the 'indirect pathway', indirectly stimulating the GPi through the external globus pallidus (GPe) and subthalamic nucleus (STN). The GPi then sends inhibitory projections to the ventral anterior and ventral lateral nuclei of the thalamus and, depending upon the balance between the stimulatory indirect pathway and inhibitory direct pathway, will then either inhibit or disinhibit motor outputs through stimulation of the motor cortex (Figure 5.13). Within the SN pars compacta (SNc) OXY can decrease the firing of dopamine neurons (Xiao *et al.*, 2017). OXTRs are primarily expressed on a population of local GABA neurons in the dorsolateral region of the SNc, an area that also receives the majority of PVN to SNc oxytocinergic projections (Xiao *et al.*, 2017). Therefore, OXY activity in

the SNc can indirectly inhibit SNc dopamine neurons, through OXTR activity on GABA neurons, reducing movement (Xiao *et al.*, 2017). Consistent with this hypothesis, increasing oxytocinergic activity in the SNc through direct OXY infusions results in decreased LMA, while decreasing oxytocinergic activity through either OXY-saporin injection or OXTR antagonism in the SNc increases LMA in rats (Angioni *et al.*, 2016). Therefore, it is possible OXY could reduce PCP-hyperactivity through facilitating GABAergic activity and reducing dopaminergic tone in the nigrostriatal pathway following s.c. OXY (Figure 5.13). However, it should be noted that in contrast to Xiao et al. (2017), Angioni et al. (2016) found OXTR expression on both dopaminergic and glutamatergic neurons in the SN. OXTR activity on dopamine neurons is unlikely to account for reduced LMA seen following OXY administration, given OXY facilitates dopaminergic activity in the SNc (Xiao *et al.*, 2017). However, Angioni hypothesised that OXY may reduce movement by facilitating GABAergic efferents to the thalamus and motor cortex. Stimulating OXTRs on glutamatergic terminals in the SN pars reticulata (SNr) releases glutamate to GABAergic neurons, increasing the inhibitory tone and reducing excitatory thalamic projections to the motor cortex (Figure 5.13). It is well documented that glutamatergic activity in the SN can modulate GABAergic and dopaminergic projections to the thalamus, therefore this may also explain how OXY can reduce PCP-hyperactivity (Angioni *et al.*, 2016). The V1aR is also expressed in the SN (Vaccari *et al.*, 1998). Although the phenotype of the neurons it is expressed on are unknown, it may be possible OXY is also acting through the V1aR to attenuate PCP-induced hyperactivity; potentially explaining why OXY was no longer able to attenuate PCP-hyperactivity following administration of the V1aR antagonist SR 49059. It is likely that the mechanisms mentioned above are not mutually exclusive, and all contribute to OXY reductions of LMA following PCP.

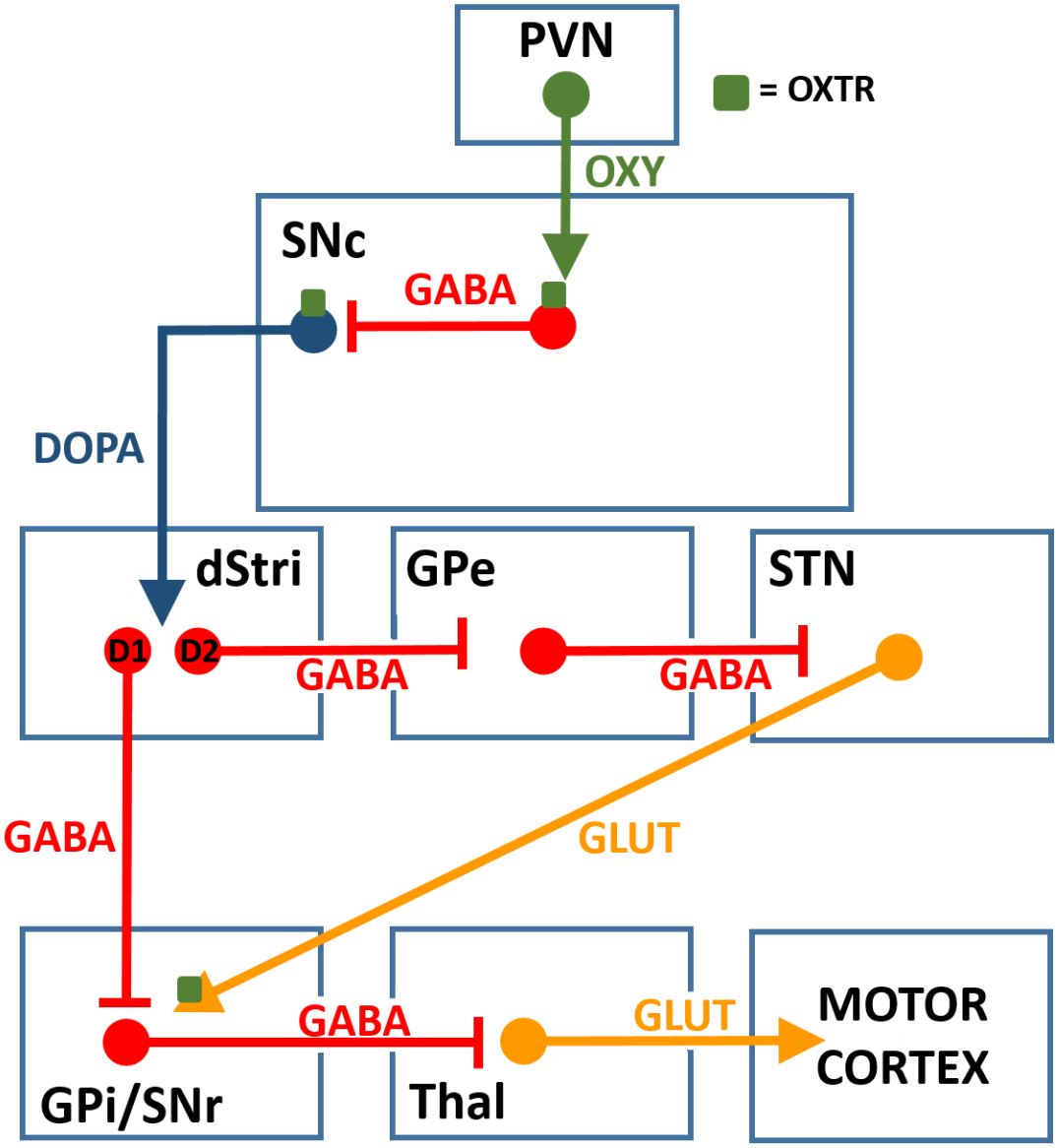

Figure 5.13: Diagram demonstrating the potential mechanisms through which OXY could inhibit PCP-hyperactivity through substantia nigra activity. Following s.c. administration OXY levels increase in the PVN, an area with oxytocinergic projections to the substantia nigra. Within the SNc OXTRs are expressed on local GABA neurons. This inhibits nigrostriatal dopamine neurons (DOPA) projecting to the dorsal striatum (dStri). Reduced dopaminergic activity in the dStri then disinhibits the GPi, resulting in increased inhibition of the thalamus (Thal). Inhibition of the thalamus reduces excitatory outputs to the motor cortex; explaining how OXY could act in the SN pars compacta (SNc) to reduce PCP-hyperactivity. OXTRs are also expressed in the SN pars reticulata (SNr) on glutamatergic (GLUT) terminals originating from the subthalamic nucleus (STN). Stimulation of OXTRs on glutamatergic terminals increases glutamatergic tone, increasing GABAergic efferents to the thalamus, reducing excitatory outputs to the motor cortex.

5.5.2 Effect of VTA OXY-saporin on OXY enhancement of social interaction

During this study we again showed s.c. OXY can enhance social interaction between two novel conspecifics, predominantly through increased body sniffing and anogenital sniffing, consistent with previous work in this thesis and Kohli *et al.* (2019). Immunohistochemistry confirmed OXY-saporin injections in the VTA caused significant neuronal loss. Following VTA OXY-saporin injection s.c. OXY elevation of social interaction was not seen, suggesting a critical role for VTA OXTR expressing neurons in OXY enhancement of social interaction. Although VTA OXY-saporin prevented OXY enhancement of social interaction, it did not reduce levels to lower than that of the control groups.

Given previous findings in this thesis it is very likely OXY is enhancing social interaction through modulation of mesolimbic dopamine, with removal of OXTR expressing neurons in both the VTA and NAc preventing OXY enhancement of social behaviour. S.c. OXY administration increases OXY in the PVN; an area which has oxytocinergic axonal projections to the VTA (Hung *et al.*, 2017; Xiao *et al.*, 2017; Yamamoto *et al.*, 2019). PVN oxytocinergic neurons that project to the VTA form monosynaptic connections with mesolimbic dopamine neurons, acting at post synaptic $G\alpha_q$ coupled OXTRs to increase mesolimbic neuron activity. These dopaminergic neurons then project to the NAc, causing phasic dopamine release and acting upon D_1 NAc MSNs (Hung *et al.*, 2017; Xiao *et al.*, 2017). Activation of D_1 MSNs in the NAc signals reward, reinforcing behaviours and driving longer periods of social interaction (Gunaydin *et al.*, 2014). Xiao *et al.* (2017) found approximately 55% of VTA dopaminergic neurons express the OXTR. Therefore, removal of OXTRs in the VTA would remove a large proportion of OXTR expressing NAc projecting dopamine neurons, reducing NAc dopamine release, and thus reducing the rewarding aspects of social interaction. This is supported by previous work in our laboratory that showed 0.1mg/kg s.c. OXY increased NAc dopamine microdialysates (Kohli *et al.*, 2019). Resendez *et al.* (2020) also recently demonstrated that activation of PVN oxytocinergic neurons is sufficient to increase social preference in mice, and

endogenous activity of these neurons is required for the normal expression of social behaviours. In addition, the presentation of a social stimulus activates PVN oxytocinergic neurons in a distinct manner from when presented with a non-social stimulus, suggesting OXY PVN neurons play a critical role in encoding social behaviour (Resendez *et al.*, 2020).

As well as expression on dopaminergic neurons in the VTA, Peris *et al.* (2017) also found OXTR expression on glutamatergic neurons in mice, although this is in contrast to Xiao *et al.* (2017). VTA glutamatergic neurons project to a variety of regions including the NAc, amygdala, and PFC, as well locally within the VTA (Dobi *et al.*, 2010; Taylor *et al.*, 2014). Dobi *et al.* (2010) demonstrated local VTA glutamatergic transmission can alter the activity of VTA dopamine projection neurons. Therefore, OXTR activation of glutamatergic neurons in the VTA may be another mechanism through which OXY can modulate social behaviour via mesolimbic activity.

It is of note that neither VTA OXY-saporin nor NAc OXY-saporin reduced basal levels of social interaction in control conditions. This may suggest although OXY can increase social interaction through mesolimbic dopamine, this is not the neural mechanism that underlies social interaction in the absence of administered OXY. Numerous other brain regions have been implicated in the neural mechanisms controlling social interaction. For example, the medial amygdala is part of a neural network that processes olfactory cues in rodents and can regulate behaviours such as social recognition and social memory (Bergan *et al.*, 2014; Guthman & Vera, 2016; Halpern & Martínez-Marcos, 2003; Noack *et al.*, 2015). The medial amygdala is also part of the SBNN, a group of interconnected brain regions thought to regulate a diverse range of social behaviours in rodents, such as aggression, sexual, and parental behaviours (Newman, 1999). Other brain regions in this network include the lateral septum, medial preoptic area, and the ventromedial and anterior hypothalamus. Differential activation of different nodes within the SBNN are thought to lead to the expression of different forms of social behaviours (Dumais & Veenema, 2016; Newman, 1999; O'Connell & Hofmann, 2012). Therefore, the administration of s.c. OXY in Lister-hooded rats is likely to be enhancing the rewarding aspects of social behaviours and subsequently driving longer periods of interaction, while activity in

the SBNN likely contributes to normal social interaction in the absence of exogenous OXY application.

To further validate our findings and gain a greater insight to OXTR activity in the VTA it would be of interest to decipher the phenotype of neurons lost following VTA OXY-saporin injection and confirm whether they are dopaminergic or otherwise. Financial constraints prevented further immunohistochemical analysis of brains and the conduction of other experiments, such as PCR analysis, which could have been used to determine neuronal phenotypes. These experiments would give more of an insight into the activity of OXY within the VTA, and either strengthen or contradict the aforementioned hypothesis.

While there is an abundance of evidence demonstrating the prosocial role of mesolimbic dopamine and OXY interactions, aversive experiences such as foot shocks and social defeat also increase dopamine release in the NAc and VTA dopaminergic activity (Brischoux *et al.*, 2009; Greenberg *et al.*, 2015; Tidey & Miczek, 1996). In addition, aggressive interactions between two rats can significantly increase NAc dopamine release and VTA dopamine firing patterns (Anstrom *et al.*, 2009). Therefore, phasic dopamine neurotransmission in the mesolimbic pathway may also be involved in non-prosocial interactions, through modulating the salience of social interactions and heightening the processing of social stimuli. It may be that OXY is able to enhance the salience of both positive and negative social interactions through mesolimbic activity, having differential effects upon social behaviours depending on the context. For example, OXY may increase prosocial behaviours in a positive environment such as the social interaction task, but also increase aggression in negative environments, such as models of social stress. This would have important translational implications, with personality traits and environmental factors all contributing to the behavioural effects of OXY. For example, although OXY may enhance the salience of positive, prosocial interactions, it may also enhance the prominence of negative social interactions, having undesirable and negative effects upon social behaviour, for example increasing aggression in anti-social situations (Shamay-Tsoory & Abu-Akel, 2016).

5.5.3 Effect of VTA OXY-saporin on production of USVs

In this chapter we saw no effect of either OXY or VTA OXY-saporin on the production of 50 kHz prosocial USVs during social interaction; consistent with both previous work in this thesis and Kohli *et al.* (2019). There was also no significant correlation between the duration of prosocial behaviours and the emission of FM prosocial calls, consistent with work in both Sprague Dawley and Wistar rats (Manduca *et al.*, 2014a). Interestingly, Manduca *et al.* (2014b) found 50 kHz calls emitted during social interaction do not necessarily correlate with the performance of social behaviours, and are also emitted during grooming and exploration of the arena. This may explain why no correlation was observed in this thesis.

As discussed in chapter 4.5.2, emission of USVs are likely to be controlled by a distinct neural mechanism to the one that controls OXY enhancement of social behaviour, for example by modulation by glutamatergic neurotransmission (Hamed & Kursa, 2020). If s.c. OXY does increase prosocial behaviours by enhancing mesolimbic dopamine, it is unlikely FM 50 kHz USVs are also modulated in this manner, due to the lack of treatment effects and lack of correlation between emission and prosocial behaviours in this thesis.

5.6 Summary

In summary, this chapter has demonstrated OXTR expressing neurons in the VTA are critical for OXY enhancement of social interaction in male Lister-hooded rats; likely through enhancing mesolimbic dopamine and subsequent social reward. In addition, neither OXY nor VTA OXY-saporin had any effect upon USVs during social interaction. This suggests emission of 50 kHz USVs during social interaction are unlikely to be modulated by OXY and the mesolimbic system. This chapter also repeated previous findings showing s.c. OXY can attenuate PCP-hyperactivity in rats. In contrast to the effect on social behaviour, injection of OXY-saporin into the VTA had no effect on the ability of OXY to attenuate PCP-hyperactivity; suggesting OXY enhancement of social

behaviour and attenuation of PCP-hyperactivity are controlled by distinct neural mechanisms.

6. General Discussion

6.1 Summary of findings

The work in this thesis has contributed to improve our understanding of the neural mechanisms which promote s.c. OXYs prosocial and antipsychotic-like effects in male Lister-hooded rats. Firstly, in chapter two we established that conjugation of OXY to the novel cell-penetrating peptide, GET, hinders biological activity at the OXTR, and therefore was unsuitable for intranasal administration or for use in this thesis. A low dose of s.c. OXY was subsequently selected for the behavioural experiments performed, due to the fact it can modulate brain OXY levels without having any profound concomitant peripheral effects, such as hypothermia or sedation (Kohli *et al.*, 2019; Yamamoto *et al.*, 2019).

In chapter 3 we demonstrated s.c. OXY can both enhance social interaction between two male rats, and attenuate PCP-induced hyperactivity; indicative of an antipsychotic-like effect and consistent with previous work from this laboratory (Kohli *et al.*, 2019). Utilising selective V1aR and OXTR antagonists, SR 49059 and L-368,899 respectively, we demonstrated activity at the OXTR was responsible for OXY enhancement of social behaviour following systemic administration. However, neither OXY, nor either antagonist, had any effect upon USVs and social communication during the social interaction task. In contrast, activity at both the OXTR and V1aR are involved in the ability of OXY to reverse PCP-induced hyperactivity, such that both antagonists reduced OXY-attenuation of PCP-hyperactivity. Future work could examine the effect of combined administration of OXTR and V1aR antagonists on the OXY attenuation of PCP-hyperactivity to see if this could totally prevent this effect.

Having established the receptors involved in each behaviour we identified the NAc as an area in which OXY may be acting to enhance both social behaviour and reverse PCP-hyperactivity, due to its role in maternal behaviours, pair bonding, and the ability of OXY to increase NAc dopamine overflow (Kohli *et al.*, 2019; Lei *et al.*, 2017; Ross *et*

al., 2009a; Shahrokh *et al.*, 2010). In addition, the NAc has high OXTR expression, as well as outputs to the VTA and subsequent motor areas (Hosp *et al.*, 2019; Nguyen *et al.*, 2018; Smith *et al.*, 2017a). To explore the effect of NAc OXTRs on social behaviour and PCP-induced hyperactivity we used bilateral micro-injections of the OXTR targeted toxin, OXY-saporin, to remove NAc OXTRs. OXY was no longer able to increase social interaction following toxin micro-injection, presumably due to the removal of NAc OXTRs; strongly implicating mesolimbic dopamine in the prosocial effect of s.c. OXY and consistent with work suggesting mesolimbic dopamine can enhance social reward and social interaction (Dölen *et al.*, 2013; Gunaydin *et al.*, 2014). However, removal of NAc OXTR expressing neurons had no effect on the ability of OXY to attenuate PCP-hyperactivity, suggesting that NAc OXTRs are not involved in OXYs antipsychotic-like effects. This is consistent with previous work demonstrating NMDA-R antagonists are still able to cause hyperactivity following chemical lesions of NAc projecting dopaminergic neurons (Carlsson & Carlsson, 1989).

The final experiment in this thesis aimed to determine the role of VTA OXTR expressing neurons on social interaction and OXY attenuation of PCP-hyperactivity; another brain region with high OXTR expression and a key node in the mesolimbic system (Peris *et al.*, 2017; Xiao *et al.*, 2017). Removal of OXTR expressing neurons in the VTA also prevented OXY enhancement of prosocial behaviours but had no effect on USVs during social interaction or on OXY's attenuation of PCP-induced hyperactivity. The involvement of the VTA in OXY-mediated social behaviours further supports the role of mesolimbic dopamine in the enhancement of social behaviour following s.c. OXY. However, as with the NAc, the VTA is unlikely to be involved in OXY-attenuation of PCP-hyperactivity.

In summary, we demonstrated s.c. OXY increases social interaction between two male rats through OXTR activity in the NAc and VTA. A possible explanation for this is that following s.c. administration OXY is likely transported into the brain by RAGE, as increased levels of OXY are observed in the PVN following s.c. OXY administration (Yamamoto & Higashida, 2020; Yamamoto *et al.*, 2019). Although the PVN is inside the BBB it is highly vascularised, which may aid RAGE-dependant entry of OXY to the

PVN (Badaut *et al.*, 2000; Duan & Ju, 1998). The PVN has axonal projections to both the NAc, where it can modulate OXTR expressing MSNs (Dolen & Malenka, 2014; Moaddab *et al.*, 2015; Peris *et al.*, 2020; Williams *et al.*, 2020), and the VTA, where it releases OXY onto dopaminergic neurons (Xiao *et al.*, 2017). Thus, s.c. OXY is likely to increase the activity of PVN OXY projections to both the NAc and VTA. VTA dopaminergic neurons project to the NAc, where they release dopamine onto D_1 and D_2 expressing MSNs (Figure 6.1) (Gunaydin *et al.*, 2014; Hung *et al.*, 2017; Melis *et al.*, 2007). Increased activity of D_1 MSNs are associated with increased reward and can modulate and increase social behaviours (Gunaydin *et al.*, 2014; Kopec *et al.*, 2018). Our experiments with OXY-saporin likely produced toxic apoptosis to either OXTR expressing NAc MSNs (following NAc OXY-saporin injection) or VTA-NAc dopaminergic projections (following VTA OXY-saporin injection); both of which prevented OXY enhancement of social behaviour. In addition, our dose of s.c. OXY is known to increase NAc dopamine efflux (Kohli *et al.*, 2019). The associations of NAc dopamine with reward suggest that OXY may enhance social interaction by increasing the feelings of reward associated with social interaction. Increased feelings of reward are therefore likely to drive longer bouts of interaction, explaining how the dose of OXY used increased social interaction. However, there are likely to be other neural mechanisms that modulate social interaction in the absence of OXY, given removal of VTA and NAc OXTR expressing neurons did not reduce social interaction in control conditions, such that rats displayed normal levels of social behaviours.

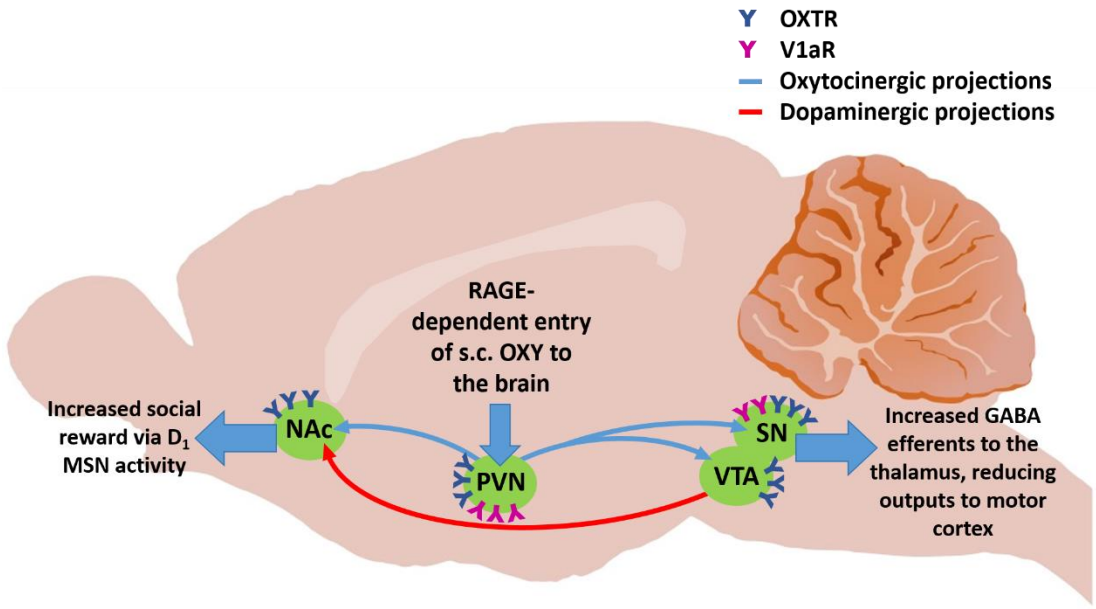

Figure 6.1: The proposed mechanisms though which s.c. OXY can increase social behaviours and attenuate PCP-induced hyperactivity, as demonstrated in this thesis. Following s.c. administration OXY is likely transported into the brain by the RAGE, increasing OXY at the PVN. The PVN has axonal projections to both the NAc, where it can increase MSN activity, and the VTA, where it releases OXY onto dopaminergic neurons. VTA dopaminergic neurons project to the NAc, where they release dopamine onto D_1 and D_2 expressing MSNs. Increased activity of D_1 MSNs is associated with increased reward, and can modulate and drive social behaviours. As well as PVN projections to the VTA, the PVN also has direct OXY axonal projections to the SN; an area heavily involved in movement. OXY activity in the SN can reduce movement through increasing GABAergic efferents to the thalamus and reducing outputs to the motor cortex; a possible mechanism through which OXY attenuates PCP-hyperactivity.

Although this thesis found robust evidence for s.c. OXY increasing social interaction, there was no effect upon the emission of 50 kHz prosocial calls during social interaction, nor an effect of either NAc or VTA OXY-saporin injection, nor a correlation between the duration of prosocial behaviours and 50 kHz call emission. This suggests that USVs during social interaction are modulated by an alternative neural mechanism to social interaction, and that the increased social interaction we saw following OXY was not the result of increased communication. It is therefore unlikely USVs emitted during social interaction are modulated by mesolimbic activity. However, glutamatergic activity may contribute to USV emission. Recent research has shown social interaction-induced 50 kHz USVs coincide with changes in glutamate

levels in the amygdala and VTA (Hamed & Kursa, 2020). However, it is of note that Manduca *et al.* (2014b) found 50 kHz calls emitted during social interaction also occur in the absence of social behaviours during social interaction tasks; such as when self-grooming. Therefore, this may explain why we saw no correlation between 50 kHz call emission and the duration of prosocial behaviours.

In addition to the prosocial effects of OXY, we also robustly demonstrated that s.c. OXY can attenuate PCP-induced hyperactivity, likely through activity at both the OXTR and V1aR. NAc and VTA OXY-saporin injections had no effect on attenuation of PCP-induced hyperactivity; therefore, suggesting mesolimbic dopamine is unlikely to be involved. Furthermore, as OXTRs are also expressed on VTA glutamatergic neurons it is unlikely VTA glutamatergic activity is responsible for OXY attenuation of PCP-induced hyperactivity (Peris *et al.*, 2017). However, although we saw a 43% and 66% reduction in the number of neurons following OXY-saporin administration into the NAc and VTA respectively, due to the absence of any commercially available selective OXTR antibody we were not able to determine whether there were still OXTR expressing neurons remaining following OXY-saporin micro-injection. Therefore, it is possible there may be sufficient expression of OXTR neurons remaining to enable OXY to reduce PCP-hyperactivity through modulation of VTA and NAc activity, despite preceding toxin administration.

As previously discussed, s.c. OXY can increase OXY in the PVN. As well as PVN projections to the VTA, the PVN also has direct OXY axonal projections to the SN; an area heavily involved in the modulation of movement (Xiao *et al.*, 2017). OXY activity in the SN can decrease LMA (Angioni *et al.*, 2016), therefore the dose of OXY used may be reducing PCP-hyperactivity through promoting PVN to SN OXY activity and reducing movement by increased GABAergic activity in the nigrostriatal pathway (Figure 6.1). The V1aR is also expressed in the SN (Vaccari *et al.*, 1998). Although the phenotype of the neurons that V1aRs are expressed on are unknown, it may be possible OXY is also acting through the V1aR to attenuate PCP-induced hyperactivity in the SN. It should be noted that although we have hypothesised that increases in the activity of PVN oxytocinergic projections to the VTA, NAc, and SN, and the subsequent effects on mesolimbic and nigrostriatal activity, drive s.c. OXYs prosocial

and antipsychotic-like effects, the PVN also has numerous other oxytocinergic projections to other brain areas. Therefore, s.c could also be enhancing OXY activity in other regions of the brain, which also contribute to the prosocial and antipsychotic-like effects of OXY.

6.2 Future work and limitations

To expand upon work in this thesis it would be of interest to firstly determine the phenotype of the neurons lost in the NAc and VTA following OXY-saporin injection, through immunohistochemistry. This would enable us to determine which neurons are critical in OXY enhancement of social behaviour. In addition, microdialysis or voltammetry studies to determine the neurochemical activity in the VTA during social interaction, and following s.c. OXY administration, would be of great interest to either add strength to or refute the aforementioned hypothesis.

An additional area to explore to enhance our understanding of s.c. OXYs effects upon social behaviour would be to examine specific aspects of social behaviours. Social interaction is a generalised model, incorporating numerous domains of social behaviour, such as social reward, social recognition, and social approach, and, in this thesis, social communication. Although social interaction has great strengths and can enable the identification of prosocial pharmacological substances, further experiments are needed to analyse specific aspects of social behaviour. We have hypothesised that in this thesis OXY is driving social interaction through increased social reward. Therefore, in addition to social interaction it would be of great interest to observe the effects of s.c. OXY in preclinical assays looking specifically at social reward, through social conditioned place preference (Calcagnetti & Schechter, 1992; Dölen *et al.*, 2013). By using assays that focus on one specific aspect of social behaviour we may learn more about s.c. OXYs mechanism of action. It may be that OXY is also enhancing the ability of the rodents to recognise each other as novel, also contributing to increased social interaction. Thus, determining the effects of s.c. OXY

on both social reward and social recognition would be insightful, as s.c. OXY may enhance both social reward and social recognition through different mechanisms.

Although social interaction has great strengths in that: it provides a more naturalistic and complex social environment than assays looking at one specific domain, it measures behaviours that naturally occur in rodents, and actions are taken to reduce anxiety (such as low light and closely weight-matched conspecifics), this paradigm does not consider some additional important aspects of social behaviour. For example, despite care being taken to ensure rats were novel, and weight- and treatment-matched, this paradigm does not take into consideration social hierarchy, where evidence suggests the subordinate rat will sniff and explore the conspecific at a reduced frequency compared with the dominant rat (Wesson, 2013). Furthermore, it is unknown whether behaviours observed between two rodents following drug administration can be generalised to social behaviours within groups. However, these criticisms can be applied to the majority of tasks measuring social behaviours. The recent advancement of technologies such as radio-frequency identification (RFID) can allow us to overcome this (Mitchell *et al.*, 2020; Peleh *et al.*, 2019). RFID alongside video tracking enables automatic extensive monitoring of social behaviours in multiple rodents over a long period in their home cages, and provides a more ethologically relevant measure of social behaviour (Peleh *et al.*, 2019).

It should be noted that while the findings in this thesis indicate a potential antipsychotic-like effect of s.c. OXY, the extrapolation of attenuating PCP-hyperactivity in rats to the multifaceted human disorder of schizophrenia is speculative. To further strengthen the research indicating an antipsychotic-like effect of OXY it would be of interest to determine whether OXY can also reverse the working memory and prepulse inhibition deficits produced by acute PCP (Bakshi *et al.*, 1994; Egerton *et al.*, 2005; Mansbach & Geyer, 1989), which are analogous to symptoms seen in patients with schizophrenia. Although we have shown in this thesis that both the OXTR and V1aR are involved in the antipsychotic-like effect of OXY, and that the mesolimbic system is unlikely to be involved, a lot remains unknown. It would be of great interest to determine the brain regions involved in OXY reversal of PCP-hyperactivity; this could be through microdialysis studies or the use of further OXY-

saporin micro-injections, followed by the identification of neurons lost in brain regions such as the SN. In addition, whilst we have robustly shown s.c. OXY can enhance social interaction between two healthy rats of the same sex, it would be of significant interest to determine whether this dose of OXY can also ameliorate the deficits seen in social interaction following chronic PCP, maternal separation, or maternal immune activation; all of which have been used as preclinical models for schizophrenia (Jones et al., 2011). Reversal of these social deficits would further strengthen the research supporting the use of OXY in the treatment of psychiatric disorders with social deficits.

Finally, there are a few caveats that should be discussed when considering the translational applications of this work to humans. Firstly, in contrast to humans, rodents use olfaction as their primary method of social perception, while humans and non-human primates primarily rely upon visual and auditory cues (Althammer *et al.*, 2018). Moreover, although OXTR expression between rodents and primates is similar, such as high expression in the mesolimbic system, there are some substantial differences (Freeman & Young, 2016). In rodents OXTR expression is dense in olfactory processing regions, whereas in primates OXTR expression is dense in regions associated with visual processing (Freeman & Young, 2016; Grinevich *et al.*, 2016). Therefore, the ability of OXY to enhance prosocial behaviours in rodents may not entirely represent the effects of OXY in other species. Secondly, this thesis only looked at acute OXY administration. Chronic OXY is likely to cause receptor desensitisation and downregulation (Conti *et al.*, 2009; Huang *et al.*, 2014). Depending on the receptor reserve and the rate at which desensitisation occurs, this may severely limit the clinical utility of OXY. This is an area of receptor pharmacology that would require in-depth research. It may be possible to develop bias agonists that preferentially activate G-protein signalling without recruiting β-arrestin to the receptor, preventing receptor desensitisation (Andresen, 2011; Rajagopal *et al.*, 2010; Rankovic *et al.*, 2016). For example, the bias OXTR-$G\alpha_q$ antagonist/OXTR-$G\alpha_i$ agonist atosiban is able to activate the $G\alpha_i$-coupled OXTR without recruiting β-arrestin to the receptor, therefore does not cause receptor desensitisation or internalisation (Busnelli *et al.*, 2012). Finally, the rationale behind studying the neural

mechanisms of OXY was its potential for use as an adjunctive therapy in psychiatric disorders with social deficits, such as schizophrenia. The primary mechanism of action that underlies antipsychotic efficacy is D_2 receptor antagonism in the mesolimbic system (Meltzer, 1991). However, evidence in this thesis and previously published work suggests OXY enhances social behaviours through increasing mesolimbic dopamine transmission; with some evidence suggesting D_2 receptor activation is necessary for the prosocial effects of OXY (Manduca *et al.*, 2016). Therefore, the interaction between antipsychotics and OXY should be explored in future studies, as their co-administration may have counteractive effects upon behaviour.

6.3 Conclusions

The findings from this thesis confirm s.c OXY can both enhance prosocial behaviour and attenuate PCP-induced hyperactivity in male Lister-hooded rats. We demonstrated OXTRs are responsible for OXYs prosocial effects in rats, and removal of OXTR expressing neurons in the NAc and VTA both prevent OXY enhancement of social behaviour. Accompanied with evidence from published works, this suggests s.c. OXY likely promotes social interaction through enhanced mesolimbic activity. However, mesolimbic dopamine and OXY are unlikely to be involved in the production of USVs during social interaction in rodents, due to the lack of treatment effects observed throughout this thesis. Whilst removal of OXTRs in the VTA and NAc prevented prosocial effects of OXY, they had no effect upon OXY attenuation of PCP-induced hyperactivity. Activity at the both V1aR and OXTR in other regions of the brain are thus likely to contribute to these antipsychotic-like effects of OXY. Future experiments are necessary to understand the neural mechanisms that modulate OXYs antipsychotic-like effect.

Overall, this work has significantly contributed to our understanding of OXYs neural mechanisms, as well as providing robust evidence peripheral OXY can modulate behaviour through central activity. The use of OXY to treat social deficits in psychiatric disorders remains promising, and further studies are necessary to determine whether

OXY can ameliorate social deficits in rodent models of psychiatric disorders, as well as determining interactions between OXY and antipsychotics.

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Appendices

Appendix 1: Oxytocin receptor immunohistochemistry optimisation

A1.1 Introduction

Immunohistochemistry can allow receptor visualisation through exploiting specific antigen-antibody reactions. In this thesis it was hoped that by using an anti-OXTR primary antibody we could confirm the loss of OXTR expressing neurons following OXY-saporin injection to the NAc and VTA. The first successful publication reporting OXTR immunohistochemistry staining was Adan *et al.* (1995), using an OXTR antibody targeting the receptors third intracellular loop. However, since this paper there have been a surprising lack of publications using OXTR immunohistochemistry in rodents, given the popularity of OXY research. To the best of our knowledge the only papers published with successful OXTR immunohistochemistry staining in the rodent brain are detailed in Table A1.1. It is of interest Yoshida *et al.* (2009) tried five different commercially available antibodies and only detected OXTR staining in the hypothalamus. However, this staining was also present in OXTR knockout mice, highlighting the unreliability of OXTR antibodies.

Group	Animal	Primary OXTR antibody	Commercially available?
Adan *et al.* (1995)	Mouse	JV3850 (internally produced)	No
Liu *et al.* (2005)	Mouse	Monoclonal anti-OTR (Cosmo Biotechnology Inc., Japan)	Discontinued
Murata *et al.* (2011)	Rat	sc-810 (Santa Cruz Biotechnology, USA)	Discontinued
Zhao and Ai (2011)	Rat	anti-OTR (USCNLIFE Science, USA)	Discontinued
Tzabazis *et al.* (2015)	Rat	sc-810 (Santa Cruz Biotechnology, USA)	Discontinued
Mitre *et al.* (2016)	Mouse	Internally produced, targeting OXTR third intracellular loop	No
Warfvinge *et al.* (2020a) and Warfvinge *et al.* (2020b)	Rat	ab87312 (Abcam, UK) and aa43-129 (LifeSpan, Sweden)	Discontinued (ab87312) and yes (aa43-129)

Table A1.1: Studies published to date with successful OXTR immunohistochemistry in the rodent brain. All antibodies have since been discontinued or are not commercially available, except aa43-129 (LifeSpan, Sweden) used by Warfvinge *et al.* (2020a; 2020b).

The difficultly in gaining selective OXTR staining may be due to the high sequence homology between the OXTR and vasopressin receptors (Gimpl & Fahrenholz, 2001). Other techniques have since been employed to look at OXTR localisation and quantification in the brain, such as autoradiography and *in-situ* hybridisation (Bales & Perkeybile, 2012; Lukas *et al.*, 2010; Smith *et al.*, 2017a; Smith *et al.*, 2019). However, studies using *in-situ* hybridisation to map OXTR expression may be inaccurate, as the presence of OXTR mRNA does not necessarily correlate with the expression of the functioning OXTR protein. Due to the good spatial resolution and speed at which it can be performed, autoradiography has become the gold standard for studying OXTR expression; mapping expressing across ages and sexes (Smith *et al.*, 2017a; Smith *et al.*, 2019; Sóvágó *et al.*, 2001).

While OXTR immunohistochemistry has not been especially successful, many groups have successfully stained for OXY neurons in the rodent brain (Angioni *et al.*, 2016; Bratzu *et al.*, 2019; Hansson *et al.*, 2018; Kiss & Osacka, 2020). However, the presence of OXY neurons does not necessarily lead to OXTR expression, especially given the

evidence OXY can act promiscuously through the V1aR (Smith *et al.*, 2019). Furthermore, OXY can be transported through the brain by the CSF following paracrine release from magnocellular neurons (Jurek & Neumann, 2018; Ludwig & Leng, 2006). Therefore, areas without OXY fibres are likely to also express OXTRs. For the purpose of this thesis we were aiming to use OXTR immunohistochemistry to confirm the loss of OXTR expressing neurons in the VTA and NAc following OXY-saporin micro-injection. Evidence suggests in the VTA and NAc OXTRs are likely located post-synaptic to OXY nerve terminals, on dopaminergic neurons and MSNs (Williams *et al.*, 2020; Xiao *et al.*, 2017). Therefore, staining for OXY neurons would shed little insight to the neurons lost following OXY-saporin injection.

A1.2 Aims

Comparing post-mortem immunohistochemical staining of the OXTR in brain tissue can allow us to visualise changes in receptor expression following the injection of the OXTR targeting toxin OXY-saporin. Due to the lack of facilities available to perform autoradiography, we aimed to optimise a commercially available OXTR antibody for use in future experiments.

A1.3 Methods

A1.3.1 Tissue preparation

Brain tissue was prepared as described previously (Chapter 4.3.8). Uterine tissue was snap frozen upon removal from lactating female Lister-hooded rats 19-21 days post-parturition. Upon slicing 1.5cm sections of uterine tissue were encased in optimal cutting temperature compound (OCT) embedding matrix (VWR International Ltd, UK) and frozen until solid. Once frozen, slices were cut along the coronal plane at 60 µM

using a freezing microtome (Anglia Scientific, U.K.). Uterine tissue then followed the same protocol as brain tissue for the rest of the immunohistochemistry process.

A1.3.2 Standard immunohistochemistry protocol

The standard immunohistochemistry was as described in Chapter 4.3.9, with any changes detailed below. The primary anti-OXTR antibodies used to try and gain successful OXTR staining were PA5-19038 (ThermoFisher, UK), ab87312 (Abcam, UK), and ab217212 (Abcam, UK). All three antibodies are polyclonal and target the intracellular C-terminal tail of the OXTR. Both ab87312 (Abcam, UK) and PA5-19038 (ThermoFisher, UK) have been discontinued since the experiments performed in this appendix. The blocking serums and secondary antibodies used for each respective primary antibody are detailed in Table A1.2. Although the concentration of blocking serum varied between 1-5% for different optimisations, unless otherwise stated in the results section the concentration of blocking serum was 2%.

Primary	Blocking serum	Secondary	Notes
ab87312 (goat)	Donkey serum (Sigma Aldrich, UK)	Donkey anti-goat; Alexa Fluor 584 (Abcam, UK)	Donkey anti-goat; Alexa Fluor 568 was also trialled (Abcam, UK)
PA5-19038 (goat)	Donkey serum (Sigma Aldrich, UK)	Donkey anti-goat; Alexa Fluor 584 (Abcam, UK)	
ab217212 (rabbit)	Goat serum (Sigma Aldrich, UK)	Goat anti-rabbit; Alexa Fluor 568 (Abcam, UK)	Donkey Anti-Rabbit; Alexa Fluor 555 (Abcam, UK) with donkey serum blocking was also trialled

Table A1.2: Details of blocking serums and secondary antibodies used with each anti-OXTR primary antibody trialled in this appendix.

A1.3.3 Antigen retrieval

Following the first PBS washes on day one, slices were incubated in 1mL citrate buffer (10mM, pH 6.0) for twenty minutes at 95˚C. This was followed by an additional wash with PBS. The standard protocol was then resumed for the remainder of the experiment.

A1.3.4 Biotinylated and Avidin Secondary Antibodies

Day 1 was conducted as per the standard protocol. Following overnight incubation with the primary antibody slices were washed in D2 buffer three times for five minutes. The secondary antibody, either biotinylated horse anti-goat or goat anti-rabbit (Vector Laboratories, UK), dependent on the host species of the primary antibody, were prepared in D2 buffer at 1:500. Slices were incubated in the secondary antibody in the dark for one hour at room temperature. Slices were washed in D2 buffer three times for 5 minutes. The tertiary antibody, Texas Red (Vector Laboratories, UK) at 1:200, was prepared using D2 buffer. Slices were incubated in the tertiary antibody for one hour at room temperature in the dark. Following this slices were washed in D2 buffer for five minutes twice, and once with PBS. The standard protocol was then resumed for mounting and cover slipping.

A1.4 Results

A1.4.1 ThermoFisher OXTR antibody

The PA5-19038 antibody was selected due its selectivity for the rat OXTR, and its suitability for immunohistochemistry. Initial trials of the PA5-19038 antibody, at a concentration of 1:100, appeared to show successful staining in NAc. Images taken in the NAc also contain the anterior commissure; a white matter tract with no known

OXTR expression. The anterior commissure showed no OXTR staining and was comparable with control conditions. In the same slices staining was seen in the islands of Calleja (ICj); an area with known OXTR expression (Dubois-Dauphin *et al.*, 1992; Hunt *et al.*, 2011; Snijdewint *et al.*, 1989). No OXTR staining was seen in control conditions lacking the primary and secondary antibody; indicating successful staining (Figure A1.1).

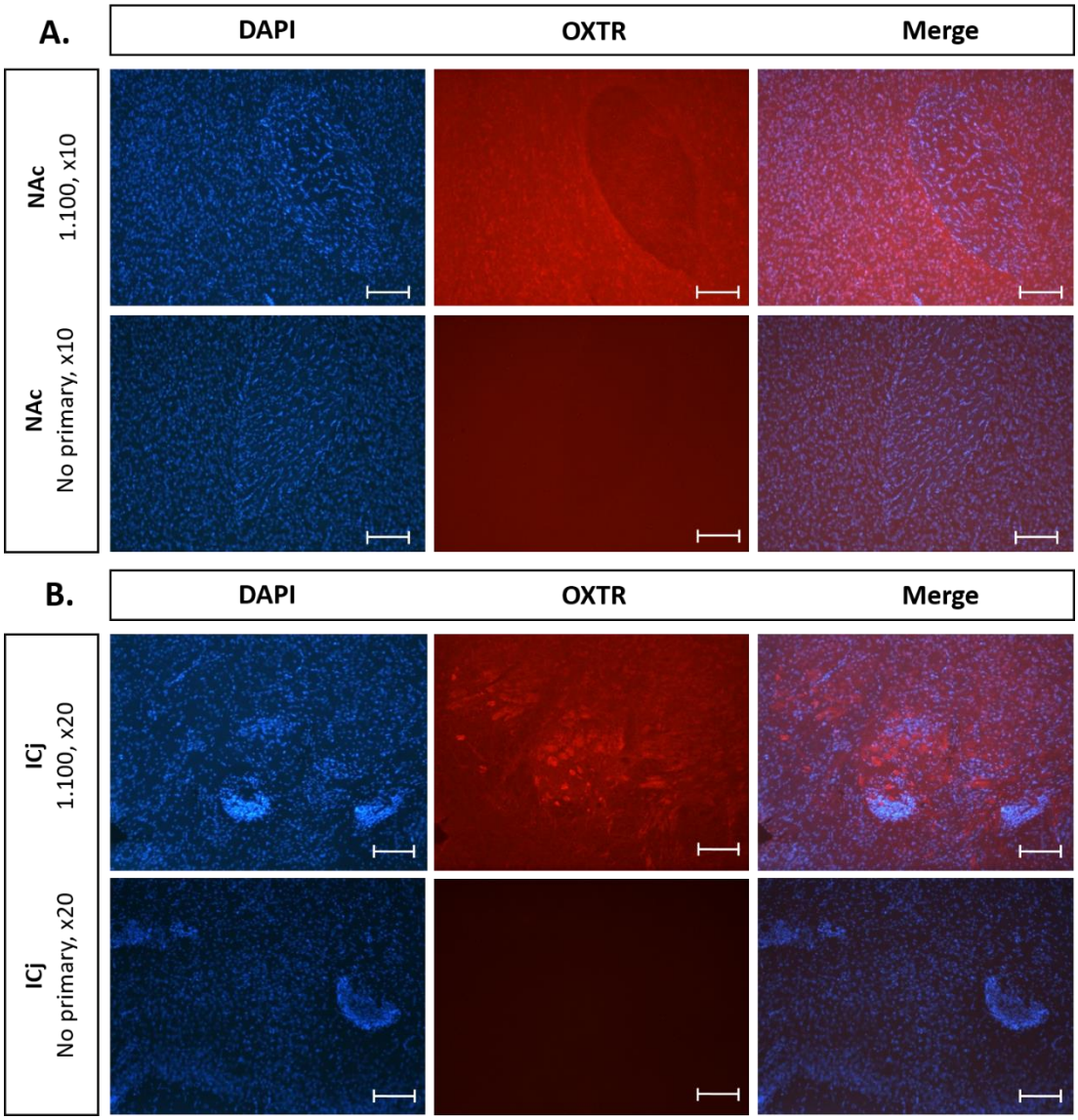

Figure A1.1: OXTR staining in the NAc (A) and ICj (B), obtained using the anti-OXTR primary antibody PA5-19038 (ThermoFisher, UK). OXTR staining appeared in both the NAc and ICj and was devoid in the control conditions lacking the primary OXTR antibody. Scale bar denotes 100µM in x10 images, and 50 µM in x20 pictures.

Immunohistochemistry was also performed in the lateral hypothalamus and on uterine tissue, a positive control (Figure A1.2). Uterine tissue has dense OXTR expression on the plasma membrane of smooth muscles within the myometrium and endometrium layers of the uterus, with these receptors having a crucial role in parturition (Engstrom *et al.*, 1998). Successful OXTR staining was seen in the uterine tissue; consistent with OXTR localisation on the smooth muscle rings in the myometrium, detailed with white arrows in Figure A1.2B. In the uterus control conditions lacking the primary antibody some markings were still present in the red channel. However, these were also present within the green channel, indicating blood in the tissue and not non-specific staining. With the exception of the blood marks the control conditions were devoid of OXTR staining, as to be expected. However, despite gaining successful OXTR straining, the PA5-19038 primary antibody was discontinued before we could order enough for future experiments. Due to the high concentration of the primary antibody required to gain successful staining we did not have enough left to perform any future staining on experimental tissue.

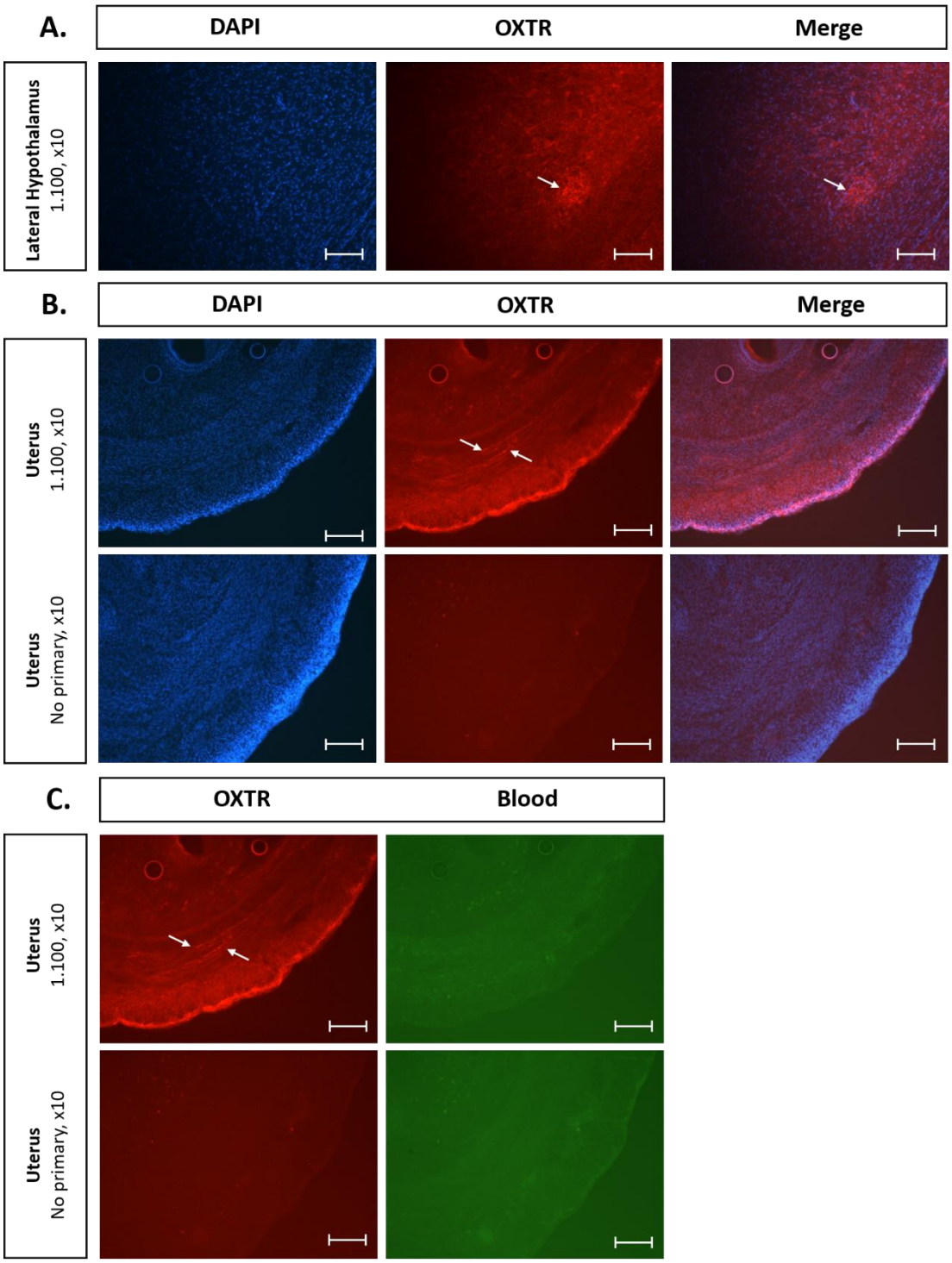

Figure A1.2: OXTR staining in the lateral hypothalamus (A) and uterus (B and C), obtained using the anti-OXTR primary antibody PA5-19038 (ThermoFisher, UK). There was a high density of OXTR staining in the lateral hypothalamus (A), an area of high OXTR expression. Uterine tissue was used as a positive control, due to the dense expression of OXTRs. OXTR expression could be seen on the smooth muscle rings of the myometrium (B), which were not present in the control conditions lacking primary antibody. Some artefacts present in red channel in the no primary control; however, these were also present in the green channel (C). This indicates these artefacts are blood, and not non-specific OXTR staining. Images taken at x10 magnification, scale bar denotes 100 µM.

A1.4.2 Abcam OXTR antibodies

Due to the discontinuation of the PA5-19038 OXTR primary antibody, two other anti-OXTR antibodies were trialled. The antibodies were selected based upon their selectivity for the rat OXTR, and suitability for use in immunohistochemistry.

Initial trials using the Acbam OXTR antibodies (ab87312 and ab217212) at concentrations recommended from the datasheet (1:150 and 1:500 respectively) showed no staining in any brain region imaged (Figure A1.3). Care was taken to stain slices containing OXTR expressing regions, such as the NAc, hypothalamus, and amygdala.

Due to the lack of initial staining observed subsequent optimisations then trialled up to 16 different conditions, including: antigen retrieval, avidin-biotin secondary antibodies, removal of Triton-X100 from buffer, varying primary antibody concentrations, and using perfusion fixed tissue. A selection of representative images taken in the amygdala are shown in Figure A1.3, using the ab83712 primary antibody. No staining was seen in any images, in any brain regions, throughout any of the optimisations for either antibody. All artefacts seen in the red filter, where the OXTR was expected to be seen, were also visible in the green filter (not shown); suggesting these artefacts can be attributed to blood or tissue damage, and not OXTR staining. Therefore, it was concluded successful OXTR staining could not be achieved with either Abcam primary antibody.

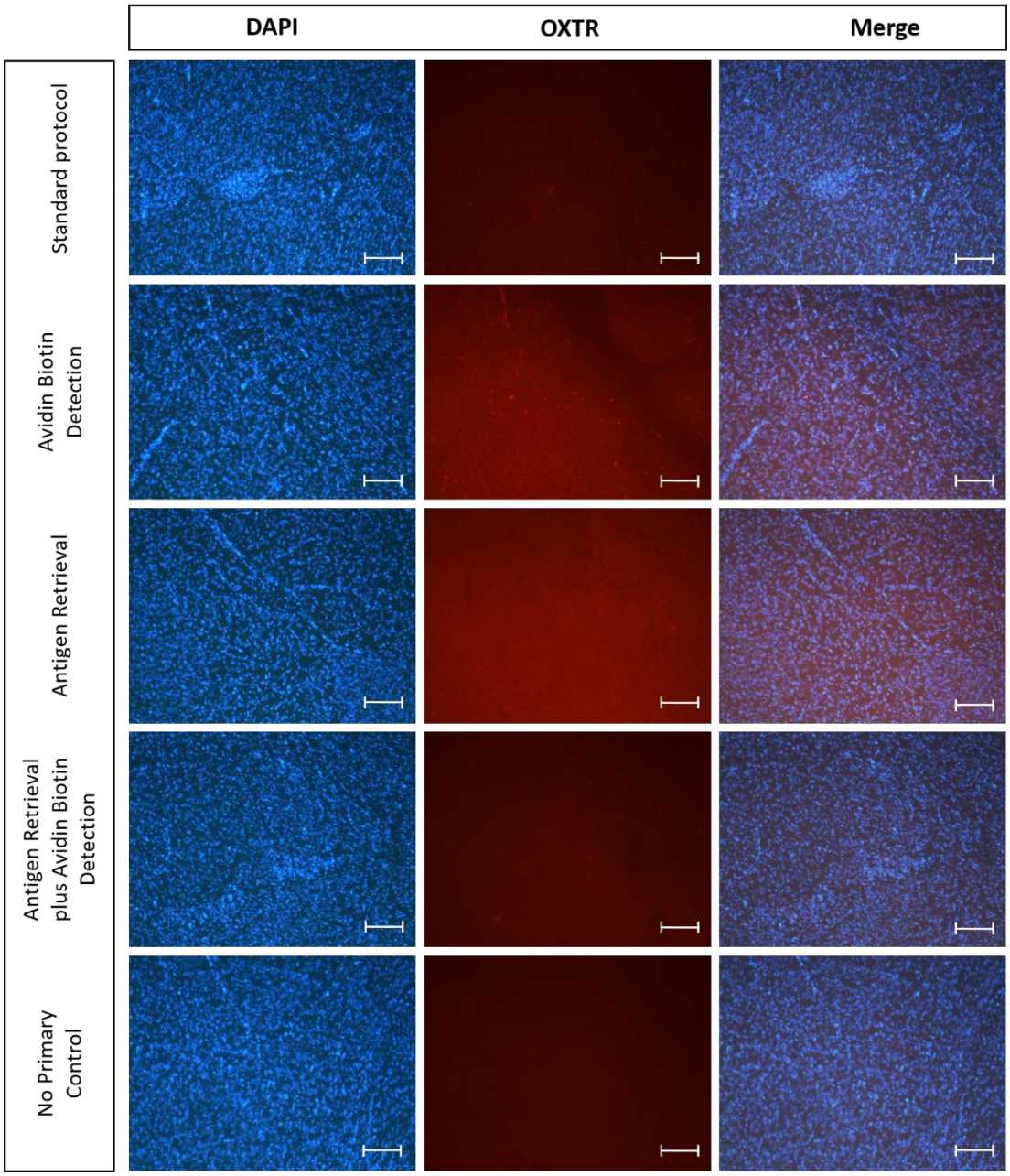

Figure A1.3: Representative images taken at x20 magnification in the amygdala, following staining with the OXTR targeting primary antibody ab87312. Numerous optimisations were trialled including: antigen retrieval, using avidin-biotin detection and varying concentrations of the primary antibody and blocking serums. No staining was observed in any condition. Scale bar denotes 50 µM.

A1.5 Discussion

Throughout the optimisation of the ThermoFisher OXTR antibody reagent controls were undertaken for each brain region, as well as the use of uterine tissue as a positive control. Control conditions lacking the primary antibody were undertaken in

all brain regions imaged, with no staining was seen in any region. This confirmed the staining seen in experimental samples was not produced from our detection system, non-specific binding of the secondary antibody. When imaging an additional step was undertaken to check the background artefacts in the tissue. All images were also taken under a green filter. Artefacts showing in the green filter can be attributed to either blood or damage to the tissue, and not OXTR staining, enabling us to determine OXTR staining and tissue artefacts. Although no slices of brain tissue displayed any artefacts in the green channel for the ThermoFisher OXTR primary antibody, the uterine tissue had multiple artefacts. The use of rat uterine tissue enabled us to perform a tissue type control. Rat uterus is known to contain high levels of OXTRs, particularly during and following parturition. There was evidence of OXTR staining in the myometrial layer; an area known to express the OXTR (Fuchs *et al.*, 1983; Ivell *et al.*, 2001). This was taken as further confirmation the primary antibody was correctly binding and staining the OXTR. It was hoped an absorption control could have been undertaken as a further conformational step to ensure the binding was specific. This process incubates the antibody with the immunogen overnight, in this case the C-terminal RRLGETSASKKSN sequence. This incubation inactivates the antibody so when used no staining should be seen, unless binding is non-specific. However, it was not feasible to undertake this step as a further control due to the lack of a commercially available pre-absorbed antibody, and the lack of facilities needed to make the specified peptide.

Dense OXTR staining was seen in the NAc, while the anterior commissure, shown in the centre of the NAc, showed no OXTR staining. These findings are consistent with previous literature. While the NAc has high OXTR expression, the anterior commissure has not previously been shown the express the OXTR (Ross & Young, 2009; Smith *et al.*, 2017a; Vaccari *et al.*, 1998). The change in staining between these brain areas was marked and obvious, further supporting the notion our OXTR immunohistochemical staining was specific for the OXTR. Other regions in which we observed OXTR staining, such as the lateral hypothalamus, were also consistent with known OXTR localisation (Smith *et al.*, 2017b). However, the antibody was

discontinued before we could carry out staining on experimental tissue, therefore other primary antibodies were trialled.

No staining was seen across any brain region imaged with either Abcam anti-OXTR antibody. Despite numerous attempts at optimisation, including using biotinylated secondary antibodies, varying concentrations of blocking serum, and varying incubation times, no successful staining was seen. Therefore, it was concluded that neither Abcam primary antibody could be used to measure OXTR staining.

The difficulty in gaining successful OXTR staining is likely due to the high sequence homology between the OXY and vasopressin receptors (Gimpl & Fahrenholz, 2001). It is of interest Mitre *et al.* (2016) created four custom OXTR targeting primary antibodies, targeting various regions of the OXTR including: the N terminus, C terminus, and the third intracellular loop. The most successful staining came from one of the N terminal tail targeting primary antibodies, an area of the OXTR that contains a residue required for high affinity binding of OXY to its receptor (Gimpl & Fahrenholz, 2001; Wesley *et al.*, 2002). Mitre *et al.* (2016) demonstrated successful staining in regions of the brain known to have high OXTR expression, as well as dense staining in the uterus. In addition, all staining was absent in OXTR knockout mice (Mitre *et al.*, 2016). Therefore, the N terminus tail may be the best location in which to target OXTR antibodies. All three antibodies used in this thesis targeted the intracellular C-terminus tail of the OXTR, perhaps indicating why we could not gain successful staining with either Abcam OXTR antibody. At the time of writing there are no commercially available OXTR primary antibodies in the UK that target the N-terminus tail of the OXTR with suitability of immunohistochemistry in rat tissue.

Due to the fact it was not possible to get successful immunohistochemical OXTR staining, and we were wanting to determine neuronal loss, we decided to stain for NeuN; a neuronal nuclear antigen that can be used as a neuronal biomarker (Mullen *et al.*, 1992). One benefit of this is that it enabled us to quantify neuronal loss, which would not have been possible using if OXTR staining as it is not a nuclear stain

Appendix 2: LMA ANOVA outputs

Figure 3.3 Further Detail

Interaction	Sphericity Correction	Sum of Squares	Mean Square	F	p
Time	Greenhouse-Geisser	893248.72	259925.666	39.548	< .001
Time ✻ ANTAGONIST	Greenhouse-Geisser	15688.505	4565.184	0.695	0.575
Time ✻ OXYTOCIN	Greenhouse-Geisser	61755.282	17970.116	2.734	0.037
Time ✻ PCP	Greenhouse-Geisser	311086.21	90522.704	13.773	< .001
Time ✻ ANTAGONIST ✻ OXYTOCIN	Greenhouse-Geisser	71319.731	20753.266	3.158	0.02
Time ✻ ANTAGONIST ✻ PCP	Greenhouse-Geisser	11480.497	3340.7	0.508	0.702
Time ✻ OXYTOCIN ✻ PCP	Greenhouse-Geisser	28619.569	8327.984	1.267	0.285
Time ✻ ANTAGONIST ✻ OXYTOCIN ✻ PCP	Greenhouse-Geisser	65401.303	19031.068	2.896	0.029
Residuals	Greenhouse-Geisser	1.581e +6	6572.463		

Post Hoc Comparisons - Time * L,368,899 * OXY* PCP

		Mean Difference	SE	t	P_{Tukey}
x75, Saline, OXY, PCP	X75, Saline, Saline, PCP	-135.233	28.977	-4.667	0.016
x80, Saline, OXY, PCP	X80, Saline, Saline, PCP	-94.764	28.977	-4.747	0.011
x70, Saline, Saline, PCP	x70, Saline, Saline, Veh	160.4	28.204	7.176	<0.001
x75, Saline, Saline, PCP	x75, Saline, Saline, Veh	214.3	28.204	7.598	<0.001
x80, Saline, Saline, PCP	x80, Saline, Saline, Veh	224.4	28.204	7.425	<0.001
x85, Saline, Saline, PCP	x85, Saline, Saline, Veh	181.6	28.204	6.439	<0.001
x90, Saline, Saline, PCP	x90, Saline, Saline, Veh	159.7	28.204	5.662	<0.001
X75, L368,899, OXY, PCP	x75, Saline, Saline, Veh	138.2	28.204	4.9	0.006
X80, L368,899, OXY, PCP	x80, Saline, Saline, Veh	151.2	28.204	5.631	<0.001
X85, L368,899, OXY, PCP	x85, Saline, Saline, Veh	129.7	28.204	4.867	0.02
X90, L368,899, OXY, PCP	x90, Saline, Saline, Veh	128	28.204	4.428	0.038
X75, L368,899, Saline, PCP	x75, Saline, Saline, Veh	132.1	28.204	4.684	0.015

Table A2.1: ANOVA outputs from Figure 3.3. The time course of PCP-induced hyperactivity in the presence and absence of OXY and the selective OXTR antagonist L368-899: 4-way repeated measures ANOVA outputs.

Figure 3.4 Further Detail

Cases	Sum of Squares	Mean	F	p
ANTAGONIST	10468.844	10468.844	0.135	0.714
OXYTOCIN	270749.444	270749.444	3.491	0.066
PCP	3.719e +6	3.719e +6	47.957	< .001
ANTAGONIST ✻ OXYTOCIN	400465.876	400465.876	5.164	0.026
ANTAGONIST ✻ PCP	23400.939	23400.939	0.302	0.585
OXYTOCIN ✻ PCP	85208.361	85208.361	1.099	0.298
ANTAGONIST ✻ OXYTOCIN ✻ PCP	301976.534	301976.534	3.894	0.050
Residuals	5.429e +6	77553.137		

Post Hoc Comparisons - ANTAGONIST * OXYTOCIN * PCP

		Mean Difference	SE	t	p_{tukey}
L368899, OXY, PCP	Saline, OXY, PCP	210.156	127.954	1.642	0.723
	L368899, VEH, PCP	83.9	124.542	0.674	0.997
	Saline, VEH, PCP	-242	124.542	-1.94	0.527
	L368899, OXY, Saline	460.9	124.542	3.701	0.01
	Saline, OXY, Saline	491.267	127.954	3.839	0.006
	L368899, VEH, Saline	428	124.542	3.437	0.021
	Saline, VEH, Saline	420.6	124.542	3.377	0.025
Saline, OXY, PCP	L368899, VEH, PCP	-126.256	127.954	-0.99	0.975
	Saline, VEH, PCP	-452.156	127.954	-3.53	0.016
	L368899, OXY, Saline	250.744	127.954	1.96	0.516
	Saline, OXY, Saline	281.111	131.278	2.141	0.4
	L368899, VEH, Saline	217.844	127.954	1.703	0.686
	Saline, VEH, Saline	210.444	127.954	1.645	0.722
L368899, VEH, PCP	Saline, VEH, PCP	-325.9	124.542	-2.62	0.167
	L368899, OXY, Saline	377	124.542	3.027	0.064
	Saline, OXY, Saline	407.367	127.954	3.184	0.043
	L368899, VEH, Saline	344.1	124.542	2.763	0.122
	Saline, VEH, Saline	336.7	124.542	2.704	0.139
Saline, VEH, PCP	L368899, OXY, Saline	702.9	124.542	5.644	< .001
	Saline, OXY, Saline	733.267	127.954	5.731	< .001
	L368899, VEH, Saline	670	124.542	5.38	< .001
	Saline, VEH, Saline	662.6	124.542	5.32	< .001
L368899, OXY, Saline	Saline, OXY, Saline	30.367	127.954	0.237	1
	L368899, VEH, Saline	-32.9	124.542	-0.26	1
	Saline, VEH, Saline	-40.3	124.542	-0.32	1
Saline, OXY, Saline	L368899, VEH, Saline	-63.267	127.954	-0.49	1
	Saline, VEH, Saline	-70.667	127.954	-0.55	0.999
L368899, VEH, Saline	Saline, VEH, Saline	-7.4	124.542	-0.06	1

Table A2.2: ANOVA outputs from Figure 3.4. The total ambulation 20 minutes post-PCP administration in the presence and absence of the OXTR antagonist L-368,899: 3-way ANOVA outputs.

Figure 3.5 Further Detail

Cases	Sphericity Correction	Sum of Squares	Mean	F	p
TIME	Greenhouse-Geisser	846449.92	234922.87	34.907	< .001
TIME ✻ ANTAGONIST	Greenhouse-Geisser	14497.989	4023.757	0.598	0.647
TIME ✻ OXYTOCIN	Greenhouse-Geisser	34022.163	9442.477	1.403	0.237
TIME ✻ PCP	Greenhouse-Geisser	273094.878	75794.48	11.262	< .001
TIME ✻ ANTAGONIST ✻ OXYTOCIN	Greenhouse-Geisser	102232.317	28373.455	4.216	0.004
TIME ✻ ANTAGONIST ✻ PCP	Greenhouse-Geisser	13904.41	3859.016	0.573	0.664
TIME ✻ OXYTOCIN ✻ PCP	Greenhouse-Geisser	23968.121	6652.088	0.988	0.409
TIME ✻ ANTAGONIST ✻ OXYTOCIN ✻ PCP	Greenhouse-Geisser	68851.011	19108.841	2.839	0.03
Residuals	Greenhouse-Geisser	1.673e +6	6729.906		

Post Hoc Comparisons - Time * L,368,899 * OXY* PCP

		Mean Difference	SE	t	P_{Tukey}
X75, SR 49059, OXY, PCP	X75, Saline, Saline, Veh	174	31.906	5.454	<0.001
X80, SR 49059, OXY, PCP	X80, Saline, Saline, Veh	139	31.906	4.357	0.045
X75, SR 49059, Saline, PCP	X75, Saline, Saline, Veh	144.9	31.906	4.541	0.027
X70, Saline, Saline, PCP	X70, Saline, Saline, Veh	160.4	31.906	5.027	0.004
X75, Saline, Saline, PCP	X75, Saline, Saline, Veh	214.3	31.906	6.717	<0.001
X80, Saline, Saline, PCP	X80, Saline, Saline, Veh	224.4	31.906	6.563	<0.001
X85, Saline, Saline, PCP	X85, Saline, Saline, Veh	190	31.906	5.692	<0.001
X90, Saline, Saline, PCP	X90, Saline, Saline, Veh	159.7	31.906	4.852	0.004

Table A2.3: ANOVA outputs from Figure 3.5. The time course of PCP-induced hyperactivity in the presence and absence of OXY and the selective V1aR antagonist SR 49059: 4-way repeated measures ANOVA outputs.

Figure 3.6 Further Detail

Cases	Sum of Squares	Mean Square	F	P
ANTAGONIST	84.225	84.225	8.467e -4	0.977
OXYTOCIN	250643.174	250643	2.52	0.117
PCP	4.194e +6	4.194e +	42.16	< .001
ANTAGONIST ✳ OXYTOCIN	415771.345	415771	4.179	0.045
ANTAGONIST ✳ PCP	384.969	384.969	0.004	0.951
OXYTOCIN ✳ PCP	135063.353	135063	1.358	0.248
ANTAGONIST ✳ OXYTOCIN ✳ PCP	219296.1	219296	2.204	0.142
Residuals	6.864e +6	99479.1		

Post Hoc Comparisons - ANTAGONIST * OXYTOCIN

		Mean Difference	SE	t	p_{tukey}
SR49059, OXY	Saline, OXY	145.061	103.812	1.397	0.505
	SR49059, VEH	32.9	101.115	0.325	0.988
	Saline, VEH	-116.35	101.115	-1.151	0.66
Saline, OXY	SR49059, VEH	-112.161	102.472	-1.095	0.694
	Saline, VEH	-261.411	102.472	-2.551	0.061
SR49059, VEH	Saline, VEH	-149.25	99.739	-1.496	0.445

Table A2.4: ANOVA outputs from Figure 3.6. The total ambulation 20 minutes post-PCP administration in the presence and absence of the V1aR antagonist SR 49059: 3-way ANOVA outputs.

Figure 3.7 Further Detail

	Sum of Squares	DF	F	p
Interaction	458089	2	0.06563	0.9366
Antagonist	14219006	2	2.037	0.1403
OXY	7856904	1	2.251	0.1393

Table A2.5: ANOVA outputs from the analysis of the distance moved during social interaction following OXY and antagonist administration

Figure 4.2 Further Detail

Cases	Sphericity Correction	Sum of Squares	Mean Square	F	p
Time	Greenhouse-Geisser	2.055e +6	428853	76.366	< .001
Time ✳ SAPORIN	Greenhouse-Geisser	31673.294	6608.75	1.177	0.32
Time ✳ PCP	Greenhouse-Geisser	779880.552	162725	28.977	< .001
Time ✳ OXY	Greenhouse-Geisser	192191.557	40101.4	7.141	< .001
Time ✳ SAPORIN ✳ PCP	Greenhouse-Geisser	20207.466	4216.36	0.751	0.581
Time ✳ SAPORIN ✳ OXY	Greenhouse-Geisser	33483.434	6986.44	1.244	0.289
Time ✳ PCP ✳ OXY	Greenhouse-Geisser	153245.975	31975.3	5.694	< .001
Time ✳ SAPORIN ✳ PCP ✳ OXY	Greenhouse-Geisser	23302.364	4862.12	0.866	0.501
Residuals	Greenhouse-Geisser	1.884e +6	5615.74		

Post Hoc Comparisons - Time * OXY* PCP

		Mean Difference	SE	t	P_{Tukey}
x65, Saline, PCP	x65, Saline, Veh	183.156	21.39	8.563	<0.001
x70, Saline, PCP	x70, Saline, Veh	223.467	21.39	10.807	<0.001
x75, Saline, PCP	x75, Saline, Veh	208.089	21.39	9.728	<0.001
x80, Saline, PCP	x80, Saline, Veh	137.417	21.39	6.424	<0.001
x85, Saline, PCP	x85, Saline, Veh	95.856	21.39	4.481	<0.001
x65, OXY, PCP	x65, Saline, PCP	-104.756	21.39	-4.897	<0.001
x70, OXY, PCP	x70, Saline, PCP	-143.217	21.39	-6.696	<0.001
x75, OXY, PCP	x75, Saline, PCP	-101.189	21.39	-4.731	<0.001

Table A2.6: ANOVA outputs from Figure 4.2. Time course of PCP-induced hyperactivity in the presence and absence of OXY, in rats that had received NAc injections of either OXY-saporin or blank-saporin: 4-way repeated measures ANOVA outputs.

Figure 4.3 Further Detail

Cases	Sum of Squares	Mean Square	F	p
SAPORIN	5.42	5.42	1.233e -4	0.991
OXY	808502.162	808502.162	18.391	< .001
PCP	4.287e +6	4.287e +6	97.521	< .001
SAPORIN ✻ OXY	2234.859	2234.859	0.051	0.822
SAPORIN ✻ PCP	14011.351	14011.351	0.319	0.574
OXY ✻ PCP	410986.956	410986.956	9.349	0.003
SAPORIN ✻ OXY ✻ PCP	2237.177	2237.177	0.051	0.822
Residuals	3.077e +6	43962.875		

Post Hoc Comparisons - OXY * PCP

		Mean Difference	SE	t	p_{tukey}
OXY, PCP	SALINE, PCP	-349.161	67.219	-5.194	< .001
	OXY, VEH	324.056	67.219	4.821	< .001
	SALINE, VEH	265.55	66.305	4.005	< .001
SALINE, PCP	OXY, VEH	673.217	68.121	9.883	< .001
	SALINE, VEH	614.711	67.219	9.145	< .001
OXY, VEH	SALINE, VEH	-58.506	67.219	-0.87	0.82

Table A2.7: ANOVA outputs from Figure 4.3. The total ambulation 15 minutes post-PCP administration, following NAc injection of either blank-saporin (control) or OXY-saporin: 3-way ANOVA outputs.

Figure 5.2 Further Detail

Cases	Sphericity Correction	Sum of Squares	Mean Square	F	p
TIME	Greenhouse-Geisser	1.386e +6	334627	49.266	< .001
TIME ✻ SAPORIN	Greenhouse-Geisser	26482.336	6395.35	0.942	0.443
TIME ✻ PCP	Greenhouse-Geisser	650008.37	156974	23.111	< .001
TIME ✻ OXY	Greenhouse-Geisser	83944.788	20272.2	2.985	0.018
TIME ✻ SAPORIN ✻ PCP	Greenhouse-Geisser	28607.49	6908.56	1.017	0.4
TIME ✻ SAPORIN ✻ OXY	Greenhouse-Geisser	9975.356	2409	0.355	0.847
TIME ✻ PCP ✻ OXY	Greenhouse-Geisser	68764.511	16606.3	2.445	0.045
TIME ✻ SAPORIN ✻ PCP ✻ OXY	Greenhouse-Geisser	12786.566	3087.89	0.455	0.775
Residuals	Greenhouse-Geisser	1.856e +6	6792.22		

Post Hoc Comparisons - Time * OXY* PCP

		Mean Difference	SE	t	P_{Tukey}
x65, Saline, PCP	x65, Saline, Veh	134.5	22.936	5.864	<0.001
x70, Saline, PCP	x70, Saline, Veh	199.889	22.936	8.715	<0.001
x75, Saline, PCP	x75, Saline, Veh	179.611	22.936	7.931	<0.001
x80, Saline, PCP	x80, Saline, Veh	162.389	22.936	7.08	<0.001
x85, Saline, PCP	x85, Saline, Veh	104.778	22.936	4.568	0.003

Table A2.8: ANOVA outputs from Figure 5.2. Time course of PCP-induced hyperactivity in the presence and absence of OXY, in rats that had received VTA injections of either OXY-saporin or blank-saporin: 4-way repeated measures ANOVA outputs.

Figure 5.3 Further Detail

Cases	Sum of Squares	Mean Square	F	p
SAPORIN	1717.206	1717.206	0.039	0.845
PCP	3.408e +6	3.408e +6	76.713	< .001
OXY	429033.39	429033.39	9.657	0.003
SAPORIN ✻ PCP	47976.625	47976.625	1.08	0.303
SAPORIN ✻ OXY	42574.778	42574.778	0.958	0.331
PCP ✻ OXY	131300.513	131300.513	2.955	0.09
SAPORIN ✻ PCP ✻ OXY	49536.738	49536.738	1.115	0.295
Residuals	2.932e +6	44428.541		

Table A2.9: ANOVA outputs from Figure 5.3. Total ambulation for the first 15 minutes following PCP administration in rats that had received bilateral VTA injections of either OXY-saporin or blank-saporin: 3-way repeated measures ANOVA output.

Appendix 3: Professional Internship for PhD Students (PIP) Reflective Statement

NOTE TO EXAMINERS:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

Host: P1vital Ltd., Wallingford, Oxfordshire

Date: 6th January 2020 - 20th March 2020

Role: Support Scientist

P1vital Ltd. is a clinical contract research organisation that specialise in CNS experimental medicine, providing a range of services from data analysis and data management, to the design and implementation of multinational clinical trials. During my placement at P1vital Ltd. I was working within the Science Team, and primarily working on two ongoing projects, both of which were for pharmaceutical clients. One project involved the design and implementation of an online study to test cognition in the elderly; this was in preparation for a clinical trial to assess a potentially cognitive enhancing compound. The other project I was heavily involved

in was the development and deployment of a reward processing task for use in an international clinical trial of a treatment for depression.

During the design and planning of an online study to test cognition in the elderly I undertook the task of researching computerised cognitive memory tests. This research was required to inform us and help us make scientifically robust decisions about the timings and content of our own computerised memory task. This involved disseminating this research to other members of the company, and with those responsible for building the online task. Throughout this project I was involved in weekly teleconferences with the clients, finalising the inclusion and exclusion criteria, and building the website where the online study will be based.

The other project I was heavily involved in was the development and deployment of a reward processing task for a multinational clinical trial. During this project I was primarily involved in the testing of the software to ensure both the user interface and task outputs were accurately built to the required specifications. This involved analysing the data outputs of the task, as well as testing the user interface of the task in over 15 languages according to a set specification, and recording all findings. Another strand of this project I assisted with was the creation of an instruction manual for clinicians at all sites to use to assist with the use of our software, and hopefully ameliorate any difficulties that they may come across. This required an in depth understanding of the study protocol, the cognitive tasks used, and the software used to platform this study.

In addition to the two main projects I was involved in I also assisted with some business development projects. For example, the creation of slides to pitch to potential clients and investors about the battery of cognitive tasks P1vital Ltd. can provide, with published research supporting the use of these products in clinical research. I also assisted the review of a paper submitted to the Journal of Affective Disorders.

During my placement I learnt huge amount about the clinical research industry and the regulations that govern it. In the first week I undertook my Good Clinical Practice qualification, enabling me to work on the clinical projects. I also undertook a small

amount of quality assurance work throughout my placement, which involved updating company Standard Operating Procedures to ensure they were still adhering to the latest rules and regulations, particularly following Brexit.

My placement exposed me to the clinical assessments and cognitive tasks used to both diagnose and assess medication effects upon neuropsychiatric disorders, such as schizophrenia. Having only previously worked in preclinical research, working in a clinical research environment enabled me to view some aspects of my PhD differently, and consider different areas of my project I had not previously thought about. I thoroughly enjoyed my placement, and it gave me real motivation to come back to Nottingham and continue with my research.

Throughout my placement I gained a lot of new skills and learnt so much about the pharmaceutical industry. I was exposed to all aspects of clinical research, from good clinical practice methods and the development of cognitive tests to use in clinical trials, to business development, marketing, and quality assurance. In addition, my placement helped me better understand the relevance of my PhD research to real-world applications. My placement with P1vital confirmed to me a career in clinical research is something I wish to pursue and has really enhanced my understanding of the pharmaceutical industry. I would like to thank the whole team at P1vital for such a wonderful, insightful placement and for being great colleagues to work alongside.