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Modulating oxytocin brain penetration via intranasal delivery and a novel conjugate peptide (OT-GET)

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Abstract

Oxytocin (OT), an endogenous hormone and neuropeptide has been highlighted for its therapeutic potential to modulate socio-behavioural deficits. However, OT's high molecular weight and hydrophilicity limits the extent of central nervous system (CNS) brain penetration, highlighting a need to improve OT brain delivery. An alternative popular route of drug delivery is intranasal administration, which allows small peptides to bypass the blood brain barrier (BBB) and access the brain more directly. Yet, the degree of OT brain penetration remains controversial and requires further improvement. As such, this thesis examined the modulation of CNS OT penetration by use of intranasal OT alone and when conjugated to a novel cell penetrating peptide: glycosaminoglycan (GAG)-binding enhanced transduction (GET; P21-LK15-8R), producing OT-GET, and its ability to affect locomotor activity and social behaviour in rats.

In vitro assays were established to determine OT-GET's bioactivity, ability to improve OT transduction across a nasal epithelial cell monolayer and potential cytotoxicity. Using a calcium fluorimetry assay, OT-GET induced robust $[Ca^{2+}]_i$ transients in OTR expressing Hs 578t breast cancer cells. Subsequent examination of OT-GET in a cell permeability assay using immortalised nasal epithelial cells RPMI 2650 saw an increased rate of OT delivery across the monolayer compared to OT alone. Only the highest GET concentration produced cytotoxicity (on cell viability) effects, which emphasised the need to identify an ideal peptide conjugation ratio in order to achieve desired effects without concomitant toxicity.

In rats, subcutaneous OT (0.1mg/kg) reversed phencyclidine (PCP)-induced hyperactivity. Intranasal OT (100µg) showed slight effect, where cumulative ambulatory counts in the 30 minutes post-PCP was not significantly different to rats pre-treated with subcutaneous OT, although counts remained higher than saline treated animals. The same dose of intranasal OT increased the time spent by weight-matched rat pairs in prosocial body sniffing. Interestingly, a separate quantification of OT levels in the olfactory bulb revealed that OT-

GET treated animals had a significant increase in OT compared to OT treated animals.

Overall, OT-GET improved OT permeation across a nasal cell monolayer *in vitro* and improved brain penetration (olfactory bulb) *in vivo* compared to OT alone. Intranasal OT-GET did not produce changes in rats' social behaviour, which may be due to OT receptor (OTR) desensitisation.

Publications

Conference proceedings

Wong S, Watson DJG, Dixon JE, King MV & Fone KCF., (2020). Intranasal oxytocin and a novel oxytocin peptide conjugate do not reverse phencyclidine-induced hyperactivity in rats. *Federation of European Neuroscience Societies (FENS) 2020 Virtual Forum.*

Wong S, Abrahams AB, Sykes DA, Veprintsev DB, Dixon JE, King MV & Fone KCF., (2019). OT-GET, a novel oxytocin peptide conjugate to enhance oxytocin delivery across the nasal epithelium*. Pharmacology 2019, British Pharmacological Society.*

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Statement of Contribution

The project was developed by my supervisors Prof. Kevin Fone, Dr. Madeleine King and Dr. James Dixon (Centre of Biomedical Sciences, University of Nottingham). Dr. James Dixon provided the GET peptide used for conjugation with OT.

Work was designed by Prof. Kevin Fone, Dr. Madeleine King, Dr. James Dixon and Sara Wong. For the *in vitro* experiments (Chapter 2), development of the calcium fluorimetry was conducted by Sara Wong and Abigail Abrahams (PhD student) with equal contribution, under the guidance of Dr. David Sykes and Prof. Dmitry Veprintsev (Centre of Membrane Protein and Receptors (COMPARE), University of Nottingham). Subsequent *in vitro* studies (cell permeability assay and cytotoxicity testing) were conducted by Sara Wong. *In vivo* experiments (Chapter 3) were conducted by Sara Wong with assistance from Dr. David Watson and Abigail Abrahams. All data analysis was performed by Sara Wong.

Abbreviations

AISS	Ambiguous Intentions Hostility Questionnaire-Abbreviated Version
ALI	air-liquid culture interface
ASD	autism spectrum disorder
ADHD	attention deficit/hyperactivity disorder
ADOS	Autism Diagnostic Observation Scale
AVP	vasopressin
BBB	blood brain barrier
BPRS	Brief Psychiatric Rating Scale
BNST	bed nucleus of the stria terminalis
cAMP	cyclic AMP
CCK	satiety peptide cholescytokinin
CGI-I/S	Clinical Global Impressions-Improvement/Severity
CNV	copy number variants
CPP	cell penetrating peptide
CSF	cerebrospinal fluid
CVLT II	California Verbal Learning Task
DA	dopamine
DAG	1,2-diacylglycerol
DANVA	Diagnostic Analysis of Non-Verbal Accuracy
DBANS	Repeatable Battery for the Assessment of Neuropsychological Status
DBC-P	Developmental Behaviour Checklist
DMEM	Dulbecco’s Modified Eagle’s Medium

DTP	direct transport percentage
ECF	extracellular fluid
eGFP	enhanced Green Fluorescent Protein
ESC	embryonic stem cells
EMEM	Eagle’s Minimum Essential Media
FCS	fetal calf serum
FEEST	Facial Expressions of Emotions Task
FITC	fluorescein isothiocyanate
FRU	fluorescent maximum-minimum
GABA	gamma-aminobutyric acid
GET	glycosaminoglycan (GAG)-binding enhanced transduction
GFP	green fluorescence protein
GnRH	gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
HBSS	Hanks Buffered Salt Solution
HEK 293T	human embryonic kidney 293T cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	hypothalamo-pituitary-adrenal axis
IAPS	International Affective Picture System
I.P.	intraperitoneal route of administration
I.C.V.	intracerebroventricular route of administration
IP_3	inositol triphosphate
IPPA	Inventory of Parent and Peer Attachment
IRSA	Interaction Rating Scale Advanced

LB	Luria-Bertani
LCC	liquid covered culture
LH	Lister-Hooded
LMA	locomotor activity
LNS	Letter Number Sequencing
LTP	long-term potentiation
MAP	methamphetamine
MeA	medial amygdala
MIRECC GAF	Global Assessment of Functioning
MRP	multidrug-resistance associated protein
MYA	million years ago
NAc	nucleus accumbens
NHP	non-human primate
NMDA	N-methyl-d-aspartate
NTS	nucleus tractus solitarius
ONF	neural fibroblast
OSN	olfactory sensory neurone
OT	oxytocin
OTR	oxytocin receptor
P_{app}	permeability coefficient
PANAS	Positive and Negative Affect Schedule
PANSS	Positive and Negative Syndrome Scale
PBS	phosphate buffered saline
PCP	phencyclidine

PEPT1	peptide-transporter system-1
PFA	paraformaldehyde
PFC	prefrontal cortex
PLC-β	phospholipase C-β
PLGA	poly-lactic-co-glycolic acid
PVN	paraventricular nuclei
RAGE	receptor for advanced glycation end-products
RBS-R	Repetitive Behaviour Scale-Revised
RCT	randomised clinical trial
RMS	rostral migratory stream
RPMI	Roswell Park Memorial Institute
SAAM	State Adult Attachment Measure
SANS	Scale for the Assessment of Negative Symptom
SAPS	Scale for the Assessment of Positive and Negative Symptoms
S.C.	subcutaneous route of administration
SD	Sprague Dawley
SFS	Social Functioning Scale
SNP	single nucleotide polymorphism
SON	supraoptic nuclei
SRS	Social Responsiveness Scale
STAI	Spielberger’s State and Trait Anxiety Inventory
TASIT	The Awareness of Social Interference Test
TD4.4	TRITC-dextran
TEER	transepithelial electrical resistance range

TRITC	tetramethylrhodamine isothiocyanate
VAS	Visual Analog Scale
VPA	valproic acid
VTA	ventral tegmental area
WGA-HRP	wheat germ agglutinin horseradish peroxidase
WHO-QOL	Quality of Life Questionnaire World Health Organization
YBOCS	Yale Brown Obsessive Compulsive Scale

Table of Contents

Table of Figures

Chapter 1 General Introduction

Chapter 3 *In vitro* assay characterisation of oxytocin-GET (OT-GET), a novel oxytocin peptide conjugate

Table of tables

Chapter 1 General Introduction

1.1 Thesis overview

Oxytocin (OT), an endogenous nonapeptide has been highlighted for its role in modulating social behaviour and thus, potential therapeutic use for central nervous system (CNS) disorders that display socio-behavioural and cognitive deficits. However, systemic drug administration methods such as intravenous or subcutaneous (s.c.) injections are not ideal as OT's molecular weight and hydrophilicity is thought to limit blood brain barrier (BBB) penetration. Intranasal delivery is a popular alternative method as it bypasses the BBB yet the extent of brain penetration remains controversial as peripheral and intranasal administration of OT sees a similar estimate of 0.002 – 0.003% of peptide reaching the brain (Mens et al., 1983, Leng and Ludwig, 2016). However, the clear advantage of intranasal delivery is the minimised risk of systemic-side effects as significant lower concentrations of OT were present in plasma with intranasal delivery compared to intravenous administration in rats, and that the sustained levels of OT in the blood was longer with systemic injection in the rat (Tanaka et al., 2018, Smith et al., 2019). As such, it is of interest to investigate ways of enhancing intranasal OT delivery to the brain.

The overall aim of this thesis is to investigate a novel cell penetrating peptide (CPP) termed glycosaminoglycan (GAG)-binding enhanced transduction (GET; P21-LK15-8R) when conjugated to OT, its bioactivity, cytotoxicity and ability to enhance OT transduction *in vitro* and subsequently, its effect on locomotor activity, social behaviour and CNS OT penetration by intranasal administration in rats.

1.2 Introduction to oxytocin

OT is a nine amino acid peptide that functions as a central neurotransmitter as well as a peripheral hormone. In the periphery, OT is best known for its role in parturition and suckling in mammals (Brunton and Russell, 2008, Leng et al., 2015). More recently, attention has turned towards its therapeutic potential for

CNS disorders that display socio-behavioural deficits, such as schizophrenia and autism spectrum disorder (ASD) (Cochran et al., 2013, Jurek and Neumann, 2018, Meyer-Lindenberg et al., 2011) for which existing therapies are very limited.

OT, and its sister peptide vasopressin (AVP) were discovered in the 1900s. Oliver and Schafer (1895) were the first to discover that extracts from the pituitary gland exhibit vasopressor effects. This was later characterised to be effects stemming from the posterior pituitary (Howell, 1898, Oliver and Schafer, 1895). In 1906, Sir Henry Dale incidentally discovered that a pituitary extract caused uterine contractions in a pregnant cat uterus (Dale, 1906). He later named this extract OT, which means “quick birth” in Greek. Other physiological effects of pituitary extracts were also exhibited: milk-ejection from the mammary gland (Ott and Scott, 1909), lowering of blood pressure in avians (Paton and Watson, 1912) and antidiuretic properties in man (von den Velden, R., 1913). In 1928, OT and AVP were separated in these extracts (Kamm, 1928).

The OT/AVP system is highly conserved in evolution with the lineage thought to have evolved from a common ancestral vasotocin peptide gene duplication event ~500 million years ago (MYA) (Acher and Chauvet, 1995). Around 400 MYA, one of the duplicated vasotocin peptide genes evolved to produce mesotocin, which further evolved into OT 100 MYA. On the other hand, AVP emerged around 200-250 MYA.

This evolution resulted in two distinct lineages – OT-like peptides and AVP-like peptides. AVP-like peptides include vasotocin (Ile^3-AVP; found in teleosts, birds, reptiles and amphibians) and AVP (found in mammals except pigs where Lys^8-AVP is expressed). OT-like peptides include isotocin (Ser^4-OT found in teleosts), mesotocin (Ile^8-OT) found in birds, reptiles and amphibians) and OT (found in mammals except primates where Pro^8-OT is expressed) (Jurek and Neumann, 2018).

As such, OT and AVP have very similar structures (Figure 1.1). The pharmacological and functional implications of this similarity is discussed in Section 1.4.

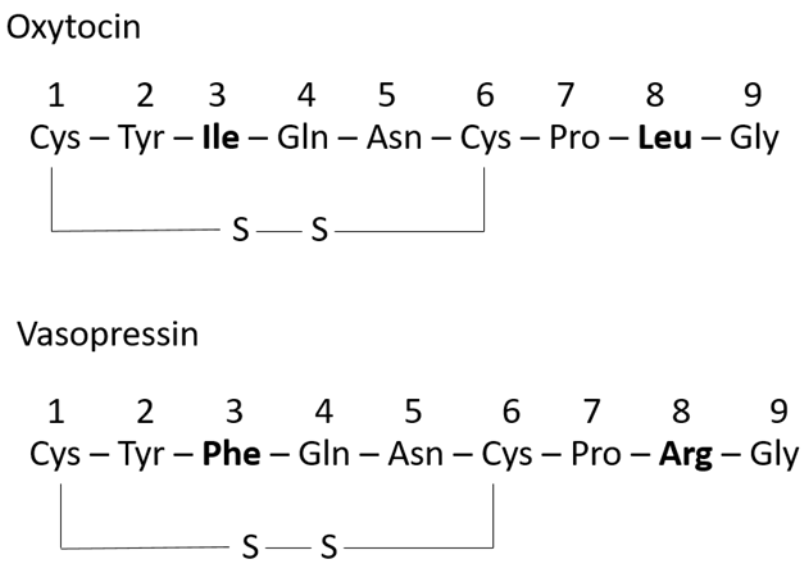


Figure 1.1 Amino acid structure of OT and AVP.
Both nonapeptides OT and AVP have a disulphide bond between the cysteine amino acids at location 1 and 6 and differ only at the third and eight amino acid. The third amino acid is isoleucine and phenylalanine and the eight amino acid leucine and arginine for OT and AVP respectively. As arginine is positively charged, this creates a difference in polarity between the two peptides, which is believed to allow for their distinct interaction with their respective receptors (Barberis et al., 1998).

1.3 Synthesis and release of oxytocin

OT is primarily synthesised in the magnocellular neurons of the hypothalamic supraoptic (SON) and paraventricular nuclei (PVN) as well as in parvocellular neurons of the PVN nuclei (McEwen, 2004). In humans, the OT gene is located on human chromosome 20 and codes for a signal peptide (allowing for transfer and packaging for posttranslational processing), nonapeptide as well as neurophysin which is believed to transport OT to the posterior pituitary (Jurek and Neumann, 2018).

Local release into the peripheral blood system occurs via axon terminals of magnocellular neurons projecting into the posterior pituitary blood system (Figure 1.4). Axonal release of OT is triggered by action potentials: Ca^{2+} ions enter via local voltage-dependent Ca^{2+} channels and in turn cause a rise in intracellular Ca^{2+} concentrations from thapsigargin-sensitive stores in OT cells (Lambert et al., 1994). This triggers exocytosis of OT containing vesicles into

neurohypophysial capillaries where it enters the peripheral circulation (Jurek and Neumann, 2018). In adult rats, posterior pituitary content of OT is estimated at 1µg while in man it is approximately 28µg (Leng and Ludwig, 2016).

Parvocellular neurones of the PVN project towards the brain stem and spinal cord (Jurek and Neumann, 2018). OT release into the central system may also occur via somatodendritic release by magnocellular neurons. Specialised magnocellular OT neurones also possess axon collaterals which project to various forebrain regions, such as the lateral septum, NAc and central amygdala (Grinevich et al., 2016, Knobloch et al., 2012). Dendritic release of OT may occur independently of electrical depolarisation although it requires mobilisation of intracellular Ca^{2+} from thapsigargin-sensitive stores. It is suggested that the release of Ca^{2+} from stores recruits dendritic vesicles in such a way that it is primed for activity-based release thereafter (Ludwig et al., 2002). Dendritic release can also be triggered by chemical signals such as OT itself, resulting in a positive feedback regulation loop (Ludwig and Leng, 2006).

For example, stimulation of the system by exogenous OT has been shown to enhance endogenous OT synthesis in prairie voles (Grippo et al., 2012).

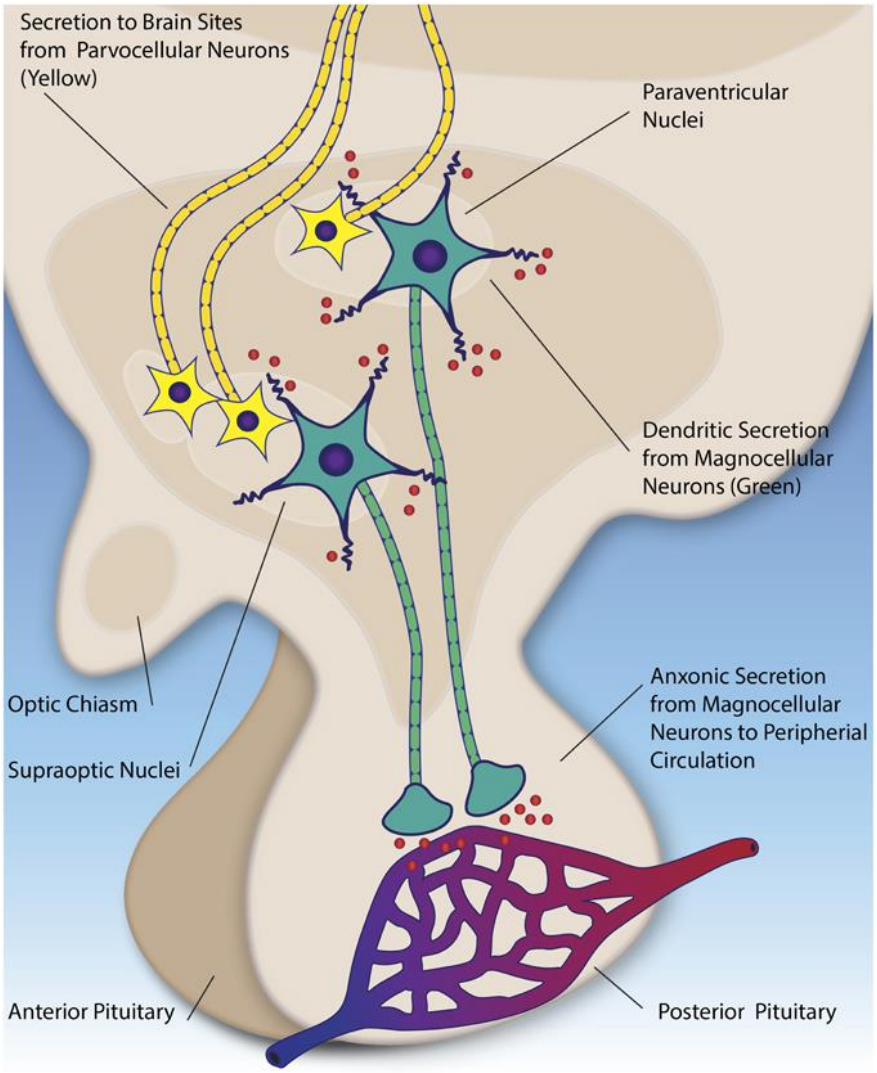


Figure 1.2 Release of OT into the peripheral circulation and brain regions.
OT is released into the peripheral circulation from magnocellular neurones' axonal terminals in the SON and PVN via the posterior pituitary. Release of OT into the brain occur via PVN parvocellular neuronal projections as well as somatodendritic release by magnocellular neurons. Figure taken from Baribeau and Anagnostou (2015).

1.4 Receptor evolution

Similar to the evolution of OT and AVP, the single vasotocin receptor ancestral gene is believed to have undergone a tandem duplication, giving rise to four vasotocin receptors (Figure 1.2). Around 400 MYA, one of the vasotocin

receptors evolved into the mesotocin receptor found in amphibians, fishes and birds. The remaining three vasotocin receptor gradually evolved into current V_{1a}, V_{1b} and V_2 receptors (previously known as AVP_{1a}, AVP_{1b} and AVP_2 receptors) and mesotocin receptor into the mammalian oxytocin receptor (OTR) 200-250MYA.

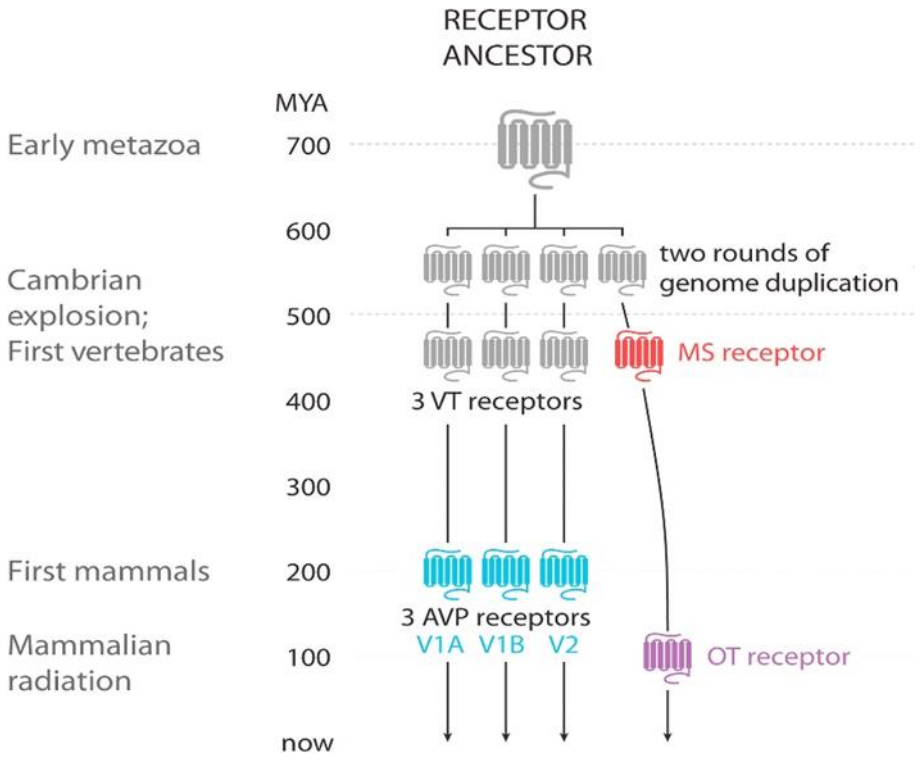


Figure 1.3 AVP and OT receptor evolution from a common vasotocin receptor ancestor 700MYA.

Over time, the single vasotocin receptor underwent genome duplication and gave rise to three AVP receptors, V_{1A}, V_{1B} and V_2 (previously known as $AVPR_{1a}$, $AVPR_{1b}$ and $AVPR_2$, respectively) as well as a single OT receptor, OTR. Figure taken from Grinevich et al. (2016).

It is believed that during the amino acid change of mesotocin to OT (isoleucine to leucine), relaxation on the functional constraints of the corresponding mesotocin receptor may have occurred (Yamashita and Kitano, 2013). The accumulation of nucleotide mutations resulting in amino acid changes that followed produced the resulting OTR, after which functional constraints were re-established (Pare et al., 2016, Yamashita and Kitano, 2013). This also happens to be when OT's most important function appeared - uterine

contractions during parturition. Interestingly, the divergence of humans and rodents is estimated at approximately 90MYA (Chinwalla et al., 2002, Hedges et al., 2006), after the re-establishment of OTR's primary function in parturition. This suggests a well-conserved oxytocinergic system in both species and thus the suitability of rodents to study the oxytocinergic system in terms of translation to man.

The gene sequence for rodent (mouse and rat) and human OTR and AVP receptors is well conserved, with approximately 80% identical amino acid residues, especially in the extracellular loops and transmembrane helices (Gimpl and Fahrenholz, 2001). As such, OT and AVP can cross bind to each other's receptors as shown in receptor binding and functional assays (Table 1.1) (Busnelli et al., 2012, Song and Albers, 2018).

	Oxytocin (K_i)				Vasopressin (K_i)			
	OTR	$V_{1a}R$	$V_{1b}R$	V_2R	OTR	$V_{1a}R$	$V_{1b}R$	V_2R
Human	0.8*	120	>1000	3500	1.7	1.1	0.7	1.2
Rat	1.0	71	294	89	1.7	2.6	0.3	0.4
Mouse	0.6	46.1	494	Not known	1.8	1.3	0.3	Not known

Table 1.1 Selectivity of OT and AVP with their respective receptors.

Affinity values are expressed as K_i (nM) where a high K_i value indicates a low affinity and vice versa. AVP has a similar affinity for its own receptors as well as OTR whereas OT has a higher affinity for OTR. * indicates selectivity for OTR over AVP receptors for that particular species. Table modified from Manning et al. (2012) and Song and Albers (2018).

As highly related peptides from an evolutionary standpoint, this has led to concomitant cross binding between receptors. As OT and AVP have consistently been linked to the regulation of social behaviour and cognition (Caldwell and Albers, 2016, Heinrichs et al., 2009), attempts have been done to tease apart and identify the role each individual neuropeptide or receptor may play in regulating behavioural effects.

For example, both peptides and their receptors have been implicated in social behaviour and cognition. In OTR knockout mice, impaired social recognition, social preference and cognitive flexibility were observed (Lee et al., 2008, Sala et al., 2011, Takayanagi et al., 2005). Social exploration and recognition were restored with intracerebroventricular (i.c.v.) OT and AVP injection but abolished when pre-treated with SR49059 (Sala et al., 2011), a selective V_{1A} receptor antagonist. In the Morris water maze task, stressed rats pre-treated with OT possessed better spatial memory than vehicle treated controls. In contrast, this effect was reversed when L368,899, a highly selective BBB penetrable OTR antagonist was given (Lee et al., 2015).

These studies provide a conflicting view whether OTR or $V_{1a}R$ is responsible for mediating prosocial behaviour. In the olfactory epithelium of rats, mRNA for $V_{1a}R$ and $V_{1b}R$ has been detected (Levasseur et al., 2004) while in the main and accessory olfactory bulb, $V1_aR$, $V_{1b}R$ and OTR have been reported (Tobin et al., 2010, Vaccari et al., 1998). As rodents utilise the olfactory system in driving social engagement and processing social cues, it is possible that AVP and OT may reinforce each other's action and a balance of both neuropeptides instead is responsible for mediating overall social behaviour. As the expression of OTR and AVP receptors are distinctly different in the same brain region (Chapter 1.3.1), it may also be possible that both peptides activate separate pathways which converge further down the biological cascade to produce the final behavioural output.

Overall, both OT and AVP appear to play important roles in regulating social behaviour and understanding this cross talk may further contribute towards our understanding of any translational relevance. Taken together, literature favours a role of OT as the main mediator for prosocial behaviour, perhaps due to AVP's more prominent role in regulating aggression in males (Caldwell et al., 2008) and OT's role in maternal behaviour, making it the preferred choice of investigation for disorders that involve social dysfunction. Therefore, this PhD focuses on the role of OT in social behaviour.

1.4.1 Oxytocin receptor distribution

OTRs occur in the myometrium, mammary gland, endometrium, reproductive organs, heart and kidney as well as in the brain (Kimura et al., 2003).

The rat is by far the most extensively investigated mammal for mapping OTR expression. In the rat brain, a series of experiments utilising autoradiography (Dumais et al., 2013, Elands et al., 1988, Freeman and Young, 2016, Tribollet et al., 1988, Klein et al., 1995, Uhl-Bronner et al., 2005) and *in-situ* hybridization (Adan et al., 1995, Yoshimura et al., 1993) have made it possible to characterise receptor distribution. Autoradiography uses radioligands to determine receptor distribution whereas *in-situ* hybridisation utilises a labelled nuclei acid/RNA/complementary DNA strand as probe, which then binds to the specific DNA or mRNA sequence in the tissue of interest. Studies using *in-situ* hybridisation however, could be inaccurate as detection of receptor mRNA does not necessarily correlate with protein expression levels. On the other hand, autoradiography studies can only be applied to whole organs and provide poor spatial definition. However, to our knowledge, there is no verified commercial OTR specific antibody available. This is mainly due to the high degree of homology between OTR and AVP receptors, raising issues of cross binding (Smith et al., 2019b).

Initial autoradiography studies utilised [tyrosyl-^{3}H]-OT and observed labelling in the olfactory system including olfactory nucleus, tubercle and islands of Calleja, hippocampus, bed nucleus of the stria terminalis (BNST), central amygdaloid nucleus, frontal, temporal and primary olfactory cortex as well as in the caudoputamen complex in the rat (Tribollet et al., 1988). However, usage of [^{3}H]-OT has been criticised due to its low selectivity and long film exposure time to attain results. More recent studies have utilised [125^{I}]-ornithine vasotocin analogue ([125^{I}]-OVTA), a highly selective iodinated OTR radioligand. Overall, OTR expression is concentrated in the olfactory pathway of the rat (which is as expected as social interaction in rodents is dependent on olfactory investigations), in regions downstream from the olfactory pathway such as BNST, central amygdala, hypothalamus and in regions involved in social information processing such as hippocampus, nucleus accumbens (NAc) and prefrontal cortex (PFC) (Dumais et al., 2013, Freeman et al., 2016,

Klein et al., 1995, Uhl-Bronner et al., 2005). *In situ* hybridisation results largely agree with those from autoradiography, although some differences include absence in central nucleus of the amygdala (Adan et al., 1995) or brainstem inferior olives (Yoshimura et al., 1993). However, both studies used differently raised antibodies: Adan et al. (1995) used antibodies raised against the third intracellular loop of the rat OT while Yoshimura et al. (1993) utilised a human OTR RNA probe.

Sex differences have also been noted in OTR expression. Male rodents generally exhibit higher OTR binding densities (Dumais et al., 2013, Uhl-Bronner et al., 2005), for example in the BNST and medial amygdala (Caldwell, 2018). Differences in social behaviour between sexes also occur in social recognition and partner preferences (Caldwell, 2018), which suggests a possible association between differential behaviour and receptor expression in males and females. Developmental differences in receptor expression are evident, for example, brain regions such as the caudate putamen, NAc core, dorsal and ventral subiculum, thalamus and olfactory nucleus subregions exhibit higher OTR density in juveniles than in adults rats (Smith et al., 2017). This might reflect development alteration in juvenile versus adult regulation of social behaviour, for example, higher risk taking behaviour and increased social motivation in juveniles.

In human post-mortem tissue, Loup et al. (1991) investigated OTR expression using [^{125}I]-OTVA and [^{3}H]-OT. Unlike rats, no specific binding was found in the amygdala or hippocampus. Intense binding was located in the basal nucleus of Meynert and nucleus of the diagonal band of Broca, both of which form part of the basal forebrain cholinergic system involved in cognitive processing in mammals (Ballinger et al., 2016). However, it appears that [^{125}I]-OTVA is only selective in rats as it binds with equal strength to V_{1a} receptors in non-human primates (NHPs) (Toloczko et al., 1997) and in the human uterus (Tence et al., 1990). Boccia et al. (2013) identified OTR binding in the amygdala, olfactory nucleus, ventrolateral septum and anterior cingulate using the monoclonal antibody 2F8. 2F8 binds to a 21 amino acid sequence at the NH_2 terminal region of human OTR and has been used to demonstrate presence of OTR in human uterus (Takemura et al., 1994), uterine vessels

(Wakasa et al., 2009) and myoepithelial cells (Kimura et al., 1998). Again, no staining was observed in the hippocampus although axonal projections are known to innervate the area (Meyer-Lindenberg et al., 2011).

In the rat, V_{1a} receptor expression has been reported in the same brain areas as the OTR, although some differences are observed: V_{1a} receptors are prominently expressed in the lateral septum and dentate gyrus of the hippocampus (Tribollet et al., 1988). There is also distinct sub-regional distribution. For example in the hippocampus, V_{1a} receptors are expressed in the dentate gyrus while OT binding sites are in the subiculum regions (Dumais and Veenema, 2016, Smith et al., 2017, Tribollet et al., 1992) while in the BNST, OTR occur in the lateral and medial subdivision whereas V_{1a} receptors in the capsular and central subdivisions (Veinante and Freund-Mercier, 1997).

1.4.2 Receptor signalling and functional effects

OTRs are G-protein coupled receptors (GPCR). Only one OT receptor has been cloned to date, termed OTR.

OTR couples to both excitatory $G_{q/11}$ proteins and inhibitory $G_{i/11}$ proteins (Figure 1.3) (Strakova and Soloff, 1997). $G_{q/11}$ activation stimulate phospholipase C-β (PLC-β) and trigger Ca^{2+} release from intracellular stores as well as recruitment of protein kinase C for subsequent protein phosphorylation via inositol triphosphate (IP_3) and 1, 2-diacylglycerol (DAG) respectively. OT can also couple to G_i, inhibiting adenylate cyclase activity and subsequent cyclic AMP (cAMP) dependent pathways.

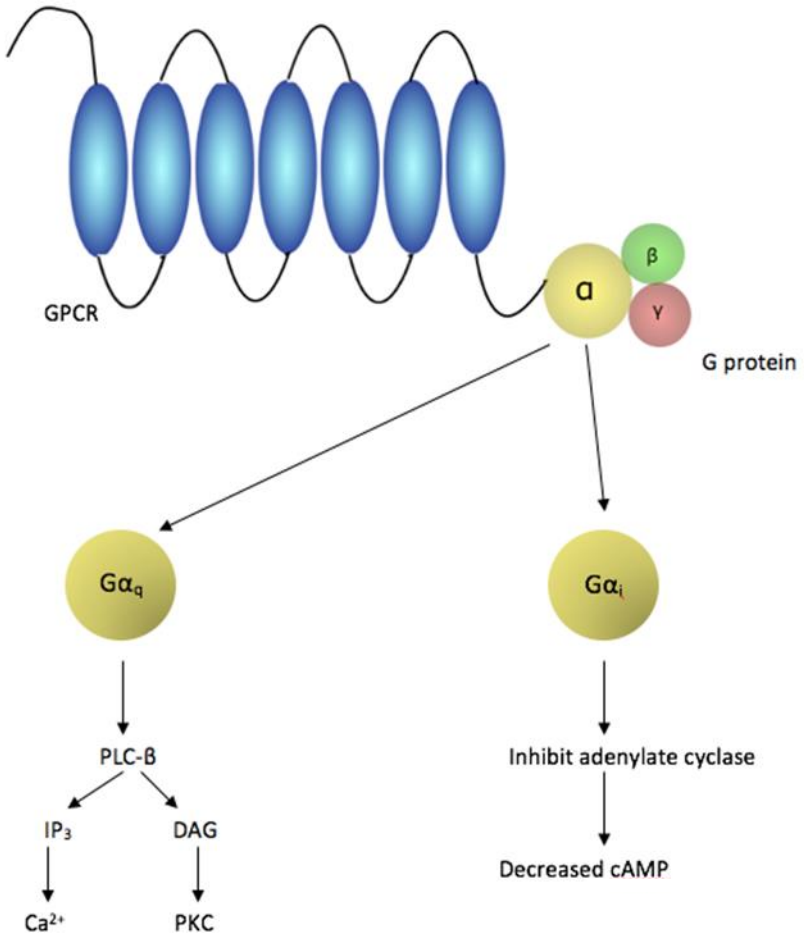


Figure 1.4 OTR is a G-protein coupled receptor (GPCR).
OT binding activates receptor G-protein which is made up of 3 subunits: α, β and γ. OT can bind to $G\alpha_q$ effector protein where via stimulation of phospholipase C-β (PLC-β) isoform, produces inositol triphosphate (IP_3) and 1, 2-diacylglycerol (DAG), triggering Ca^{2+} release from intracellular stores and recruitment of protein kinase C for subsequent protein phosphorylation respectively. OT can also bind to G_i, inhibiting adenylate cyclase activity and subsequent cAMP dependent pathways.

Coupling of OTR to the different G-proteins can produce synergistic or opposing effects. In rat myometrial cells, coupling of OTRs to $G_{q/11}$ subunit contribute to OTR mediated contractions (Zhou et al., 2007). In a gonadotropin-releasing hormone (GnRH)-positive immortalized GN11 murine cell line, OT either inhibited or stimulated potassium ions conductance via inward rectifying K^+ channels when coupled to G_q and G_i protein, respectively (Gravati et al., 2010). Using a BRET assay and dose-response curves,

Busnelli et al. (2012) found that G_q signalling is activated with an EC50 value of 2.16nM whereas $G_{i/o}$ signalling is activated with EC50 values ranging from 11.5nM to 91.8nM in human embryonic kidney (HEK) 293 cells. This suggests that $G_{i/o}$ proteins are activated at a higher concentration than needed for G_q protein activation. The ability of OT to couple to different G proteins should be kept in mind when developing OT based therapeutics in order to elicit the intended effect.

Like any other receptor, OTR function and expression is regulated via receptor desensitisation and internalisation. After constant stimulation, OTR-expressing alpha T3-1 cells (a gonadotrope-derived cell line) exhibit rapid receptor desensitisation (Gimpl and Fahrenholz, 2001). Briefly, following ligand binding, desensitisation is initiated upon phosphorylation of the receptor by G protein receptor kinases or secondary messenger kinases (Ferguson and Caron, 1998). This triggers uncoupling of the receptor with their G proteins and subsequent binding of the β-arrestin protein, leading to clathrin-coated pit dependent internalisation (Goodman et al., 1996). Dynamin, a GTPase pinches off the clathrin coated vesicle where the internalised GPCR is either recycled back to cell surface or sent to the lysosome for degradation (Marchese et al., 2008). OTR desensitisation occurs through this classical clathrin mediated pathway, with the β-arrestin 2 isoform having a higher affinity for OT activated OTR (Busnelli et al., 2012, Smith et al., 2006). Interestingly, Passoni et al. (2016) found that carbetocin, a selective G_q agonist induces OTR internalisation in the absence of β-arrestin in OTR transfected HEK293 cells. On the other hand, G_i selective agonists such as atosiban do not promote β-arrestin recruitment and thus no receptor internalisation occurs (Busnelli et al., 2012). Thus, it appears that OTR modulate desensitisation and internalisation according to the type of G protein it couples with and consequently, this affect receptor expression and their response to stimuli.

Drugs may exert their pharmacological actions via receptor or non-receptor mediated actions. Non-receptor mediated activity refers to actions attributed to the molecules' chemical action (for example antacids, which are weak bases that neutralise gastric acid (Richardson, 2004)) or physical means (such as demulcents which form a film over mucous membranes for pain and

inflammation relief (Eccles and Mallefet, 2017)). Drugs may also interact with proteins, such as cisplatin which binds to nucleic acids and induce deoxyribonucleic acid (DNA) damage in cancer cells (Dasari and Tchounwou, 2014). OT interacts with a number of neurotransmitters in the brain including dopamine, glutamate and GABA although these effects have been attributed to receptor mediated effects as OTR expression on GABAergic (Zaninetti and Raggenbass, 2000), glutamatergic (Tan et al., 2019) and dopaminergic neurones (Xiao et al., 2018) have been observed. Furthermore, OTR antagonists are able to block OT agonist induced firing of fast-spiking interneurons (Owen et al., 2013) and dopaminergic neurones (Xiao et al., 2018) in rodents. OT also has a role in regulating appetite, which has also been attributed to receptor action as lesion of OTR expressing neurones resulted in elevated food intake in rats (Baskin et al., 2010), as well as in the regulation of the hypothalamo-pituitary-adrenal axis (HPA) where OT dependent stimulation of adrenocorticotrophic hormone mediates the mobilisation of Ca^{2+} from IP_3-sensitive intracellular Ca^{2+} stores (Viero et al.. 2010), which requires activation of OTRs (Ludwig and Leng, 2006). In regards to sex steroids, OT is known to interact with estrogen: central administration of OT in rats reduced basal and stress-stimulated plasma ACTH which in turn was dependent on levels of oestradiol (Ochedalski et al., 2017). However, these changes are likely due to overall changes in OTR expression as an increase in OTR mRNA in estrogen-sensitive regions was stimulated by estrogen in rats (Jurek and Neumann, 2018). Overall, OT's receptor mediated effects are well documented, however to our understanding, the existence of any non-receptor mediated effects have yet to be reported.

1.5 Central effects of oxytocin

Beyond OT's well-established role in lactation and childbirth, studies began to reveal that OT may be involved in modulating social behaviour in mammals during the late 1970s.

The first study highlighting OT's role in regulating maternal behaviour was by Pedersen and Prange (1979). They showed that i.c.v. injection of OT induced

full maternal behaviour in virgin ovariectomised rats primed with estradiol benzoate, towards foster pups. While in rats given the OT antagonist d(CH_2)5-8-ornithine-vasotocin centrally during the gestational period or parturition, post-partum maternal behaviour was dramatically attenuated (Fahrbach et al., 1985, van Leengoed et al., 1987). However, when an OT antagonist was given five days postpartum, maternal behaviour was not inhibited. This indicates OT plays a role in the onset rather than the maintenance of maternal behaviour (Fahrbach et al., 1985). Several other studies conducted in rats have since confirmed OT's effect on maternal behaviour, with prior steroids priming essential to initiate the behaviour (Gimpl and Fahrenholz, 2001, Insel, 1992).

OT is also involved in regulating other behavioural aspects such as social interaction and cognition. Acute intranasal OT increases social behaviour in otherwise healthy rodents (Huang et al., 2014) while chronic central OT infusion enhances social interaction (Witt et al., 1992) and improves performance in an object recognition task (Havranek et al., 2015). A range of behavioural experiments using OT or OTR knockout (-/-) mice (Table 1.2) has also contributed largely to our understanding in this area.

Knockout Type	Group	Behaviour
OT -/-	Ferguson et al., 2000	Impaired social memory between familiar and novel conspecifics
	Ferguson et al., 2001	Impaired social recognition in five-trial habituation-dishabituation test
	Kavaliers et al., 2003	Normal non-social olfactory memory, impaired social odour memory for discrimination
	Macbeth et al, 2009	Impaired inter-strain social recognition in two-trial social recognition task
	Pedersen et al., 2006	Knockout dams show decreased latency to retrieve and groom pups
	Ragnauth et al., 2005	Increased infanticide rate in knockout dams
	Wersinger et al., 2008	Impaired social memory tested via Bruce effect
OTR -/-	Hattori et al., 2015	Impaired social recognition in intruder aggression task
	Lee et al., 2008	Impaired social recognition in two-trial social recognition task
	Miyazaki et al., 2016	Fewer ultrasonic vocalisation in pups
	Pobbe et al., 2012a; Pobbe et al., 2012b	Decreased social interaction and social recognition
	Rich et al., 2014	Lactation and nurturing defects in knockout dams
	Sala et al., 2011	Impaired social interaction, social recognition and cognitive flexibility
	Takayanagi et al., 2005	Fewer ultrasonic vocalisation, impaired social discrimination, elevated aggressive behaviour in male pups. Impaired maternal behaviour in female mice.

Table 1.2 Studies examining oxytocin (OT-/-) or oxytocin receptor (OTR-/-) genetic knockout mice impairment in social behaviour or cognition.

Overall, OT and OTR -/- mice exhibit impaired maternal behaviour, social memory and recognition. Impaired social memory is not due to affected olfactory ability as OT -/- mice were still able to identify predator odour (Kavaliers et al., 2003) and perform olfactory dependent tasks such as foraging and habituation (Ferguson et al., 2000). In contrast, spatial memory, while reported intact in OT (Ferguson et al., 2000) and RAGE (receptor for advanced glycation end-products) -/- mice (Sakatani et al., 2009), is disrupted in OTR knockouts (Sala et al., 2011). RAGE, expressed on capillary endothelial cells of the BBB, is a possible transporter of OT across the BBB (Yamamoto et al., 2019). RAGE knockout mice have decreased cerebrospinal fluid (CSF) OT levels following s.c. OT injection and exhibit locomotor hyperactivity and maternal bonding deficits, both of which have been attributed to OT imbalance/deficits (Kohli et al., 2019, Pedersen et al., 2005, Qi et al., 2008, Ragnauth et al., 2005). Thus, it appears spatial memory depends on OTRs. OTRs are highly expressed in pyramidal neurones and a small portion of GABA-ergic interneurons in the hippocampus of rats (Dumais et al., 2013, Klein et al., 1995, Lin et al., 2017, Tribollet et al., 1988), a known region responsible for spatial memory formation (Eichenbaum et al., 1999).

In prairie voles, central administration of OT induces formation of a partner preference (Williams et al., 1994) that is prevented by the OT antagonist d(CH_2)$_5$,Tyr(Me)^2,Thr^4,Tyr-NH_2($_9$)]ornithine vasotocin treatment (Insel and Hulihan, 1995), which is OTR selective in rodents (Greenwood and Hammock, 2017, Greenwood and Hammock, 2019). In CRISPR/Cas9 OTR knockout prairie voles, an increased tendency of marble burying (an ASD relevant phenotype (Angoa-Perez et al., 2013)), and impairments in social recognition but not social interaction was observed (Horie et al., 2019). Social interaction and recognition in rats and mice have been attributed to both V_{1A} receptor and OTR activation (Bielsky et al., 2005, Landgraf et al., 2003, Pobbe et al., 2012a, Pobbe et al., 2012b, Ramos et al., 2016). Rodents utilise the olfactory system in processing social cues and V_{1A} receptor and OTR have been reported in the main and accessory olfactory bulb (Tobin et al., 2010, Vaccari et al., 1998). Thus, it is possible that the two peptides reinforce each other's action and a balance of both is responsible for mediating social behaviour reliant on

olfaction. As Horie et al. (2019) was the first to investigate social recognition and/or interaction in prairie voles, the extent of how much influence V_{1A} receptor and OTR have over mediating these social behaviour is unknown and may be different to that of mice and rats.

Social behaviour aside, OT also plays a role in reducing appetite. For example, lesion of OTR expressing neurones in the nucleus tractus solitarius (NTS) where OTRs are expressed (Loup et al., 1989) reduced the ability of satiety peptide cholecystokinin (CCK) to reduce food intake and prevented OTR antagonism action to stimulate food intake (Baskin et al., 2010). Similarly, suppressed food intake was observed upon optogenetic activation of PVN OT neurones (Atasoy et al., 2012) and OT -/- and OTR -/- mice are known to develop late-onset obesity (Camerino, 2009, Takayanagi et al., 2008). OT also plays an inhibitory role in drug addiction and dependence via action in the NAc: systemic OT administration attenuated methamphetamine (MAP)-induced cFos expression in the accumbens core (Carson et al., 2010) and formation of MAP-induced conditioned place preference (Baracz et al., 2012) while i.c.v. injection of OT into the NAc decreased MAP seeking behaviour in rats (Baracz et al., 2016). OT is believed to modulate dopaminergic activity in the mesolimbic system as Lee et al. (2019) observed a potentiation of methylphenidate induced DA release while concurrently preventing methylphenidate self-administration after systemic OT pre-treatment. As such, it may be that OT acts to prime mesolimbic dopamine activity in an effort to reduce subsequent psychostimulants use.

However, the expression of OTRs in the peripheral system requires careful consideration when it comes to developing OT as a potential therapeutic for central use. OT has a role in regulating cardiovascular and gastrointestinal functions (Gutkowska et al., 2014, Welch et al., 2014) and peripheral administration of acute OT observed hypothermia and bradycardia in rats (Hicks et al., 2014, Kohli et al., 2019) while chronic administration of s.c. OT accelerated angiotensin induced hypertension and cardiac hypertrophy in mice (Phie et al., 2015). This highlights the need for robust dose-response studies as intranasal delivery of OT (discussed in Section 1.7 and Chapter 3), which allows for better brain penetration, sees a concurrent rise of peptide in the

plasma (Neumann et al., 2013, Striepens et al., 2013, Smith et al., 2019a), which may lead to unwanted peripheral effects in an already vulnerable clinical population where compliance may be an issue.

1.5.1 Preclinical animal models of ASD/schizophrenia

OT's unequivocal role in modulating social behaviour and cognition has made it an increasingly popular drug of investigation for human neurodevelopmental disorders that display socio-behavioural deficits, such as ASD and schizophrenia. Currently, there are no efficacious medical treatments for social behaviour and cognition deficits aside from behavioural interventions and risperidone, which have little efficacy and a concerning side-effect of weight gain in ASD patients (Masi et al., 2017, Stepanova et al., 2017). In patients with schizophrenia, there is no convincing evidence that any antipsychotics ameliorate negative and cognitive symptoms (Kane et al., 2001, Naber and Lambert, 2009).

Inbred mouse strains BALB/c and C58 are commonly used to replicate idiopathic ASD models as they show robust ASD-relevant behaviour. For example, BALB/cByJ strains exhibit anxiety like behaviour, C58/J strains show repetitive behaviour and both strains observe impaired social interaction and preference (Moy et al., 2007, Ryan et al., 2010, Varghese et al., 2017). The Grin1 knockout genetic mice model (N-methyl-d-aspartate (NMDA) receptor NR1 subunit under expression) also recapitulates many ASD features including behavioural deficits, impaired prepulse inhibition and hyperactivity (Duncan et al., 2004, Gandal et al., 2012, Teng et al., 2016). Sub-chronic intraperitoneal (i.p.) injection of OT increased sociability tendency to explore an unfamiliar stranger mice in inbred adolescent mouse strains BALB/cByJ and C58/J at 1mg/kg (Teng et al., 2013) as well as in adult Grin1 knockdown mice at 2mg/kg (Teng et al., 2016). By exposing neonatal or foetal rats to valproic acid (VPA), a teratogen known to increase the risk of the foetus developing schizophrenia to replicate social deficits and repetitive behaviour and movement characteristic of ASD (Varghese et al., 2017), a single intranasal dose of 20μg OT during adolescent or repeated dosing postnatal also improved sociability in the same manner (Dai et al., 2018).

In a chronic-PCP rat model for schizophrenia, intra-amygdala infusion of 1000ng OT increased social interaction (Lee et al., 2015). The chronic-PCP model behavioural changes akin to the positive and negative symptoms of schizophrenia; positive symptoms reflected by locomotor sensitisation to subsequent PCP exposure and negative symptoms such as reduced social interaction are attenuated by existing antipsychotics (Jones et al., 2011, Phillips et al., 2001, Sams-Dodd, 1998). OT s.c. also decreased PCP-induced hyperactivity (believed to mirror positive symptoms of schizophrenia (Jones et al., 2011)) and increased social interaction (Kohli et al., 2019) in healthy adult male rats, the latter of which may have translational relevance to the negative symptoms of schizophrenia (Watson et al., 2012).

1.5.2 Human clinical studies

These preclinical observations have resulted in numerous human studies. For instance, healthy volunteers administered intranasal OT exhibit increased trust (Kosfeld et al., 2005) and ability to interpret social cues (DeWall et al., 2014), improved social recognition (Domes et al., 2007, Guastella and MacLeod, 2012) and partner bonding (Ditzen et al., 2009, Scheele et al., 2013).

Clinical trials administering intranasal exogenous OT to investigate therapeutic potential in patients with socio-behavioural deficits however, yield inconsistent results. Collective results from five meta-analyses examining OT's effect in patients with socio-behavioural deficits suggest OT may improve higher cognitive functions. Williams and Burkner (2017) analysed eight randomised clinical trials (RCTs) which administered OT daily to patients with schizophrenia. OT had no significant superiority over placebo in reducing positive symptoms, negative symptoms, general psychopathology, or overall symptomology. However, the authors did not break down the different negative symptoms into subcategories nor did they analyse effects of OT on cognition. Leppanen et al. (2017) and Keech et al. (2018) found no significant effect of intranasal OT on lower level social cognition such as emotion recognition, similar to Ooi et al. (2017) who analysed 12 RCTs involving ASD patients. However, Burkner et al. (2017) and Keech et al. (2018) reported significant improvement in emotion interpretation (classed as high-level cognition), as opposed to Leppanen et al. (2017). This may be due to the different patient

criteria and dosage routine. Burkner et al. (2017) included 12 RCTs with schizophrenia patients while Keech et al. (2018) included 17 RCTs with patients suffering from neurodevelopmental disorders. Both authors included studies with a range of single/repeated OT dose. Leppanen et al. (2017) on the other hand, analysed single intranasal OT dose in a large clinical population (33 studies) suffering from mental disorders which included ASD, schizophrenia, borderline personality disorder, frontotemporal dementia, anorexia nervosa, bulimia nervosa and post-traumatic stress disorder. Overall, clinical trials examining intranasal OT on socio-behavioural deficits and cognition show mixed findings, which may be put down to the heterogeneity of the population itself.

1.6 Intranasal drug delivery

The intranasal route of drug administration has become increasingly popular as a valuable technique for therapeutics to access the brain more directly, bypassing the BBB. This allows many advantages: it is non-invasive, avoids first pass metabolism and has a reduced risk of systemic side-effects otherwise afforded by high levels administered systemically.

The nasal cavity consists of three regions. The vestibular region is located at the anterior and is enclosed by nose cartilage. The respiratory region which covers the lateral walls of the nasal cavity, has the largest surface area and is the most vascular, making it an ideal site for systemic absorption of drugs (Arora et al., 2002). It is also innervated by the maxillary branch of the trigeminal nerve (TGN), derived from the pons in the brainstem (Crowe et al., 2018). TGNs also innervate the olfactory region and a small portion is found to terminate in the olfactory bulb (Dhuria et al., 2010). The olfactory region lies at the most posterior end and represents the main target area for drug absorption into the CNS.

Mechanisms are divided into intracellular and extracellular routes. Figure 1.5 summarises the possible transport mechanisms upon intranasal drug delivery.

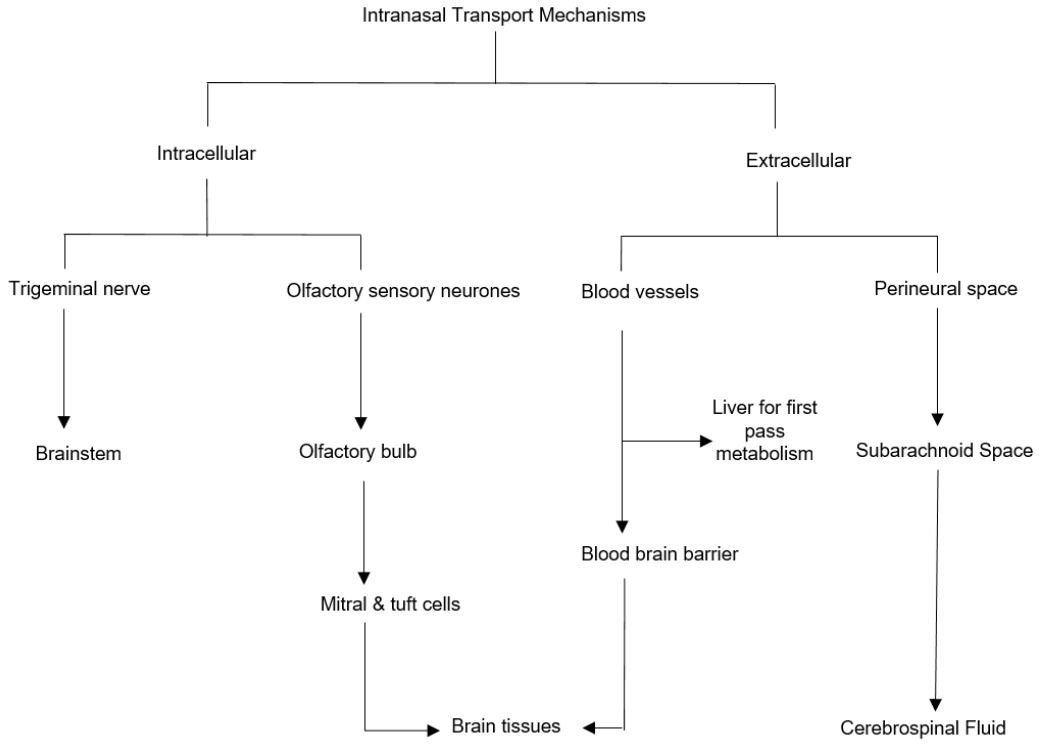


Figure 1.5 Possible transport mechanisms that drugs may use for transportation from the nasal cavity to CNS.

Intracellular routes include internalisation of drug by olfactory sensory neurones or the trigeminal nerve via receptor mediated endocytosis, adsorptive endocytosis or fluid phase endocytosis. Extracellular routes involve absorption into the systemic circulation via blood vessels occurring in the respiratory and olfactory region or transport to the brain via perineural spaces along the length of olfactory and trigeminal nerve axons and perivascular spaces via bulk flow mechanism.

1.6.1 Intracellular routes

Upon uptake by olfactory sensory neurones (OSNs) or TGNs, a drug is contained within endosomes and transported along the axon to the glomeruli layer of olfactory bulb or brainstem respectively. Studies utilising horseradish peroxidase, albumin and wheat germ agglutinin horseradish peroxidase (WGA-HRP) as tracers in rodents found labelled endosomes within primary olfactory neurons as well as in the trigeminal brainstem nuclear complex (Broadwell and Balin, 1985, Kristensson and Olsson, 1971). Olfactory nerve axons and glomeruli of olfactory bulb were also labelled with tracers within 6 – 24 hours (Broadwell and Balin, 1985, Kristensson and Olsson, 1971, Shipley, 1985). Unlike the other studies, Shipley (1985) found staining of mitral cells in

the olfactory bulb and the staining extended along the neuroanatomical line to the caudal pole of the cerebral hemisphere, which is a known projection from the olfactory bulb (Kosel et al., 1981). However, they implanted gelfoams directly in the nasal cavity, which would allow direct access to the olfactory epithelium as compared to the other studies that applied tracers as drops into the nostrils and would have to transverse the respiratory epithelium first.

The intracellular route is unlikely to be the only or even the primary route of transport. Staining of the olfactory neurons was only observed at least 6 hours after administration whereas tracer containing endosomes within axons were found only after a minimum of 24 hours post-administration. This is in contrast to behavioural studies that show a positive effect within 5 – 30 minutes of intranasal OT administration (Calcagnoli et al., 2015, Huang et al., 2014, Lukas et al., 2012). Furthermore, there is a high chance of proteolysis during axonal transport.

1.6.2 Extracellular routes

1.6.2.1 Perineural space pathway

Olfactory sensory neurones (OSN) in the olfactory epithelial have dendritic cell bodies dispersed amongst surrounding supporting and basal cells (Figure 1.6). The dendrites are exposed to the environment of the nasal cavity while their axons project through cribriform plates of the ethmoid bones and synapse on the glomeruli layer of the olfactory bulb (Thorne et al., 2004). The axons are ensheathed by “Schwann-like cells” termed olfactory ensheathing cells (OECs) and covered by an additional layer of neural fibroblasts (ONFs), forming a cavity termed the perineural space (Field et al., 2003). As the ONF layer is continuous with the meninges layer of the brain, this means the extracellular fluid of the nasal lamina propria is continuous with the cerebrospinal fluid of the subarachnoid space (Li et al., 2005).

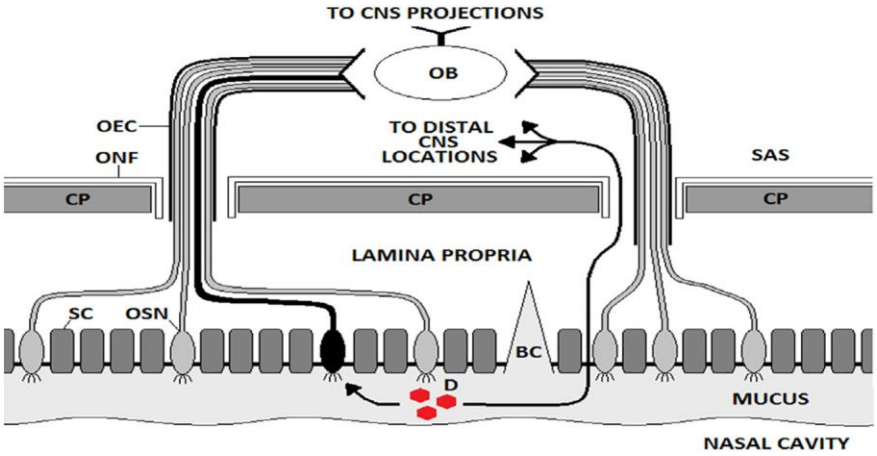


Figure 1.6 Intracellular and extracellular pathways for intranasal drug delivery. Drugs may be taken up via intracellular (darkened) or extracellular pathways. In the extracellular pathway, transport of drugs can occur paracellularly via tight junctions between cells (as shown above) or transcellularly across cells via channels and pores. Once the lamina propria is reached, drugs encounter the cribiform plate before entering the brain through the perineural space, a space formed between olfactory axons and neural fibroblasts. Support cells (SC), olfactory sensory neurons (OSN), mucus-secreting Bowman's capsule (BC), cribriform plate (CP), olfactory ensheathing cells (OEC), olfactory nerve fibroblasts (ONF), subarachnoid space (SAS), and olfactory bulb (OB). Figure taken from Crowe et al. (2018).

Upon arriving in the subarachnoid space adjacent to the olfactory bulb and brainstem where OSNs and TGNs terminate, drugs can be distributed via bulk flow in perivascular spaces. These are spaces adjacent to the many arteries that run alongside the olfactory axons (Crowe et al., 2018). Fluid moves more rapidly in this space compared to the rest of the perineural space. This phenomena is explained as the “perivascular pump” where fluid movement occurs due to arterial pulsations (Bilston et al., 2003).

Tracers have been found marking the epithelium of blood vessels supporting this suggestion (Kristensen et al., 2016, Thorne et al., 2004). Furthermore, Bender et al. (2015) and Thorne et al. (2004) found higher concentration of radioactivity at rostral and caudal brain regions in rats given iodine-125 [^{125}I] intranasally. Decreasing concentration gradients of the radioactive tracers in directions caudal from the olfactory bulb and in both direction from the brainstem where the OSNs and TGNs terminate were also seen (Thorne et al., 2004, Johnson et al., 2010). Observations that the olfactory bulb and

brainstem possess highest radioactivity or fluorescence intensity within 5-10 minutes of peptide administration are consistent across studies (Bender et al., 2015, Falcone et al., 2014, Thorne et al., 2004, Johnson et al., 2010, Oviedo et al., 2017). Other areas such as the hypothalamus, hippocampus, caudal cortex, thalamus and cerebellum show moderate labelling, with radioactive or fluorescent intensity peaking at different timings. Dahlin et al. (2000) found that upon intranasal administration of radiolabelled dopamine, the dopamine concentration peaked twice in the olfactory lobes; first at 30 minutes and again at 4 hours. They suggest that the first peak occurs due to extracellular pathways while the second is due to axonal transport.

Despite being described as a more direct route for brain penetration, doses of intranasal OT required for behavioural changes are extremely high: an increase of at least five folds from the basal pituitary content are usually seen (Jurek and Neumann, 2018). Furthermore, levels of OT that penetrate the brain successfully are controversial, with estimation of only a 0.002% - 0.005% increase observed in CSF of humans and NHPs (Leng and Ludwig, 2016).

1.6.2.2 Vascular pathways

The nasal mucosa is highly vascular. The respiratory mucosa receives blood from the maxillary artery whereas the olfactory mucosa receives blood from the ophthalmic artery (Dhuria et al., 2010). The respiratory region has the largest surface area and a higher density of blood vessels compared to the olfactory region (DeSesso, 1993, Watelet and Van Cauwenberge, 1999). This makes the former an ideal location for absorption of drug from the nasal cavity into the systemic system. However, drugs that enter the systemic circulation would have to subsequently cross the BBB, which is impermeable to large hydrophilic therapeutics. Supporting the notion that vascular routes are unlikely to contribute to the rapid behavioural effect observed after intranasal drug administration, Falcone et al. (2014) found that only 4% of albumin applied intranasally entered the bloodstream.

1.6.2.3 Rostral Migratory Stream (RMS)

The RMS is a migratory route whereby neuroprogenitor cells form a long chain and translocate from the subventricular zone to the olfactory bulb where they

then detach and disperse radially (Lois et al., 1996). This migration has been found to be closely associated with blood vessels in the granule layer (Bovetti et al., 2007). A study by Scranton et al. (2011) suggests it plays an important role, as surgical transection of the RMS decreased intranasal drug delivery to the brain by approximately 80%. However, the study was carried out in mice where the RMS is well developed, while the presence of a RMS analogue in humans remains controversial (van Strien et al., 2011).

1.7 Thesis aims

Given the enormous potential and clinical interest in the use of OT to treat social disorders and the controversial benefits of intranasal OT delivery to enhance CNS delivery of the peptide for such conditions, methods to improve OT brain penetration as well as dose-response studies to identify efficacious doses without causing any undesirable peripheral side-effects are clearly required. Many attempts have been carried out to improve intranasal OT brain penetration, mainly via novel drug carrier systems. Ideally, a drug to carrier ratio should be identified *in vitro* which would allow a starting point for dose-response studies to be carried out *in vivo* in order to identify concentrations that affect behaviour as intended without side-effects. As such, this thesis aims to investigate the ability of the novel OT peptide conjugate OT-GET (GAG-binding enhanced transduction; GET) versus OT alone to improve the level of brain penetration and behaviour in male Lister-hooded rats of OT when delivered intranasally. Chapter 2 aims to investigate the ability of OT-GET to improve OT transduction across a nasal epithelium cell membrane (RPMI 2650) as well as determine the optimum OT: GET ratio to bring forward to *in vivo* studies. Chapter 3 then compares the ability of OT-GET vs OT alone in reversing PCP-induced hyperactivity and improving social interaction in rats as well as the level of OT brain penetration in the presence or absence of the GET carrier.

Chapter 2 Intranasal OT in the clinic: a review

2.1 Introduction

Neurodevelopmental disorders refer to a group of multifaceted illnesses with an early onset during the developmental period, producing deficits such as impaired cognition, communication and/or behaviour that typically last into adulthood. Examples of disorders that fall under this description include autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD), neurodevelopmental motor disorders and schizophrenia (American Psychiatric Association, 2013, (Owen et al., 2011, Reiss, 2009). Although categorised into discrete groups based on their clinical presentation, there is substantial overlap in symptoms and genetic epidemiology. Patients with schizophrenia and ASD both exhibit impaired social communication and cognition (Sugranyes et al., 2011) and are associated, for instance, with similar copy number variants such as the 2p16.3 region deletion and microduplication of 16p11.2 region (Viñas-Jornet et al., 2014, McCarthy et al., 2009). Considering the significant phenotype-genotype overlap between disorders, it is hardly surprising that the task of finding curative treatments for each specific neurodevelopmental disorder remains a huge challenge. Although symptomatic treatments are successful, e.g. positive symptoms in patients with schizophrenia are well managed by current antipsychotics, (Kane et al., 2001), the social behavioural and cognitive deficits accompanying these disorders remain poorly responsive to existing medications (De Crescenzo et al., 2017, Masi et al., 2017, Stepanova et al., 2017, Naber and Lambert, 2009). However, it is these symptom domains that prevent patients from reintegrating into society and therefore produce the highest economic and social burden. The cost of supporting a patient with ASD with intellectual disability over a lifespan is 1.6-fold more than that of a patient without intellectual disability (Buescher et al., 2014), and changes in negative and cognitive symptoms in schizophrenia better predict therapeutic outcome, quality-

of-life and successful integration of patients back into society (Napolitano et al., 2014). Thus, the need to discover efficacious treatments remains urgent.

OT is a popular candidate for its potential application in neurodevelopmental disorders due to accumulating evidence of its prosocial behavioural effects in both human clinical and animal preclinical trials (as discussed in detail in Chapter 1, Introduction). Additionally, there is mixed evidence suggesting that the dysfunction of the oxytocinergic system in these disorders may contribute to the social dysfunction observed. For example, plasma OT levels are significantly lower in schizophrenia (Keri et al., 2009), ASD (Green et al., 2001a, Modahl et al., 1998, Taurines et al., 2014) and ADHD patients (Demirci et al., 2016, Sasaki et al., 2015) compared to healthy controls, although some studies have observed the opposite effect (Beckmann et al., 1985, Jansen et al., 2006). The precise correlation of plasma OT levels to CSF or brain concentrations remains a controversy (Carson et al., 2015, Kagerbauer et al., 2013). However, clinical studies have shown significant correlations between plasma OT levels and symptomatic scores in patients (Keri et al., 2009, Sasaki et al., 2015, Taurines et al., 2014). Furthermore, there is evidence that SNPs of the OTR are associated with increased risk of schizophrenia and linked to both pathophysiology and negative symptom domains (Montag et al., 2013). A recent meta-analysis also found significant associations between ASD and the SNPs rs7632287, rs237887, rs2268491 and rs2254298 of the OXTR (LoParo and Waldman, 2015). OT precursor peptide and OT carrier proteins are raised in children with ASD and patients with schizophrenia respectively, suggesting abnormal peripheral OT processing occurs in the brain.

This Chapter reviews the potential of intranasal OT as a treatment for social cognition deficits in two neurodevelopmental disorders: schizophrenia and ASD, chosen due to their high prevalence in society and because impaired social behaviour and cognition remain untreatable features of both. This chapter also discusses the translational relevance

of animal studies to the effects of OT on these human symptoms in the clinical setting.

2.2 Schizophrenia

Schizophrenia affects approximately 1% of the population (National Institute for Health and Care Excellence (NICE), 2014) with an incidence of ~1.5 per 1000 people (McGrath et al., 2004). There are three main symptom domains: positive (hallucinations, delusions, thought disorder), negative (social withdrawal, anhedonia, avolition) and cognitive (impaired executive function, memory and attention) (Andreasen, 1995). Schizophrenia typically occurs in late adolescence and early adulthood: men have a peak onset between 21-25, while for women between 25-30 and after 45 years of age (Li et al., 2016), with more men diagnosed at a median incidence ratio of 1.4:1 (McGrath et al., 2008). In addition to one's genotype, a multitude of environmental factors can occur during the perinatal period and subsequent adolescent period, increasing the risk of the first psychotic episode which usually emerges during late adolescence or early adulthood (Huttenlocher, 1984, Paus et al., 2008). The disorder is subsequently diagnosed (symptoms may or may not subside upon pharmacological intervention) and recurrent psychotic episodes may occur alongside persistent negative and cognitive symptoms. Throughout the disorder, adverse environmental triggers may further aggravate the gravity of the disorder (Millan et al., 2016). Although the aetiology is largely unknown, underlying neurotransmitter dysfunction is well established: hyperdopaminergic mesolimbic structures cause overactivation of D_2 family receptors which is thought to be associated with manifestation of the positive symptoms of schizophrenia. In contrast, negative symptoms appear to be linked to regionally specific hypodopaminergic activity in the prefrontal cortex, leading to reduced D_1 family receptor activation (Davis et al., 1991). There is also dysfunction of NMDA glutamate receptor, in particular on inhibitory GABA interneurons (Homayoun and Moghaddam, 2007), resulting in the disinhibition of excitatory pyramidal neurones with an increase in AMPA

receptor mediated glutamate signaling as a form of compensation (Moghaddam et al., 1997). This glutamate-GABA interaction is proposed to have a role in causing negative and cognitive symptoms of schizophrenia due to resulting excitotoxicity in the hippocampus and prefrontal regions. Evidence of this hypothesis includes a reduction in glutamatergic neuronal spine density and synaptophysin expression as well as a reduction of parvalbumin containing GABA neurones in these areas (Hashimoto et al., 2003, Hu et al., 2015, Glantz and Lewis, 2000). Of interest in relation to the predictability of preclinical models for schizophrenia, loss of hippocampal and pyramidal parvalbumin containing GABA neurones and alterations in cortical glutamate function are also very common features in many models including isolation rearing and chronic phencyclidine administration (Jones et al., 2011). The general brain morphology observed across patients with schizophrenia includes decreased whole brain volume as well as in subcortical regions like the hippocampus and enlarged ventricles (Steen et al., 2006, Vita et al., 2006, Wright et al., 2000). However, these changes are not biomarkers of the disorder and can only be reliably quantified by meta-analysis of patients compared to controls. Although current antipsychotics are relatively effective in treating the positive symptoms of schizophrenia, they offer little accompanying restoration of the negative and cognitive symptoms. The Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) and the Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study (CUtLASS) both failed to show any significant difference in terms of treatment discontinuation, improvement of psychosis or quality-of-life between First-Generation Antipsychotics and Second-Generation Antipsychotics.

Is there a role for OT in schizophrenia specifically? Clozapine, an atypical antipsychotic which is the atypical antipsychotic thought to produce the greatest attenuation of negative symptoms of schizophrenia (Brar et al., 1997, Khan and Zaidi, 2017, Llorca et al., 2000), increases plasma OT and cFos activation in hypothalamic PVN and SON nuclei (the site of OT containing neurones) which is not seen with the typical antipsychotic,

haloperidol (Kiss et al., 2010, Uvnas-Moberg et al., 1992b), which has less effect on negative symptoms. SNPs variations in the OTR gene have been linked to increased risk for schizophrenia, with several variations being associated with the severity of general, positive and negative symptoms of schizophrenia (Bartholomeusz et al., 2015). This evidence supports an interaction of the oxytocinergic system in relation to the negative and cognitive deficits found in schizophrenic patients.

The first suggestion to utilise OT as a treatment in man for schizophrenia was in the early 1970s, preceding any preclinical animal research (Bujanow, 1972). Bujanow's clinical observation was that 6-10 injections of OT intravenously or intramuscularly was sufficient to produce rapid therapeutic effects, reducing the need for hospitalization. However, these early studies did not use standardized scales to measure therapeutic outcome. Since then, clinical trials utilizing exogenous OT have been rigorously designed and conducted in schizophrenic patients to examine their therapeutic potential (Table 2.1).

No	Study	Participants	Study design	OT dose	Length of treatment	Outcomes measured	Results
1	Feifel et al. (2010)	15 patients	Double-blind, placebo-controlled, crossover	20IU for 1st week, 40IU for 2nd and 3rd week, twice daily	3 weeks	PANSS, CGI-S, CGI-I	Improved PANSS total and positive symptom scores and CGI-I scores
2	Pedersen et al. (2011)	20 patients (11OT, 9 placebo)	Double-blind, placebo-controlled	24IU twice daily	2 weeks	Brüne Theory of Mind Picture Stories Task, Trustworthiness Task, Paranoia Scale	Accurate identification of second order false belief in the Brüne Task, reduction in PANSS total, positive subscale and Paranoia Scale
3	Averbeck et al. (2012)	1st experiment: 30 patients, 29 controls 2nd experiment: 21 patients	Double-blind, placebo-controlled, crossover	24IU	acute (50 mins before task)	Hexagon emotion discrimination task	Experiment 1: OT showed no improvement compared to control group Experiment 2: OT improved overall emotion recognition but not on any specific emotion
4	Feifel et al. (2012)	15 patients	Double-blind, placebo-controlled, crossover	20IU for 1st week, 40IU for 2nd and 3rd week, twice daily	3 weeks	CVLT II, LNS	OT improved some CVLT measures: total recall, short delayed free recall, total recall discrimination

5	Modabbernia et al. (2013)	40 patients	Double-blind, placebo-controlled, parallel-group	20IU for 1st week, 40IU for next 6 weeks, twice daily	8 weeks	PANSS	Improved PANSS total score from Week 4, positive and negative and general psychopathology subscale score from Week 6
6	Lee et al. (2013)	28 patients (13 OT, 15 placebo)	Double-blind, placebo-controlled	20IU twice daily	3 weeks	BPRS, SANS, CGI-S	At week 3, OT improved total SANS score and motivation-pleasure subscore for inpatients but not outpatient or general group
7	Fischer-Shofty et al. (2013)	35 patients, 46 controls	Double-blind, within-subject crossover	24IU	acute (45 mins before task)	PANSS, CGI, Interpersonal Reactive Index (kinship and intimacy category chosen, measures empathy)	OT improved kinship recognition (but not intimacy) in the patient group
8	Davis et al. (2013)	23 patients (11 OT, 12 placebo)	Double-blind, placebo-controlled	40IU	acute (30mins before task)	PANSS, CGI, MIRECC GAF	Improved performance in high level social cognition.
9	Gibson et al. (2014)	14 patients (8 OT, 6 placebo)	Double-blind, placebo-controlled, randomised	24IU twice daily	6 weeks	Emotion Recognition-40, Theory of Mind Picture Stories Task, The Eyes Test, The Interpersonal	Improved fear recognition, ToM total score and deception detection subscore, reduction in hostility bias, decrease in all PANSS subscores

						Reactivity Index, The Trustworthiness Task, The AIHQ, PANSS	
10	Davis et al. (2014)	27 patients (13 OT, 14 placebo)	Double-blind, placebo-controlled, randomised	40IU twice weekly	6 weeks	Facial affect recognition, Profile of Nonverbal Sensitivity, Empathic Accuracy Task, MSCEIT (manging emotions component), TASIT	Improved empathic accuracy both 1 week and 1 month after study completion
11	Woolley et al. (2014)	29 patients 31 controls	Double-blind, placebo-controlled, crossover	40IU	acute (30mins before task)	RMET, TASIT (emotion evaluation test, social inference minimal, social inference enriched)	OT had no effect on lower level automatic social cognition for composite or any subscales, but improved higher level controlled cognition i.e. performance in SI-E 'think' verbal and 'say' subsccale in patients

12	Horta de Macedo et al. (2014)	20 patients 20 controls	Double-blind, placebo-controlled, crossover	48IU	acute (50mins before task)	facial emotion-matching task, PANSS-negative, BPRS	No improvement in facial emotion matching task
13	Michalopoulou et al. (2015)	21 patients (10 OT, 11 placebo)	Double-blind placebo-controlled, crossover	24IU	acute	Working memory using digit span task (storage and maintenance, info maintenance and manipulation), Digit Symbol Coding (processing speed)	OT improved the 'executive' component of working memory
14	Guastella et al. (2015b)	22 patients (11 OT 11placebo)	Within-subject, double-blind, crossover, randomised	24IU	acute (45 mins before task)	lower order cognition: DANVA, Facial Expressions of Emotions Task, RMET High order cognition: False Belief Picture Sequencing Task (assess theory of mind) Neurocognition: RBANS	No improvement in lower order cognition except in the accuracy for detecting vocal intonations; improved higher order cognition overall and on tasks that requires appreciation of social nuances in communicative exchanges
15	Lee et al. (2016)	28 patients (13 OT, 15 placebo)	Double blind, randomised	20IU twice daily	3 weeks	BPRS, SANS	OT had no effect

16	Brambilla et al. (2016)	31 patients	Double blind, crossover, placebo controlled	40IU	4 months	MSCEIT (emotional intelligence), RMET, emotional priming paradigm	OT improved MSCEIT 'Understanding Emotions' and decreased response time in facial affect recognition
17	Jarskog et al. (2017)	68 (35 OT, 33 placebo)	Double-blind, placebo controlled, randomised	24IU twice daily	3 months	emotion recognition task, Brune ToM Stories Task, RMET, Trustworthiness Task, Ambiguous Intentions Hostility Questionnaire Secondary measures: Specific Levels of Functioning Scale, Social Skills Performance Assessment, PANSS	No improvement
18	Buchanan et al. (2017)	58 patients (17 OT 20 galantamine 21 placebo)	Randomised, double blind, placebo controlled	24IU twice daily	6 weeks	MATRICS Consensus Cognitive Battery, Rapid Visual Information Processing Test (sustained attention task), UCSD Performance-	OT did not show improvement compared to placebo group

						Based Skills Assessment	
19	Halverson et al. (2019)	68 (35 OT, 33 placebo)	Double blind, randomised	24IU twice daily	12 weeks	Introspective accuracy: Specific Level of Functioning Scale, Interpersonal Perception Task empathy: Interpersonal Reactivity Index (IRI) social symptoms: Liebowitz Social Anxiety Scale, Green et al. Paranoid Thoughts Scales	OT improved IRI Perspective Taking in OT patients, compared to placebo.
20	Bradley et al. (2019b)	150 (70 patients, 80 controls)	Placebo-controlled, crossover, randomised	40IU	acute (90 mins before task)	LNS (working memory)	OT did not improve working memory
21	De Coster et al. (2019)	48 (23 patients, 25 controls)	Double-blind, placebo-controlled, crossover	40IU	acute (45 mins before task)	verbal ToM tasks: False Belief task, Person Description task	OT improved ToM accuracy

22	Wynn et al. (2019)	47 patients	Randomized, double-blind, placebo-controlled cross-over	8, 12, 24, 36, 48, 60, 72, or 84 IU	acute (30mins before task)	EEG for mu suppression, facial affect pupillometry	36 and 48IU dose enhanced mu suppression to biological motion stimuli
23	Bradley et al. (2019a)	33 patients	Placebo-controlled, double-blind, crossover	40IU	acute (50 mins before task)	eye-tracking, PANSS, Experiences in Close Relationships-Relationships Structures	OT improved eye fixation time
24	Bradley et al. (2020)	93 (39 patients, 54 controls)	Randomised, double blind, placebo controlled	40IU	acute (30 mins before task)	Auction Game that quantifies preferences for monetary v. social reward	OT increased overbidding - an increased motivation to reward?
25	Bradley et al. (2021)	64 females (26 patients, 38 control)	Randomized, placebo-controlled, double-blind, crossover	40IU	acute (45 mins before task)	Social Inference–Enriched (SI-E) sub-section of The Awareness of Social Inference Test	OT did not improve mentalizing

Table 2.1 List of randomised clinical trials carried out in patients with schizophrenia and examining intranasal OT effect on negative symptoms and social cognition. AISS: Ambiguous Intentions Hostility Questionnaire-Abbreviated Version; BPRS: Brief Psychiatric Rating Scale; CGI-I/S: Clinical Global Impressions-Improvement/Severity; CVLT II: California Verbal Learning Task; DANVA: Diagnostic Analysis of Non-Verbal Accuracy; DBANS: Repeatable Battery for the Assessment of Neuropsychological Status; FEEST: Facial Expressions of Emotions Task; LNS: Letter Number Sequencing; MIRECC GAF: Global Assessment of Functioning; MSCEIT: Mayer–Salovey–Caruso emotional intelligence test; PANAS: Positive and Negative Affect Schedule; PANSS: Positive and Negative

Syndrome Scale; RMET: Reading the Mind in the Eyes Test; SANS: Scale for the Assessment of Negative Symptom; SAPS: Scale for the Assessment of Positive and Negative Symptoms; SFS: Social Functioning Scale; TASIT: The Awareness of Social Inference Test; VAS: Visual Analog Scale

Studies utilising intranasal OT in patients with schizophrenia range from acute (task carried out between 30 – 90 mins after drug administration) to long-term studies (2 weeks to 4 months, most common being 3 weeks). Doses range from 20IU to 40IU. Results from these studies are mixed: some studies report an improvement in negative symptoms (Feifel et al., 2010, Pedersen et al., 2011, Modabbernia et al., 2013, Lee et al., 2013, Gibson et al., 2014, Bradley et al., 2019a) while others did not (Brambilla et al., 2016, Buchanan et al., 2017, Jarskog et al., 2017). These investigations of impact on social cognition looked at a range of tasks from lower order emotion recognition to higher order Theory of Mind, empathy recognition and working memory. Emotion recognition generally shows no improvement with OT treatment (Davis et al., 2013, Woolley et al., 2014, Horta de Macedo et al., 2014, Guastella et al., 2015a, Jarskog et al., 2017, Buchanan et al., 2017, Wynn et al., 2019) whereas higher order cognition tasks tended to report more favorable outcomes following OT (Pedersen et al., 2011, Fischer-Shofty et al., 2013, Davis et al., 2013, Davis et al., 2014, Gibson et al., 2014, Woolley et al., 2014, Guastella et al., 2015b, Halverson et al., 2019, De Coster et al., 2019). Cognitive and executive functioning, which are also significantly impaired in patients with schizophrenia, have been less explored, although trials investigating OT's effect on working memory seem mostly negative (Buchanan et al., 2017, Bradley et al., 2019b). These observations are largely in line with meta-analysis studies reporting inconsistent evidence for intranasal OT improving high-level social cognition (Burkner et al., 2017) and a lack of OT superiority overall in comparison to placebo in terms of cognition and total psychopathology (Zheng et al., 2019). However, Martins et al. (2021b) and Zheng et al. (2019) saw a small but significant improvement in general psychopathology and positive symptoms with an 80IU dose (as seen in Feifel et al., (2010), Feifel et al., (2012) and Modabbernia et al. (2013)) suggesting the requirement for more intricate dose response studies. Indeed, only one study examined a range of eight doses and found only mid-range doses improved social stimuli interpretation (Wynn et al., 2019). Although surprisingly the 80IU dose did not improve social cognition, this may be due to low sample size (Wynn et al. (2019) only

had n=6/dose). Clearer information about efficacious doses would also help in guiding treatment length as OT use in the clinic would likely be long-term and studies utilising lower 20-24IU doses lasting between 3 to 12 weeks show little to no symptom improvement (Buchanan et al., 2017, Halverson et al., 2019, Jarskog et al., 2017, Lee et al., 2016) compared to studies that utilised higher doses of 40IU (Brambilla et al., 2016, Davis et al., 2014).

In animal studies, OT administered to otherwise normal rodents without social deficits increased social interaction (Calcagnoli et al., 2015, Huang et al., 2014, Kent et al., 2016). In animal models for schizophrenia, intra-amygdala infusion of 1000ng OT in a chronic-PCP rat model increased social interaction (Lee et al., 2015) while in an acute-PCP rat model, 0.1mg/kg of subcutaneous OT decreased PCP-induced hyperactivity and increased social interaction (Kohli et al., 2019). Mechanisms of action may involve OT projections to the amygdala and nucleus accumbens (Lee et al., 2015, Kohli et al., 2019). Intranasal OT attenuates amygdala activity and strengthens amygdala resting state functional connectivity in patients with schizophrenia (Shin et al., 2015a) while in healthy volunteers, enhances connectivity in striatal (reward) pathways (Jiang et al., 2021, Zhao et al., 2019), showing translational relevance of these pharmacological models. Social cognition in rodents is measured using social recognition and motivation tasks (requires animal to recognize another conspecific as familiar or unfamiliar and be motivated to approach and interact) and OT delivered to the brain of healthy rats facilitates social recognition in a dose-dependent manner (Popik and van Ree, 1991). Although impaired social recognition has been shown in some models such as the dual hit neonatal PCP and social isolation model (Hamieh et al., 2021) and the MK-801 (NMDAr antagonist) pharmacological model (Deiana et al., 2015), to the best of my knowledge, it has not been tested whether OT is able to reverse these deficits. Overall, the preclinical models predict successful use of OT in the clinic, and indeed intranasal OT appears to produce efficacious results in patients although more emphasis should be placed on

identifying suitable dose and length of treatment for further clarity. The lack of dose-response studies also remains a less explored domain in preclinical studies.

2.3 Autism spectrum disorder (ASD)

Autism spectrum disorder is an umbrella term referring to a heterogenous group of disorders characterised by common behavioural features such as persistent deficits in social interaction and communication and restricted repetitive behaviour, interests, or activity. The diagnostic criteria for ASD are behavioural and typically diagnosed by the age of three (Sauer et al., 2021).

The prevalence for ASD vary between regions: the Centre for Disease Control and Prevention Agency, USA estimate a prevalence of 15.6 per 1,000 children aged 4 years in 2016 (Shaw et al., 2020) while results in Europe fluctuate dramatically between countries with an estimate of 12.6 per 1000 children in Denmark to 4.8 per 1000 in South-Eastern France in children aged 7-9 years in 2015 (Delobel-Ayoub et al., 2015). In Asia, a meta-analysis looking at studies from 2009 – 2018 reported a prevalence as high as 51 per 1000 in East Asia while in South Asia the estimate was only of 3.1 per 1000 (Qiu et al., 2020). This discrepancy across and within geographical areas is likely due to the source of data from which cases were detected which are largely based on administrative data and questionnaires (Chiarotti and Venerosi, 2020) as well as social and cultural differences which may impact diagnosis as diagnostic criteria are developed primarily based on Western participants (Bernier et al., 2010). A gender difference is also observed, with males diagnosed four times more than females (Fombonne, 2009) although it is suggested that there exist diagnostic biases against girls as well as protective effect of the female sex on genetic risk (Robinson et al., 2013) and the true ASD diagnostic ratio of male to females is likely lower at 3:1 (Loomes et al., 2017).

The aetiology of ASD has a strong genetic component but with impact from the environment. ASD has a strong heritability risk: a meta-analysis of twin study puts an estimate at 64% - 91% (Tick et al., 2016) while a large population based multinational cohort study found a heritability risk of approximately 80% (Bai et al., 2019). Currently, identified genetic causes of ASD include cytogenic chromosomal abnormalities (~5% (Vorstman et al., 2006)), single-gene disorders (~5% (Miles, 2011)) and copy number variants (CNVs). Indeed, de novo CNVs has a strong association in the aetiology (Sebat et al., 2007) while the impact of common variants, if any, is less substantial (Devlin et al., 2011). The fact that some CNVs associated with ASD have also been associated with other disorders such as schizophrenia (Sebat et al., 2009) suggest secondary insults such as a rare variant (Vrijenhoek et al., 2008) or that environmental factors may be required to determine the final phenotype. Prenatal exposure, primarily maternal hypertension, previous fetal loss and maternal medication have been shown to have significant associated risks (Gardener et al., 2009) although later disputed in a meta-analysis (Yip et al., 2018). Other environmental risk factors that have been associated with higher risk of ASD include advanced parental age, birth complications and exposure to toxic elements such as heavy metals like mercury and lead (Modabbernia et al., 2017). In-utero exposure to sodium valproate (an antiepileptic) during pregnancy also increases the absolute risk of ASD in children (Christensen et al., 2013). This neurodevelopmental effect was later examined in mice and used to produce the valproate model of autism, where exposed pups display stereotypic behaviour and social behaviour deficits with similar neural/molecular changes to that of patients (Tartaglione et al., 2019). This emphasises the importance of communication between the preclinical and clinical setting if we wish to further improve on translational aspects in this area.

Many CNV studies have been informative in revealing brain abnormalities in patients namely synaptic formation and maintenance, cellular proliferation and signalling as well as neurogenesis (Pinto et al.,

2010, Gilman et al., 2011). Neuroimaging studies have also shown hyperextension of cortical surface areas with larger brain volumes in children later diagnosed with ASD (Hazlett et al., 2017), as well as aberrant development of white matter pathways (Andrews et al., 2019) and structural and function connectivity (Lewis et al., 2014, O'Reilly et al., 2017). The current gold standard treatment consists of behavioural interventions (Eldevik et al., 2009). There are currently no effective medications to treat ASD but antipsychotics (such as risperidone) can be prescribed to treat symptoms of aggression, stereotypic behaviour, and hyperactivity (McCracken et al., 2002, McPheeters et al., 2011), although side-effects such as weight gain and hyperprolactinemia may lead to treatment discontinuation (Anderson et al., 2007), just as it does when they are used to treat schizophrenia (Montag et al., 2013). Selective serotonin reuptake inhibitors (SSRIs) may also be prescribed for the repetitive/stereotyped behaviour and co-occurring depression/anxiety as changes in the serotonin system have been linked to ASD: hyperserotonemia which is present in more than 25% of children with ASD was the first identified biomarker and variants in the 5-HT transporter gene have been associated with ASD (Muller et al., 2016, Veenstra-VanderWeele et al., 2012). However, evidence for SSRIs as an effective treatment is scarce (Williams et al., 2013). As of now, there is no known medication to treat the social dysfunction.

There is evidence for OT system disturbance in children with ASD: lower plasma OT levels have been found in prepubertal and adult males with ASD (Al-Ayadhi, 2005, Andari et al., 2010, Modahl et al., 1998) and abnormal OT peptide processing was found in children with ASD (Green et al., 2001b). Single nucleotide polymorphisms (SNPs) in the OTR gene have also been associated with autism (Jacob et al., 2007, Liu et al., 2010, Wu et al., 2005). OT is also responsible for the GABAergic excitatory (during fetal and postnatal periods) to inhibitory (adult period) current switching during development by reducing intracellular chloride levels through the NKCC1 chloride inward transporter (Tyzio et al., 2006). This perinatal excitatory-to-inhibitory switch is abolished in the fragile X

and valproate rodent models of ASD as well as by administering a selective OTR antagonist in naiive rodents and produced similar behavioural deficits (reduced ultrasonic vocalisations), stressing the importance of the OT-GABA link (Tyzio et al., 2014). These association has led to OT being a popular investigation for the core social cognition deficits seen in patients with ASD.

The first studies utilising exogenous intravenous synthetic OT (Pitocin) in adult patients showed a decrease in the severity of repetitive behaviour and affective speech comprehension, placing a highlight on OT's potential to reduce core behavioural symptoms severity (Hollander et al., 2003, Hollander et al., 2007). A flurry of randomised clinical trials utilising exogenous intranasal OT soon followed (Table 2.2).

No	Study	Participants	Age range (years)	Study design	OT dose	Length of treatment	Outcomes measured	Results
1	Guastella et al. (2010)	16 adolescents (ASD or Asperger's Syndrome)	12 to 19	Double-blind, placebo-controlled crossover	18-24IU	acute (45mins before task)	RMET	OT improved RMET performance compared to placebo
2	Andari et al. (2010)	33 adults (20 ASD and Asperger Syndrome, 13 controls)	17 to 39	Double-blind, placebo-controlled, within-subject	24IU	acute (50mins before task)	Social interaction (computerised ball-tossing game), face perception task	OT enhanced cooperation in ball tosses with 'good' partners and increased feelings of trust, increased eye gaze duration compared to placebo (but not healthy controls)
3	Anagnostou et al. (2012)	19 adults (ASD or Asperger's Syndrome)	18 to 60	Double-blind, placebo-controlled, parallel design	24IU twice daily	6 weeks	Repetitive behaviour (YBOCS-compulsion), RBS-R, Social Responsiveness Scale, CGI,	OT decreased lower order repetitive behaviour only (e.g. stereotypy) but not higher order behaviour (e.g. compulsive-like)

							RMET-Revised, DANVA-2	
4	Domes et al. (2013)	28 adults (14 Aperger Syndrome 14 controls)	mean age 24	Double-blind, placebo-controlled, , crossover	24IU	acute (45mins before task)	Face Discrimination Task	OT did not improve task performance although MRI showed an increased amygdala activity to social stimuli
5	Dadds et al. (2014)	38 children (19 OT 19 placebo)	7 to 16	Double-blind, placebo-controlled	12IU, 24IU	4 days	Parent-Child Interaction, UNSW Facial Emotion task, social interaction, repetitive behaviour	OT did not improve any aspect
6	Aoki et al. (2014)	20 adults (10 OT 10 placebo)	mean age 29.6	Double-blind, placebo-controlled crossover	24IU	acute (40mins before task)	Theory of Mind (1st and 2nd order false belief)	OT improved 2nd order of false belief and increased brain activity in right anterior insula

7	Auyeung et al. (2015)	74 adults (37 patients 37 controls)	18 to 56	Double-blind, placebo-controlled within-subject	24IU	acute (45 mins before task)	Eye tracking	OT increased duration and number of eye fixation compared to placebo. Within OT group, OT also enhanced eye fixation duration for individuals who had lower than average baseline scores compared to those with higher scores
8	Guastella et al. (2015a)	50 adolescents (26 OT 24 placebo)	12 to 18	Double-blind, placebo-controlled	18IU, 24IU twice daily	8 weeks	SRS, CGI-I,RBS, Developmental Behaviour Checklist, RMET, emotion recognition, DANVA	No improvement in any aspect although parents who belief OT has been administered report greater improvement in social responsiveness and behavioural/emotional problems in their child
9	Watanabe et al. (2015)	18 adults (9 OT 9 placebo)	18 to 55	Double-blind, placebo-controlled crossover	24IU twice daily	6 weeks	ADOS, CARS2, Autism Spectrum Quotient, SRS, RBS, STAI state	OT improved ADOS social reciprocity scores

10	Althaus et al. (2015)	62 adults (32 patients 30 controls)	18 to 31	Double-blind, placebo-controlled crossover	24IU	acute (45 mins before task)	STAI, IAPS	No changes in empathy scale or state anxiety
11	Kosaka et al. (2016)	60 adults	mean age 23.9	Double-blind, placebo-controlled, parallel design	32IU, 16IU	12 weeks	CGI, IRSA, Aberrant Behavior Checklist, Zung Self-Rating Depression Scale, STAI, eye tracking	32IU OT improved CGI scores compared to placebo and increased duration of eye gaze
12	Yatawara et al. (2016)	31 children	3 to 8	Double-blind, placebo-controlled crossover	3-12IU then 6 -24IU (increasing progressively over the week)	5 weeks	SRS, RBS, ADOS, DBC-P, CGI	OT improved SRS and CGI scores

13	Kanat et al. (2017)	60 adults (29 Asperger's 31 control)	8 to 17	Double-blind, placebo-controlled crossover	24IU	acute (45 mins before task)	Social Interaction Anxiety Scale, Autism Spectrum Quotient, Wortschaztest (verbal intelligence)	OT improved social stimulus attention in individuals with high social anxiety
14	Quintana et al. (2017)	17 adults	18 to 35	Double-blind, placebo-controlled 3-period crossover	8IU, 24IU	acute (50mins before task)	Overt emotion sensitivity task, RMET, emotional dot probe (covert emotional salience), emotional face-morphing (speed of recognition)	8IU enhanced perception of happiness faces compared to placebo
15	Parker et al. (2017)	32 children (14 OT 18 placebo	6 to 12	Double-blind, placebo-controlled	24IU twice daily	4 weeks	SRS, repetitive behaviour, associated symptoms	OT increased SRS total score. Interestingly, individuals with lowest OT baseline

								concentration improved the most.
16	Strathearn et al. (2018)	32 children and adolescence (16 patients 16 control)	8 to 19	Double-blind, placebo-controlled crossover	12IU, 16.8IU, 24IU (age dependent)	acute (30mins before task)	Eye tracking, Systemizing Picture Task (gauge gaze preference)	OT reduced patients' tendency to fixate on highly systemised pictures while control receiving OT became more likely to fixate.
17	Yamasue et al. (2020)	106 adults (53 OT 53 placebo)	18 to 48	Double-blind, placebo-controlled, parallel-group, multicentre	48IU	6 weeks	ADOS, repetitive behaviour, eye gaze,	OT reduced ADOS score and increased eye gazing duration (within group) but not compared to placebo. Repetitive behaviour was decreased by OT compared to placebo group.
18	Kruppa et al. (2019)	39 adults (15 patients 24 controls)	18 to 25	Double-blind, placebo-controlled crossover	20IU	acute (45mins before task)	Probabilistic reinforcement learning task	OT enhanced social learning and the correlation of Reward Prediction Error signals with activation of the nucleus accumbens in patients

19	Owada et al. (2019)	103 adults (51 OT 52 placebo)	18 to 54	Double-blind, placebo-controlled, parallel-group, multicentre	24IU twice daily	6 weeks	Facial expression intensity	OT increased neutral facial expression variations in patients with the maximum effect at 2 weeks post treatment
20	Bernaerts et al. (2020a)	40 adults (22 OT 18 placebo)	18 to 35	Double-blind, randomized, placebo-controlled, parallel	24IU	4 weeks	SRS, RBS, SAAM (measures feelings of avoidance toward others), IPPA, WHO-QOL	OT improved social responsiveness compared to baseline immediately after treatment and 1-month post-treatment although not significant compared to placebo. Repetitive behaviour and attachment avoidance was improved up to 1 year post treatment
21	Borowiak and von Kriegstein (2020)	32 adults (18 patients 18 control)	mean age 30.7	Double-blind, placebo-controlled, within-subject, cross-over	24IU	acute (45mins before task)	Voice-identity recognition experiment	OT did not alter speaker recognition accuracy and MRI recording did not see any enhanced responses of the right posterior superior

								temporal sulcus/gyrus as opposed to an enhanced response in the control group
22	Bernaerts et al. (2020b)	38 adults (21 OT 17 placebo)	mean age 24	Double blind, placebo-controlled, parallel	24IU	4 weeks	Emotion recognition task	Acute OT enhanced brain activity in the posterior superior temporal sulcus and chronic OT saw a significant enhancement post one-year follow up indicating long-lasting neural changes in social brain regions. Amygdala activity was also attenuated and associated with self-reported improvement in avoidant attachment feelings in the one month follow up session.

23	Sikich et al. (2021)	221 children and adolescents (114 OT 107 placebo)	3 to 17	Double-blind, parallel, placebo-controlled	8IU to 80IU (increased over weeks)	24 weeks	ABC modified Social Withdrawal subscale, SRS-2 Social Motivation, Sociability Factor and SB5 Abbreviated IQ	OT did not improve any measures

Table 2.2 List of randomised clinical trials carried out in patients with ASD and examining efficacy of intranasal OT on symptom severity. ADOS: Autism Diagnostic Observation Scale; DANVA-2: Diagnostic Analysis of Nonverbal Accuracy; DBC-P: Developmental Behaviour Checklist; IAPS: International Affective Picture System; IPPA: Inventory of Parent and Peer Attachment; IRSA: Interaction Rating Scale Advanced; RBS-R: Repetitive Behaviour Scale-Revised; SAAM: State Adult Attachment Measure; SRS: Social Responsiveness Scale; STAI: Spielberger's State and Trait Anxiety Inventory; WHO – QOL: Quality of Life Questionnaire World Health Organization; YBOCS: Yale Brown Obsessive Compulsive Scale

Compared to trials of patients with schizophrenia, the dosage used in ASD trials are relatively homogenous at 24IU for adults. Intranasal OT improved social interaction (shown via an increased duration and tendency to fixate on the eye area as active avoidance of the eye region is a classic hallmark of ASD (Kliemann et al., 2010) and clinical assessments conducted by trained professionals) in adults (Andari et al., 2010, Auyeung et al., 2015, Kosaka et al., 2016, Watanabe et al., 2015, Yamasue et al., 2020) and children aged 3 – 12 (Parker et al., 2017, Yatawara et al., 2016). However Andari et al. (2010) and Yamasue et al. (2020) only saw an improvement within group comparison and not compared to placebo. Eye Interestingly, studies that did not report an improvement were conducted in children and adolescents, aged 7 -19 (Dadds et al., 2014, Guastella et al., 2010, Strathearn et al., 2018). Might there be an effect of age and/or medication interaction? The body goes through a large hormonal change during adolescence and patients are on a range of medication from antipsychotics to antidepressants in addition to OT. OT is known to interact with monoamines (DA: (Xiao et al., 2017, Peris et al., 2017), glutamate: (Eliava et al., 2016) and estrogen (Jurek and Neumann, 2018)) and estrogen levels have been shown to rise during puberty in boys which is the main gender examined in these trials (Klein et al., 1996). Epigenetic methylation of the OTR gene, in particular the CpG site-934, is well documented and have been linked to socioemotional qualities (Moerkerke et al., 2021) and changes in gene methylation in general has been linked to the adolescent period (Han et al., 2019). However, whether this applies to the OTR gene specifically is unclear, although a recent study showed that the level of OTR methylation was associated with greater attachment avoidance in young adults more than older adults (Ebner et al., 2019). Other core symptom domains of ASD such as repetitive behaviour (Dadds et al., 2014, Guastella et al., 2010, Parker et al., 2017, Watanabe et al., 2015, Yatawara et al., 2016, Yamasue et al., 2020) and social cognition showed mixed effects: tasks examining Theory of Mind are mostly positive (Dadds et al., 2014, Althaus et al., 2015) while emotional recognition show contradictory results: improvement (Kruppa et al., 2019, Owada et

al., 2019, Sikich et al., 2021); and no effect (Dadds et al., 2014, Guastella et al., 2015b). Meta-analysis confirmed the lack of effect of OT on repetitive behaviour (Ooi et al., 2017, Zhou, 2021 #921) although Peled-Avron et al. (2020) suggested otherwise. Brain imaging studies carried out during cognition tasks show that OT administration increased the neural response to social versus non-social stimuli (Andari et al., 2016, Kruppa et al., 2019), suggesting OT does enhance the saliency of social stimuli, but perhaps doses administered are not enough to produce behavioural (meta-analysis also show a lack of effect of OT on social cognition in ASD patients (Ooi et al., 2017, Peled-Avron et al., 2020)).

In the valproate rodent model of ASD, intranasal and peripheral OT rescued social interaction and social cognition deficits (Dai et al., 2018, Hara et al., 2017). Similar rescue of social deficits is seen in genetic ASD models such as the Chd8 haploinsufficiency mouse (Cherepanov et al., 2021) and SHANK3-deficient rat (Harony-Nicolas et al., 2017). Sub-chronic i.p. injection of OT also increased sociability tendency in inbred adolescent mouse strains BALB/cByJ and C58/J which have low sociability (Teng et al., 2013) and adult Grin1 knockdown mice (Teng et al., 2016). The socio-behavioural improvements induced by OT seem largely successful in animals compared to the more inconclusive clinical results described above. This may be due to single factor manipulation in the animal models where resulting improvement is by OT exerting effects in the specific altered area. For example, SHANK 3 mutation in mice causes deficits in synaptic transmission and plasticity accompanied by impaired social interaction and social recognition (Uchino and Waga, 2013) and OT rescues both behavioural and synaptic deficits (Harony-Nicolas et al., 2017). As such, OT effects may be masked in a patient with ASD, who would likely have contributions from multiple gene variants and thus multiple neuronal/molecular/behavioural deficits. The given dosage may not be strong enough as clinical studies have robustly show that although OT induces neural changes, the doses may not translate behaviourally.

2.4 Discussion

Overall, intranasal OT's effect on improving social behaviour and cognition deficits in patients remains controversial. OT seems more promising for the treatment of patients with schizophrenia than ASD which appears puzzling as several symptom categories clearly overlap. Schizophrenia trials hinted at the possibility that only higher-order cognition is improved and meta-analysis encompassing larger clinical populations suggest the same (Burkner et al., 2017, Keech et al., 2018). This effect may be masked in ASD trials due to the larger age range of the clinical population. Other main factors that may contribute to the unclear effects of OT are the heterogeneity of trial design and patient population as well as poor translation of current animal models.

2.4.1 Heterogeneity

Clinical trials carried out are often of small sample sizes and different intranasal dosages (especially in trials for schizophrenia). There is a severe lack of dose-response studies investigating efficacious doses with chosen doses usually based on ranges that do not produce adverse effects as commonly seen in trials with ASD patients of different ages e.g. Sikich et al. (2021), where doses were adjusted based on tolerance rather than efficacy. Trial designs also differ from patient criteria (age range, inclusion criteria, comorbidities, outpatient/inpatient recruitment) to time between administration of OT and the start of behavioural tasks. Administration protocols may also result in different doses absorbed across the nasal membrane and into the brain: for example, OT may be administered under observation or by a trained technician (Feifel et al., 2010, Modabbernia et al., 2013), but some are self-administered without supervision (Cacciotti-Saija et al., 2015, Pedersen et al., 2011). Considering that patients with schizophrenia and ASD may show poor compliance to a strict treatment regime, it may be possible that unsupervised patients do not actually administer the full dose as intended. Patients are also on a range of medication, as OT in trials is given as an adjunct, ranging from antipsychotics to antidepressants which may confound effects of OT. For instance, lower antipsychotic

doses have been associated with better improvement in certain social cognition tasks (Woolley et al., 2014). The oxytocinergic system is also known to interact with monoamines such as dopamine (Xiao et al., 2017, Peris et al., 2017) and serotonin (Lefevre et al., 2017, Pagani et al., 2015) which are the primary targets of these medications. Aside from these obvious differences, the heterogeneity of symptom manifestation remains the largest confounding factor as symptom severity differs between individuals, ASD in particular is an umbrella term – the inclusion of patients diagnosed with Aspergers Syndrome e.g. (Andari et al., 2010, Anagnostou et al., 2012, Domes et al., 2007, Kanat et al., 2017) which previously had its own discrete diagnostic category under the DSM-IV and diagnosis requires an exclusion of language delay and cognitive deficits, is now diagnosed as ASD under DSM-V (Hosseini et al., 2021). The large age range in ASD trials (considering ASD is diagnosed at a very young age) may also be a confounding effect as the oxytocinergic system has been shown to be sensitive to developmental experiences in animal studies (Bales and Perkeybile, 2012). A meta-analysis by Huang et al. (2021) showed that age had a significant effect on social functioning, with OT improving symptoms in adults but not children/adolescents (although the lack of effect in the latter population may be due to its small sample size). There are also inter-individual differences which may affect response to OT treatment, for example the extent of DNA methylation of the OTR gene is associated with variable effects on human sociability (Haas et al., 2016). Mice, which are highly amenable to genetic manipulation, would be a useful model to use to shed light on whether a more personalised approach may be more successful.

The difference in behaviour observed between individuals also brings us to the social salience theory. This is touched on in Chapter 5.3 (Discussion: Social salience theory of oxytocin) and has more recently been taken into consideration for treatment purposes. From the studies summarised above, a trend is noticed where the more severe the deficits in social cognition, the greater the impact of OT treatment. For example,

an improvement in emotion recognition was seen only within the patient subgroup but not when compared to healthy controls (Pedersen et al., 2011) and in the negative symptom domain within inpatient groups but not outpatients (Lee et al., 2013). The same observation is picked up in ASD trials: an increase eye duration is observed only in patients who had a lower-than-average baseline score (Auyeung et al., 2015) and patients with lower baseline plasma OT concentrations improved the most on the Social Responsiveness Scale, an indicator for social interaction functioning (Parker et al., 2017). Overall, this suggests that possible beneficial effects of OT may be masked by the heterogeneity of patient population, be that due to symptom severity, age, or even genetic makeup. Kosaka et al. (2016) identified 19 SNPs in the OTR gene in their patient population that predicted stronger improvement in CGI score with 32IU dose and a different SNP predictive of 16IU dose and resulting CGI score), highlighting the need for a more personalised design.

2.4.2 Criticism of animal models

The usage of animals to model neurodevelopmental disorders is essential as *in vitro* models, although useful in dissecting molecular mechanisms, are unable to recapitulate drugs' effects on behaviour nor allow in depth understanding on how genes and/or the environment may contribute to a disease. Rodent models are invaluable in this field due to their financial and ethical advantages compared to non-human primates, and the fact that they are amendable to genetic manipulation as the aetiology of neurodevelopmental disorders are multifactorial. Yet, translational success has been poor despite the positive prosocial effects OT has in many preclinical animal models. One main reason may be publication bias as null results often go unpublished, but it also raises the question of the validity of animal models. The challenge in fulfilling the triad of validity (Figure 4.1), in particular, construct and face validity in neurodevelopmental disorders is considerable. The main criticism is that the neurodevelopmental factor is rarely considered and that models are often produced from a single manipulation of either genetic/environmental/pharmacological insult despite disorders being

multifactorial, and interventions usually applied during adult age, and not mirroring the age of onset of the disorder: schizophrenia during the adolescent period and ASD in early childhood.

For schizophrenia, for example the chronic PCP model is a popular pharmacological approach. Rats administered chronic PCP appear to exhibit some symptoms and neurochemical changes akin to those of patients: sensitization of the limbic circuit expressed as locomotor sensitization to subsequent PCP exposure (a translational index to positive symptoms), enduring cognitive impairments and reduced social interaction (Jentsch and Roth, 1999, Jones et al., 2011), reduced dopamine turnover in the prefrontal cortex (Jentsch et al., 1997a, Jentsch et al., 1997b) as well as reduced number of cortical and hippocampal parvalbumin-immunoreactive neurones (McKibben et al., 2010). However, in this model pharmacological intervention typically is given to the adult rat. Models that try to take into consideration the neurodevelopmental hypothesis also pose challenges: post-weaning isolation of pups produces relevant behavioural alterations such as hyperactivity in a novel arena, cognitive and sensorimotor gating impairments (Fone and Porkess, 2008, Marsden et al., 2011) and produces alteration in dopamine neurotransmission in the prefrontal cortex (Hall et al., 1998) and ventral tegmental area (Fabricius et al., 2010) as well as glutamatergic changes such as altered NMDA receptor expression in the prefrontal cortex which contributes to cognitive deficits (Melendez et al., 2004). However, resulting behavioural effects are highly sensitive and the magnitude of any behavioural change depends on precise isolation procedure used. Variation in the magnitude of change between cohorts of animals also constitutes a problem for replication, that can add doubt into experimental interpretation. Dual-hit models of schizophrenia have been attempted to produce more robust changes over single interventions to overcome this, such as combining neonatal PCP injection and subsequent post-weaning social isolation (Gaskin et al., 2014, Hamieh et al., 2021). This dual hit model showed in addition to hyperactivity and social cognition deficits, which was reversed by

clozapine (Hamieh et al., 2021), an increased impairment in prepulse inhibition (of translational relevance to sensorimotor gating deficits (Braff and Geyer, 1990)) and working memory deficits (Gaskin et al., 2014), suggesting a right step in the direction of producing more robust animal models for schizophrenia. For ASD, animal models lack construct validity as most originate from genetic manipulation of a single gene or chromosome region while the genetic causes of ASD are due to addition and accumulation of a variety of common and rare variants. This multi-factorial aetiology is hard to recapitulate in a single animal model considering each patient's genetic makeup is unique. Rather, the genetic manipulation in animals focuses on producing known neuronal alterations (for example aberrant synapse formation in SHANK3 mice which display social interaction deficits (Peça et al., 2011)).

The next question is whether the behavioural tasks employed in rodents are suitable readouts to be translated to the clinic. In this thesis, the acute PCP model was used as a short-term pharmacological model to assess the effect of intranasal OT on reversing the induced hyperactivity as an index of positive symptoms of schizophrenia. Construct validity in this model has been (rightly) questioned as it does not take into consideration the neurodevelopmental hypothesis and the acute PCP model do not produce as wide a range of cognitive impairments as the chronic PCP model (Jones et al., 2011). Face validity is also questioned: although acute PCP produces temporary hyperactivity, decreased social interaction and cognition impairments (Jentsch and Roth, 1999, Mouri et al., 2007), hyperactivity as an index for positive symptoms has been criticised (see Chapter 4.3, PCP model for schizophrenia) However, examination of the underlying neuronal mechanisms supports the dopaminergic hypothesis as isolation reared rats exhibit consistent increased activity in terms of horizontal activity and rears, which is reduced by both dopamine D_3 and D_2 receptor antagonists (which do not reduce locomotor activity in group housed control) as well as by antipsychotics such as haloperidol and risperidone (Jones et al., 2011). As such, considering it is impossible to confirm nor measure psychosis

in rodents, the isolation-induced locomotor hyperactivity remains the most useful positive symptom readout index with high predictive validity. Reduced social interaction (representing social withdrawal) and social cognition deficits are overlapping symptoms seen in both schizophrenia and ASD patients and laboratory rodents are used as a model for these behaviours due to their natural social nature displayed as 'huddling' behaviour and robust sociability towards members of their own group (although male mice have a tendency towards higher aggression and territorial behaviour (Kondrakiewicz et al., 2019)). Pharmacological and neurodevelopmental rodent models robustly produce social interaction deficits (Gururajan et al., 2010, Neill et al., 2010), and social interaction behaviour in humans and rodents show neurobiological similarities, such as involvement of the amygdala and prefrontal cortex, as well as alterations in neurotransmitter systems including the dopaminergic, GABAergic, serotonergic, glutamatergic and more recently, the oxytocinergic system (File and Seth, 2003, Ike et al., 2020, Wilson and Koenig, 2014). However, the social assays designed to test interaction are carried out in highly controlled environments and often within dyads (File and Seth, 2003, Silverman et al., 2010) that do not replicate the complex repertoire of human social interaction. Furthermore, considering that OT works to regulate salience of social cues, rather than increase general prosocial behaviour, we must stop and question to what extent the results seen in these assays are truly translational? Research groups have worked to advance these behavioural paradigms. For instance Anpilov et al. (2020) utilised optogenetics to study behaviour of mice in a group setting, in a social arena equipped with food, water and shelter, and show that activation of OT neurones increased prosocial behaviour initially, in agreement with literature (Calcagnoli et al., 2015, Huang et al., 2014), but increased aggressive behaviour on subsequent days. These are behaviours that would be missed upon acute manipulation and emphasises the importance of long-term observation, especially when human clinical trials often utilise chronic OT administration and patients would require long term treatment if OT was to be a medication option. Social behaviour is also a complex paradigm beyond the drive to seek

interaction and higher order cognition requires adaptive cognitive flexibility to recognise social situations and be able to attribute mental states beyond oneself. Social paradigms in rodents have been created to explore these domains such as social empathy (Panksepp and Lahvis, 2011), for example increased allogrooming of a cage mate exposed to stress (Lu et al., 2018) and to free cage mates from a restrained environment (Ben-Ami Bartal et al., 2011), even forgoing a food reward. Are these actions truly due to the ability to empathise and console another in an unfavourable state, or a stress response/drive of curiosity to explore, as social interaction triggers the reward processing system (rats have been shown to choose social interaction over drug self-administration (Venniro et al., 2018)). Thus, results of these social tasks should be approached with an open mind. Although the outcome seems to translate, the motivation driving these actions may be very different and thus differently impacted by OT.

2.5 Conclusion

In conclusion, although OT clearly plays an important role in social behaviour and cognition, its potential to be marketed as a therapeutic drug for neurodevelopmental disorders is far from being established. The social salience hypothesis of OT is an aspect that should be given more consideration for future studies, be it preclinical (for example designing more fluid social interaction tasks in a natural environment rather that a controlled rigid environment) or clinical (genetic screening of patients/establishing personality traits to predict more favourable outcomes). Trial designs should also be standardised and done to ensure there is sufficient power to detect changes (Quintana et al., 2021) as this is a long holding criticism of clinical trials. Lastly, we should move away from trying to create the ‘perfect’ model (a near impossible task for such highly heterogenous disorders), and rather, focus strictly on what we wish to examine, for example the effects of OT on social withdrawal specifically. Working backwards, we can use information from human neuroimaging/post-mortem studies to identify brain regions and neuronal

circuits involved, for example social withdrawal in humans involves changes in cortical top-down inhibition of the amygdala and altered mesolimbic reward system including reduced NAc activation (Gellner et al., 2021) and apply this understanding to animal models. PCP administration in rats produces social withdrawal and decreased neuronal cortical activation, increased c-fos activation in the anterior amygdala and reduced c-Fos in the VTA which projects to the NAc (Matricon et al., 2016), akin to changes in patients with schizophrenia and ASD where reduced prefrontal cortical activation is observed during social cues based tasks (Baas et al., 2008, Pinkham et al., 2008), and reduced ventral striatum activation in patients with schizophrenia in response to reward predicting cues (Juckel et al., 2006, Nielsen et al., 2012). Thus, by examining OT's effect on these brain regions in the PCP model for example, may better shed light on whether OT may be beneficial for social withdrawal symptoms and may identify the precise location for pharmacological manipulation.

Lastly, we emphasize here that the acute pharmacological manipulation of the rat in this thesis was mainly to assess OT's effect on an index of antipsychotic-like behaviour, rather than employing a full-blown model for schizophrenia to thoroughly examine OT on behavioural changes across all symptom categories. This is also why intranasal OT was administered to healthy rats to assess increased prosocial behaviour, rather than in an animal model for schizophrenia, which would be a suitable next step.

Chapter 3 *In vitro* assay characterisation of oxytocin-GET (OT-GET), a novel oxytocin peptide conjugate

3.1 Introduction

Intranasal delivery of OT, although presumed to bypass the BBB to achieve direct brain penetration, is also limited in the concentration that reaches the brain. It is thus of interest to investigate if this transport may be improved by conjugation with a novel cell penetrating peptide (GET). Before proceeding to *in vivo* studies, it is important to first characterise this conjugate peptide (OT-GET) in terms of its bioactivity, potential cytotoxicity and whether it is able to improve OT transduction across an *in vitro* cellular membrane.

3.1.1 Blood brain barrier (BBB)

The BBB is a semipermeable membrane formed by endothelial cells of the cerebral blood vessels that prevents solutes in blood entering the extracellular fluid of the CNS. It consists of tight junctions and highly selective transport proteins to tightly regulate entry of ions, molecules and cells into the CNS. The BBB is vital for regulating CNS homeostasis as well as preventing entry of potential neurotoxin and pathogens (Daneman and Prat, 2015). Only highly lipid soluble molecules less than 400Da are able to penetrate the BBB via passive diffusion (Pandey et al., 2016). Polar hydrophilic small molecules such as glucose and larger biomolecules such as insulin, are taken up via selective transporter systems and receptor mediated endocytosis respectively (Pardridge, 2012). However, OT is non-polar and has a molecular weight of approximately 1007Da, so is unlikely to cross the BBB to any extent by passive diffusion. The only evidence of possible OT transporters on the BBB are vascular RAGE, a member of the immunoglobulin superfamily of pattern recognition receptors (Yamamoto et al., 2019). The presence

of RAGE transporters could explain the observed central effects after peripheral administration of OT. In rodents, only approximately 0.002% of 5µg OT administered subcutaneously passed the BBB to reach the CNS after 10 minutes in rats (Mens et al., 1983) while Yamamoto et al, (2019) found an OT bioavailability of approximately 0.2% in the CSF after s.c. injection of 30ng in mice. These differences may be due to different treatment protocols or species. However, it is clear that only a very small amount of OT crosses the BBB.

As mentioned in Chapter 1, OTRs are expressed in both CNS and the peripheral system, such as the mammary tissue and uterus (Hoover, 1971), gastrointestinal tract (Ludwig and Leng, 2006) as well as the heart (Gutkowska et al., 2014). As such, the concurrent rise in plasma OT after peripheral administration (Neumann et al., 2013, Striepens et al., 2013, Smith et al., 2019a) may elicit off-target effects, affecting regulation of cardiovascular and gastrointestinal function (Gutkowska et al., 2014, Welch et al., 2014). Although vital under normal circumstances, the presence of higher than usual OT concentrations may affect these functions negatively, which in turn may cause compliance difficulties in an already vulnerable group that display socio-behaviour deficits. On the other hand, high doses of OT injected directly into the brains of rats decreased core body temperature and locomotor activity (Angioni et al., 2016, Uvnas-Moberg et al., 1992a) suggesting a possible 'sedative-like' effect. Intranasal delivery of OT, an attractive alternative as it bypasses the BBB and allows for direct delivery to the brain, may help decrease peripheral side-effects. However, optimal drug doses would have to be determined to minimise the other central side-effects mentioned as the primary goal of OT treatment in this case is to improve social behaviour and cognition.

Intranasal delivery also poses its own problems. Peptides with molecular weight 1kDa and above are poorly absorbed across the nasal epithelium (Fisher et al., 1987, McMartin et al., 1987). Bioavailability of OT after intranasal delivery are estimated to be low: in rats, Tanaka et al. (2018) found a low nasal bioavailability of 1.41% upon a 500µg intranasal dose.

Many peptides are also susceptible to metabolic degradation in the nasal mucosa and have a short retainment time on the mucosal surface for permeation before mucociliary clearance (Al Bakri et al., 2018). Some approaches around these problems include chemical modification or formulation changes to stabilise and improve transmembrane permeability. However, this may alter biological activity of the original peptide and will require extra toxicological and pharmacological studies. In contrast, an alternative method such as the utilisation of a carrier peptide may be able to enhance OT permeation, and reduce degradation without loss of bioactivity while also minimising peripheral side-effects by reducing the dose required to cross the BBB and achieve optimal brain penetration. Many nanocarrier-based strategies have been studied to improve intranasal delivery. These include liposomes, exosomes, nanoparticles modified to increase drug residence time in the nasal cavity, lectin and lactoferrin modified nanoparticles to improve cell interaction via binding to existing receptors and cell penetrating peptides to enhance translocation (for full review see Fan et al. (2018)).

3.1.2 Oxytocin-GET (OT-GET)

An ideal drug carrier should be efficacious but pharmacologically inert at the concentration used, as well as non-toxic or irritant. The conjugation between carrier and transport protein should be transient or reversible and if co-transported across the membrane with the drug, must be stable or the resulting metabolites should be non-toxic. As such, any carrier peptide needs to be tested rigorously before advancing into clinical trials, with a balance struck between its absorption promoting ability and any toxic effect. CPPs, which are short peptide sequences that aid in efficient receptor-independent translocation of cargoes into cells (Raucher and Ryu, 2015) are a popular avenue in translational research. They enhance drug delivery in hard to access cell or tissue compartments and have low cytotoxicity (Guidotti et al., 2017). CPPs traditionally penetrate membranes via transient pore formations or membrane destabilisation (Layek et al., 2015). The mechanism of how the resulting endosome releases its cargo however remains to be determined. An important class

are arginine rich CPPs, whose most prominent peptides include transactivator of transcription (TAT)-derived peptides (Park et al., 2002, Vives et al., 1997) and polyarginines. Cationic amino acids are important for cellular uptake, with arginine residues critical for successful transduction (Tunnemann et al., 2008, Wender et al., 2000). However, endocytosis triggered by CPPs mediated protein delivery (Wadia et al., 2004) restricts localisation and activity of the endosome contained cargo protein. Furthermore, a high *in vivo* extracellular concentration of CPP protein is required in order for bioactive protein to escape the endosome, enter the cytosol, and elicit the intended effect at the required target (Guidotti et al., 2017).

The GAG-binding enhanced transduction (GET) peptide is a novel CPP (P21-LK15-8R) developed by the Dixon laboratory (Centre for Biomolecular Sciences, University of Nottingham). The addition of P21, a short 21-amino acid heparin-binding domain sequence from heparin binding epidermal growth factor that interacts with cell membrane heparan sulphates, to the polyarginine (8R) CPP helps stimulate cell interactions (Dixon et al., 2016). This modification allows for more efficient small molecule transduction and thus higher number of peptides available for endosomal release. GET-mediated transduction utilises lipid-raft mediated endocytosis, more specifically, micropinocytosis as NIH3t3 mouse embryonic cells pre-treated with micropinocytosis disruptors such as amiloride and cytochalasin D, resulted in a dose-dependent reduction in cellular transduction, measured via successful GET-Cre recombinase activity in enhanced green fluorescent protein (eGFP) expressing NIH3t3cells (Dixon et al., 2016). Although specific experiments were not carried out by Dixon et al. (2016) to investigate the type of interaction between GET and its conjugated peptides, the finding that heparin is able to strongly inhibit transduction and cell-surface binding of GET *in vitro,* suggests that soluble GAGs act as competitive molecules for P21 and a reversible noncovalent molecular interaction is likely to exist between GET and corresponding peptide. Electrostatic

interactions are a possible interaction, bearing in mind that heparin is highly negative and P21 highly positive.

Conjugation of GET to nucleic acids (plasmid DNA, modified nucleotide mRNA and small inhibitory RNAs modified to express green fluorescence protein upon transfection) demonstrated significant transfection activity of human mesenchymal cells, visualised via fluorescence microscopy of GFP expression (Dixon et al., 2016). This GET transfection efficiency was compared to that of Lipofectamine 2000 (a commercial standard transfection reagent) using flow cytometry, and transfection activity was retained even when Lipofectamine 2000 was inhibited. GET of transcription factor NANOG (Chambers et al., 2003) was able to promote self-renewal of mouse embryonic stem cells (ESCs) CGR-8Z, in the absence of leukaemia inhibitory factor (which stimulates mouse ESCs self-renewal (Nicola and Babon., 2015), measured via changes in pluripotency-associated alkaline phosphatase activity and levels of cell proliferation. GET of MYOD myogenic factor (Bichsel et al., 2013) on the other hand, successfully drove human ESCs HUES7 myogenic differentiation, measured via immunolabelling of multinucleated myogenin-positive myotubes and expression of endogenous MYOD and skeletal muscle-specific ACTA1 using quantitative polymerase chain reaction.

These apparent appropriate factors justified selection of GET to attempt to produce a conjugate with OT that would enhance OT penetration across the BBB. Before attempting to use OT-GET *in vivo* in rats, it was important to assess the pharmacodynamics of OT-GET *in vitro*. As such, suitable assays and *in vitro* models were established as described in this Chapter to examine OT-GET's pharmacodynamics, including its bioactivity, cytotoxicity and ability to improve OT delivery across an epithelial barrier. It is to be noted that this is the first example of using GET to enhance peptide delivery across a nasal epithelial cell monolayer and subsequently into the brain (Chapter 3).

3.1.3 Aims of Chapter

This chapter aims to:

1. Validate use of a calcium fluorimetry assay to assess the bioactivity of OT and OT-GET
2. Validate use of the human nasal epithelium cell line, RPMI 2650, to investigate rate of OT transduction across a cell monolayer with or without GET and any toxicity of OT-GET on membrane integrity

3.2 Materials and methods

3.2.1 Cell culture

In order to achieve an OT bioassay, three cell lines were used for assay optimisation: Human embryonic kidney 293T cells (HEK293T; ATCC, UK) and breast cancer cell lines MCF-7 (generously donated by Professor Nigel Mongan; The University of Nottingham) and Hs 578t (University of Nottingham Cancer Research Nottingham (CRH) NCI-60 cell line facility).

HEK 293 cells are widely used for transfection protocols, as they are robust, easily maintained, amenable to transfection and possess post-translational modification machineries to generate functional proteins (Thomas and Smart, 2005). HEK 293T cells are a derivation of the HEK 293 cell line, which express a large SV40 T antigen (DuBridge et al., 1987). The expression of the T antigen is important to replicate plasmids containing SV40 to a higher copy number in the transfected cell than its parental HEK293 cell line (Pear et al., 1993) and thus used for transient expression of OTRs in the present study. MCF-7 and Hs 578t cells were used as they endogenously express OTRs (Amico et al., 2002, Cassoni et al., 2006, Copland et al., 1999, Schiffmann and Gimpl, 2018), thus likely to provide more reproducible responses across replicates in comparison to transfected HEK293T cells, which may vary in OTR expression in each batch. In this case, both breast cancer cells line was used to compare biological responses to OT and the cell line with the most robust response was brought forward for further studies. For OT permeability studies, the human nasal epithelium cell line RPMI 2650 was chosen as it is the only immortalised human nasal cell line available for studying drug transportation (Bai et al., 2008).

HEK 293T were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich, UK) + 10% heat-inactivated Fetal Calf Serum (FCS; Sigma Aldrich, UK). MCF-7 were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (Sigma Aldrich, UK) + 10% heat-inactivated FCS. Hs 578T were cultured in DMEM + 10% heat-inactivated

FCS containing 0.1% bovine insulin (Sigma-Aldrich, UK). RPMI 2650 cells (ATCC, UK) in culture flasks and transwell filter inserts were cultured in Eagle's Minimum Essential Media (EMEM; ATCC, UK) supplemented with 10% non-heat inactivated FCS (ATCC, UK) + 1% penicillin/streptomysin antibiotics (Invitrogen, UK). Cells were maintained at 37^{o}C in a 5% humidified CO_2 incubator (Thermo Scientific, U.K.) and split with 0.25% trypsin EDTA (Invitrogen, UK) when a confluence of 80% - 90% was attained.

3.2.2 Generation of a transient oxytocin receptor expressing HEK293T cell line

OTR expressing pCDNA3.1 (2µL, Wheatley laboratory, University of Birmingham) was added to 50µL of Top 10 competent cells (Invitrogen, UK), incubated on ice for 20 minutes and heat shock at 42^{o}C for 2 min. Cells were spread on Luria-Bertani (LB) agar plates with 0.1mg/mL ampicillin and grown overnight at room temperature. A single cell colony was picked the following day, transferred into a flask containing 3mL LB media + 3µL 0.1mg/mL ampicillin and incubated at 37^{o}C in a shaker overnight. Purification and elution of resulting DNA was carried out using a Plasmid Midi Kit (Qiagen, UK) according to manufacturer's instructions. Briefly, harvested cells were centrifuged (6000g, 15 minutes, 4°C) and bacterial pellet resuspended in buffer before lysing in LyseBlue reagent. Resulting solution was centrifuged twice (6000g, 15 minutes, 4°C), to obtain supernatant containing the plasmid DNA. DNA lysate was washed using a vacuum manifold before washing with elution buffer to obtain a pure DNA yield. Resulting DNA was measured using a nanodrop machine (NanoDrop™ 2000/2000c Spectrophotometers, Thermo Fisher, UK). This was thereafter used as the stock OTR source (890ng/µL).

HEK 293T cells were transfected at 60-70% confluency with 3µg/mL polyethylenimine (Thermo Fisher, UK) and 1µg/mL stock OTR DNA made up in optiMEM 1X Reduced Serum Medium (Thermo Fisher, UK). Total volume of 250µL or 500µL of optiMEM + PEI mix was added to a suspension of 2.4 million cells in 6mL of cell growth medium in T75cm^2

flasks. Cells were incubated at 37°C in a 5% CO^2 humidified incubator (Thermo Scientific, U.K.)

3.2.3 Calcium fluorimetry

OTRs are G_q coupled receptors and upon receptor activation, release intracellular [Ca^{2+}] as explained in Chapter 1. As such, a calcium fluorimetry assay was developed to investigate bioactivity of OT and OT-GET.

A common drawback of calcium flux assays is the possible production of artefacts due to interference of the fluorescent compounds. However, the assay kit used in this study: Fluo-4 NW Calcium Assay Kit (Thermo Fisher Scientific, UK) uses Fluo-4 as its fluorescent indicator, which is non-fluorescent (until mobilised) and remains stable in pH7-7.5 buffer for hours, thus ruling out spontaneous conversion to fluorescent signals and any background fluorescence.

3.2.3.1 Assay development

OT induced calcium responses were compared in OTR transfected HEK 293T, MCF-7 and Hs 578t cells to determine which cell line would be the most suitable to be used for subsequent investigation of OT-GET bioactivity.

HEK 293T and MCF-7 cells were plated into a poly-D lysine coated (10µg/mL) 96 well black walled plate at 50,000 cells per well and Hs 578t cells were plated in poly-D lysine coated and uncoated 96 well black walled plate at 80,000 cells per well 24h prior calcium fluorimetry. Poly-D-lysine aims to promote cell adhesion to the 96 well polystyrene plate. Changes in intracellular calcium [$Ca^{2+}]_i$ were measured using the Fluo-4 NW Calcium Assay Kit (Thermo Fisher Scientific, UK). On the day of experiment, cell medium in 96 well black plate was aspirated and 100µL of 1X dye loading solution (Fluo-4 NW mix + assay buffer (1X Hanks Buffered Salt Solution (HBSS; Sigma Aldrich, UK) + 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma Aldrich, UK) solution) + 2.5mM probenecid solution) added per well. In studies investigating OTR and AVP receptor antagonism effects, 0.01mM –

0.1µM OTR antagonist L,368-899 (chemical name: (2S)-2-Amino-N-[(1S,2S,4R)-7,7-dimethyl-1-[[[4-(2-methylphenyl)-1piperazinyl]sulfonyl]methyl]bicyclo[2.2.1]hept-2-yl]-4-(methylsulfonyl)butanamide; Tocris, UK), 0.1µM V_{1a} receptor antagonist SR49059 (chemical name: (2S)-1-[[(2R,3S)-5-Chloro-3-(2-chlorophenyl)-1-[(3,4dimethoxyphenyl)sulfonyl]-2,3-dihydro-3-hydroxy-1H-indol-2-yl]carbonyl]-2-pyrrolidinecarboxamide; Sigma Aldrich, UK) and 1nM V_2R antagonist tolvapton (chemical name: (N-(4-(7-Chloro-5-hydroxy-2,3,4,5-tetrahydro-1H-benzo[b]azepine-1-carbonyl)-3-methylphenyl)-2-methylbenzamide; Sigma Aldrich, UK) were diluted in 5% DMSO (Sigma Aldrich, UK) and 1X dye loading solution and added at 100µL per well in 96 well black plate containing Hs 578t cells. These antagonists were chosen for their high receptor selectivity (Pettibone et al., 1993, Serradeil-Le Gal et al., 1993) as well as optimal brain penetrance for usage in subsequent *in vivo* studies (Hicks et al., 2016, Tribollet et al., 1999). Antagonists concentration was chosen based on literature, although application were for use in different cell lines, for example cardiomyocytes for SR 49059, as there are none recorded for the use in Hs 578t cells in current literature (Wasilewski et al., 2016, Xiao et al., 2018, Zhu et al., 2013).

Ninety-six well black plates containing cells were incubated at 37^{o}C for 40 minutes. Mean fluorescence (λex = 485 nm, λem = 525 nm) across each well was measured using a Flexstation 3 (Molecular Devices, Wokingham UK). Upon laser excitation at λex = 485 nm, Fluo-4, which binds calcium, produces an emission of λem= 525 nm, whose intensity is directly proportional to the concentration of free [Ca^{2+}]. Fluorescent reading was made every 1.6s for 90s. Agonists carbachol (1mM – 0.032µM) or OT (1mM – 1pM) was added at 16s. Carbachol was used as a positive control in HEK 293T cells as they express muscarinic receptors (Rumenapp et al., 2001) and enables successful transfection of OTR to be evaluated. All agonists were made up in assay buffer. Fluorescence readings were measured in a minimum of duplicates and values expressed in fluorescent maximum-minimum (FRU).

3.2.3.2 Bioactivity of OT-GET

Hs 578t cells were plated at 80,000 cells per well in uncoated 96 black walled plates 24h prior calcium fluorimetry. The same assay kit, temperature and incubation time as described in Section 2.2.3.1 was used. Agonists GET (0.001µM - 10 µM) or OT-GET 1 – 5 (0.1µM OT conjugated with 0.001µM - 10 µM GET, increasing 10 fold each and made fresh prior each experiment by mixing thoroughly in an eppendorf) with or without presence of 10µg/mL heparin were made up in assay buffer. A sub-maximal concentration of 0.1µM oxytocin was chosen as it is closest to the EC_{50} value observed in Hs 578t OT dose-response curves, so that a change in either direction in intracellular $[Ca^{2+}]_i$ transients induced by OT-GET could be observed. Heparin was added to unconjugate the peptide complex. 0.1µM OT and assay buffer were present in each plate to act as a positive and negative control respectively. Fluorescence readings were measured in triplicates and values expressed in fluorescent maximum-minimum (FRU).

3.2.4 RPMI 2650 permeation model

RPMI 2650 has been shown to grow, differentiate and express transepithelial electrical resistance range (TEER) as well as tight junction and transporter proteins similar to human nasal mucosa (Mercier et al., 2018b) when cultivation is carried out under air-liquid conditions. Thus, this study compared the barrier properties of RPMI 2650 cells, namely TEER and permeability value of a standard compound 4.4kDa TRITC-dextran (TD4.4), when grown under traditional liquid covered culture (LCC) or recommended air-liquid culture interface (ALI). The most suitable cultivation method was used for subsequent experiments with OT and OT-GET permeation.

3.2.4.1 Cell culture

RPMI 2650 cells were grown on Transwell® polyester filter inserts (12mm diameter, 0.4µm pore size) at 200,000 cells/1.12cm^2 in a tissue culture treated 12 well plate (Corning Costar, USA) with 0.5mL and 1mL EMEM media in the apical and basolateral chamber respectively. Seeding density and size/type of filter inserts used were chosen based on

literature as stable TEER values in reproducible range after 21 days was observed (Wengst and Reichl, 2010). Cells were shifted to ALI by removing media in the apical chamber or retained in liquid-covered culture LCC on Day 8 until Day 20-21 for permeability or cytotoxicity testing.

3.2.4.2 Transepithelial electrical resistance measure (TEER)

TEER is a quantitative technique used to measure tight junction integrity of a cellular barrier (Srinivasan et al., 2015). Briefly, the voltohmmeter applies an AC electric signal to both sides of the cellular monolayer via electrodes and the resulting resistance (ohm, Ω) calculated based on Ohm's law is displayed. TEER values of RPMI 2650 cells cultured on filter inserts were measured every 2-4 days using the EVOM2TM epithelial voltohmeter (World Precision Instruments, Sarasota, Florida, USA). When conducting TEER readings, apical and basolateral chambers were replaced with 0.5mL and 1mL fresh EMEM and values taken after 5 minutes. Blank filter values were deducted from readings. Only cultures with TEER values above 60Ω/cm^2 on Day 20-21 were used for permeability assays. This was to mimic TEER values of human nasal mucosa which ranges from 60 to 180 Ω/cm^2 (Mercier et al., 2018b).

3.2.4.3 Permeation of 4.4kDa TRITC-dextran

TRITC-dextrans are standard compounds primarily used in permeability studies as a high molecular weight paracellular marker. 4.4kDa size was chosen, as it is the closest to that of OT (1kDa) and OT-GET (6kDa). As ALI culture conditions have been shown to produce a more robust RPMI 2650 model compared to LLC cultivation (Mercier et al., 2018b), transepithelial permeability of TD4.4 as a high molecular weight paracellular marker was compared under both cultivation conditions.

On the day of the experiment, RPMI 2650 cells were incubated with fresh EMEM (0.5mL apical, 1.5mL basolateral) for 60 minutes. Media was removed from apical chamber and 500mL of 0.5mg/mL TD4.4 (made up in EMEM) added. A separate plate with empty filter inserts was run in parallel as a control to ensure filter inserts were not limiting the rate of

TD4.4 permeation. Cells were kept at 37°C in a 5% humidified CO^2 incubator throughout the experiment. 100 µL samples were drawn in triplicates, from the basolateral chamber at 1, 2, 4 and 6h (to show the time course of transfer) and filter inserts transferred to a new well containing fresh 1.5mL EMEM in the basolateral chamber each time. TD4.4 in apical chamber remained untouched. Permeability of TD4.4 to the basolateral chamber was determined using a fluorescent plate reader, λex = 550nm, λem = 580nm at 25°C. Values were expressed in apparent permeability coefficient (P_{app}) which quantifies the rate at which a molecule crosses a membrane and was calculated according to equation below (Gao et al., 2001):

$$P_{app} = \frac{dQ}{dt \cdot C_o \cdot A}$$

where dQ/dt = flux of compound across barrier (mmol/sec), C_o = initial concentration of compound in apical chamber (mmol/cm^3) and A = surface area of the cell monolayer (cm^2).

3.2.4.4 Permeation of OT/OT-GET

RPMI 2650 cells grown under ALI conditions were used for the OT/OT-GET permeation study. On the day of experiment, RPMI 2650 cells were incubated with fresh serum free EMEM media (0.5mL apical, 1.5mL basolateral) for 60 minutes. All drugs were made up in serum free EMEM so that final concentration seen by Hs 578t cells during calcium fluorimetry was 0.1µM OT and OT-GET 3 – 5 (0.1µM OT conjugated with 0.1µM - 10 µM GET, increasing 10 fold each) to match protocol mentioned in Section 3.2.3.2. OT-GET 3 to 5 were chosen for permeability testing as they presented sufficient conjugation with OT and reversibility upon heparin addition, in the bioactivity study.

Media was removed from apical chamber after 60 minutes and 0.5mL of OT or OT-GET 3 – 5 added. A separate plate with empty filter inserts was run in parallel, as a control. Cells were kept at 37°C in a 5% humidified CO2 incubator throughout the experiment. 100 µL samples were drawn in triplicates, from the basolateral chamber at 1, 4 and 6h and filter inserts

transferred to a new well containing fresh 1.5mL EMEM in the basolateral chamber each time. Media in apical chamber remained untouched. TEER values were measured at each time point to determine the cell monolayer integrity. Collected samples were kept on ice until the last time point, where calcium fluorimetry was carried out immediately according to Section 3.2.4.2.

Collected samples were added to Hs 578t cells (plated and prepped as described in Section 3.2.3.1) with or without 10µg/mL heparin. Heparin was added to enable unconjugation of OT-GET as it was unknown as to whether OT-GET would permeate the cellular barrier as a complex or dissociate to release free OT and enable subsequent OTR activation. Figure 3.1 explains the overall protocol used.

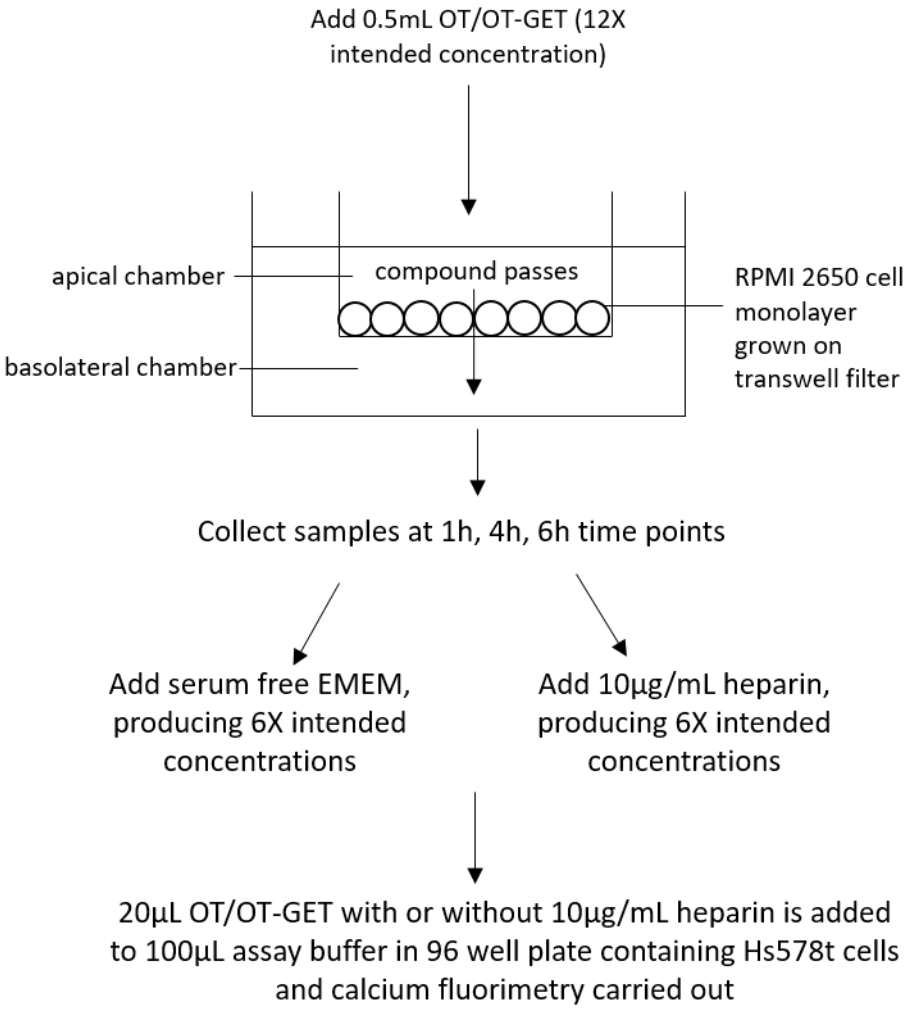


Figure 3.1 Protocol for OT/OT-GET permeability study in RPMI 2650 cells.

Resulting fluorescence reading (λex = 485 nm, λem = 525 nm) was conducted in triplicates and the fluorescence maximum-minimum (FRU) values were used to calculate free OT concentration with reference to a standard curve (0.01mM – 0.1nM OT made up in serum free EMEM). Derived results were expressed in FRU, OT P_{app} and percentage of maximum cumulative OT concentration (maximum being the value from 0.1µM OT control plate). Cumulative values were derived from adding each time point's concentration to the total concentration derived from previous time points.

3.2.4.5 PrestoBlue cell viability assay

In order to investigate potential OT-GET cytotoxicity effects on RPMI 2650 membrane integrity, the PrestoBlue® Cell Viability Reagent (Thermo Fisher, UK) was used. Viable cells, which remain metabolically active will convert rezarufin in the reagent to fluorescent rezazurin, whose fluorescence intensity is directly proportional to the number of viable cells.

RPMI 2650 cells were grown as described in Section 3.2.4.1. On day 8, cells were switched to ALI cultivation to ensure same growth conditions as the permeation study was achieved. On day 20, RPMI 2650 cells were incubated with fresh EMEM media (0.5mL apical, 1.5mL basolateral) for 60 minutes to achieve equilibration. Media was then replaced with 0.5mL of OT and OT-GET 3 – 5 (in this case, at 12x final concentration to match that seen by RPMI 2650 during permeability assay, e.g. 0.12µM OT + 0.12µM GET for OT-GET 3; all concentrations made up in EMEM). After a 6h incubation at 37ºC in a 5% humidified CO^2 incubator, OT/OT-GET was removed from apical chamber and replaced with 1x PrestoBlue™ Cell Viability Reagent (Thermo Fisher, UK) which was diluted in HBSS. Cells were further incubated for 30 minutes and 100µL samples from the apical chamber were pipetted into a 96 well black walled plate in triplicates. Fluorescence was measured at λ_{ex} 560nm, λ_{em} 590nm on a

fluorescence plate reader and results expressed as percentage of control.

3.2.5 Statistical Analysis

Statistical analysis were conducted using GraphPad Prism 7 and differences between groups assessed using unpaired t-tests, one-way or two-way analyses of variance (ANOVA) with Tukey or Dunnett's post-hoc where applicable. Data were assessed for normality using Shapiro-Wilk normality test. All test carried out were two-sided, results presented as mean ± SEM with an alpha criterion of $p<0.05$.

3.3 Results

3.3.1 Hs 578t as the most suitable cell line for calcium fluorimetry assay

OTR transient transfected HEK 293T cells as well as breast tumour cell lines MCF-7 and Hs 578t were tested for free $[Ca^{2+}]_i$ transient responses.

OTR transient transfected HEK 293T cells showed a robust Ca^{2+} response towards the positive control, carbachol (0.032µM – 1mM; Figure 3.2A) but not OT (1pM – 1mM); Figure 3.2B), suggesting that functional OTR incorporation had not occurred.

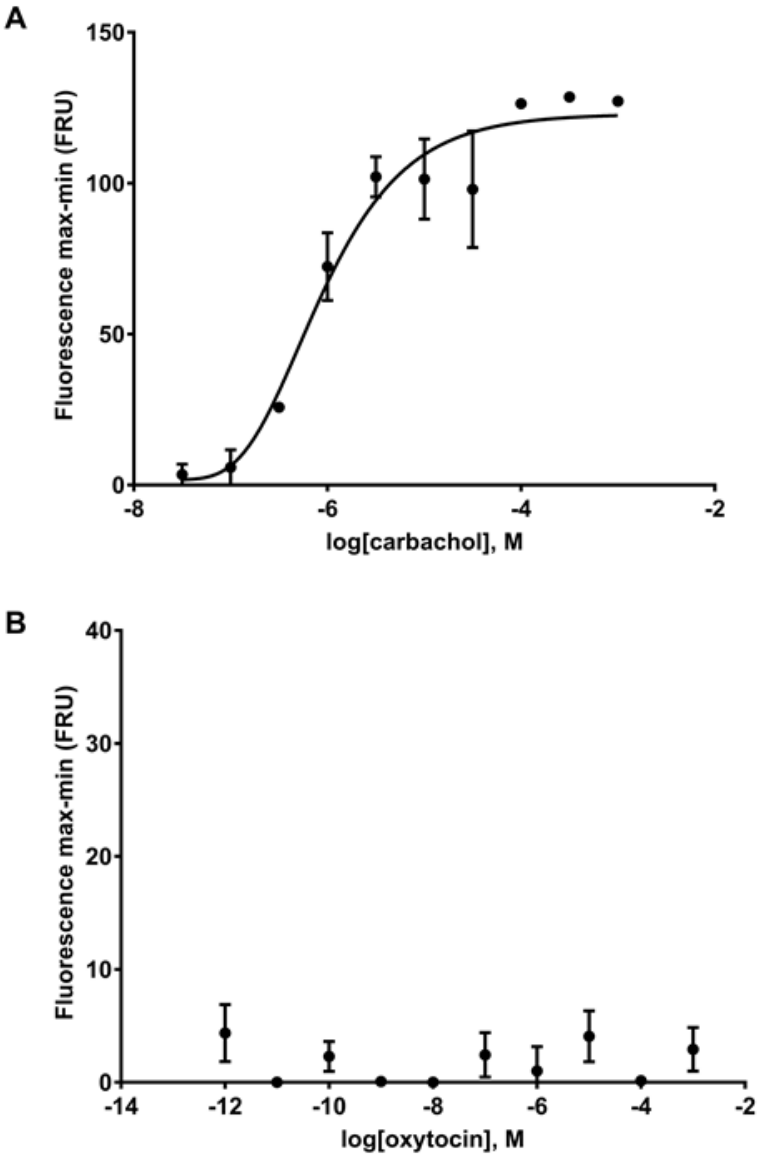


Figure 3.2 Free $[Ca^{2+}]_i$ transient dose-response curves in OTR transient transfected HEK 293T cells.

Dose-response in OTR transient transfected HEK 293T cells to (A) 0.032µM – 1mM carbachol and (B) 1pM – 1mM OT were recorded using calcium fluorimetry. Results are mean ± SEM of triplicate values, n =1. Values with no error bars displayed were due to removal of negative fluorescence values, resulting in single values**.**

MCF-7 cells grown in poly-D-lysine coated black plates showed only a weak Ca^{2+} response to increasing concentration of OT (3.16nM – 0.1mM; Figure 3.3).

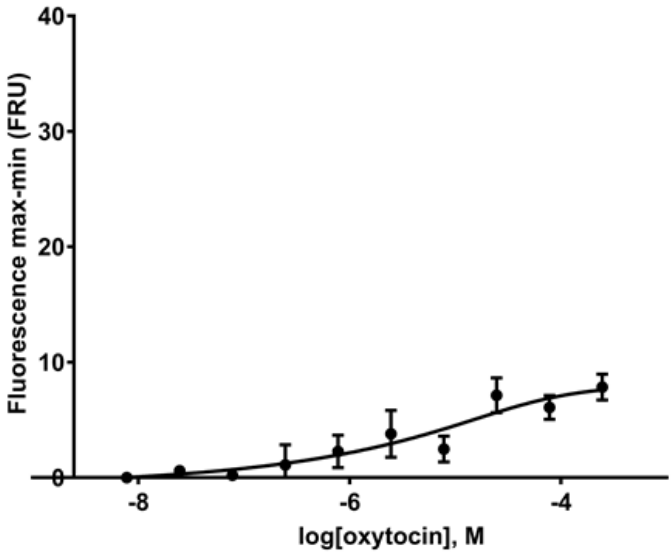


Figure 3.3 Free $[Ca^{2+}]_i$ transients dose-response curves in MCF-7 cells. Dose response to 3.16nM – 0.1mM OT in MCF-7 cells were recorded using calcium fluorimetry. Results are mean ± SEM values, n=2.

Hs 578t cells in poly-D-lysine coated plates produced a shallow Ca^{2+} activation in response to OT (0.32nM - 10µM), such that the maximal response was low at an average FRU value of 26. This maximal response was increased to 112 FRU, EC_{50} value 0.26± 1.25 µM when cells were grown in uncoated plates (Figure 3.4).

Unlike with HEK 293T cells, where carbachol was used as a positive control for successful OTR transfection, this was not carried out with MCF 7 or Hs 578t cells as they already endogenously express OTR. Instead, subsequent experimentations with selective OT and AVP receptor antagonists to establish the role of OTRs in inducing $[Ca^{2+}]_i$ transients was carried out, using Hs 578t cells grown in uncoated 96 well plates.

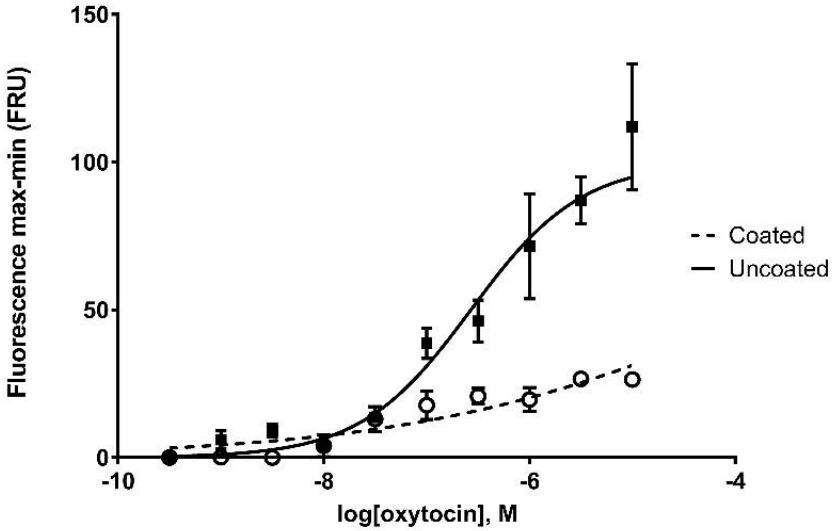


Figure 3.4 Free $[Ca^{2+}]_i$ transients dose-response curves in Hs 578t cells. Cells were plated in 10µg/mL poly-D-lysine coated (n=2) or uncoated 96 well black plates (n=4) and dose-response to 0.32nM - 10µM OT was recorded using calcium fluorimetry. Results are mean ± SEM values.

As OT has high affinity for AVP receptors (both V_{1a} or V_2) as well as OTR, the OT response seen in Hs 578t cells was repeated in the presence of OT or of either the OT, V_{1a} or V_2 receptor antagonists, namely L,368 -899 (0.01mM – 0.1µM), SR49059 (0.1µM) and tolvapton (1nM) respectively.

A parallel rightward shift of the OT dose-response curve was observed in the presence of 0.1µM - 10µM OTR antagonist, L-368,899 (Figure 3.5A) with an apparent progressive decrease in max. Higher OT concentrations were not performed to see if this was a real finding as the main aim was to establish OTRs were responsible for producing the Ca^{2+} response. As there was a clear rightward shift of each OT dose-response in response to increasing OTR antagonist dose in contrast to addition of 0.1µM V_{1a} antagonist SR49059 or 1nM V_2 receptor antagonist tolvapton which had no effect on the OT-induced Ca^{2+} fluorescent response (Figure 3.5B), this clearly showed only OTR and not AVP receptors were involved in the response.

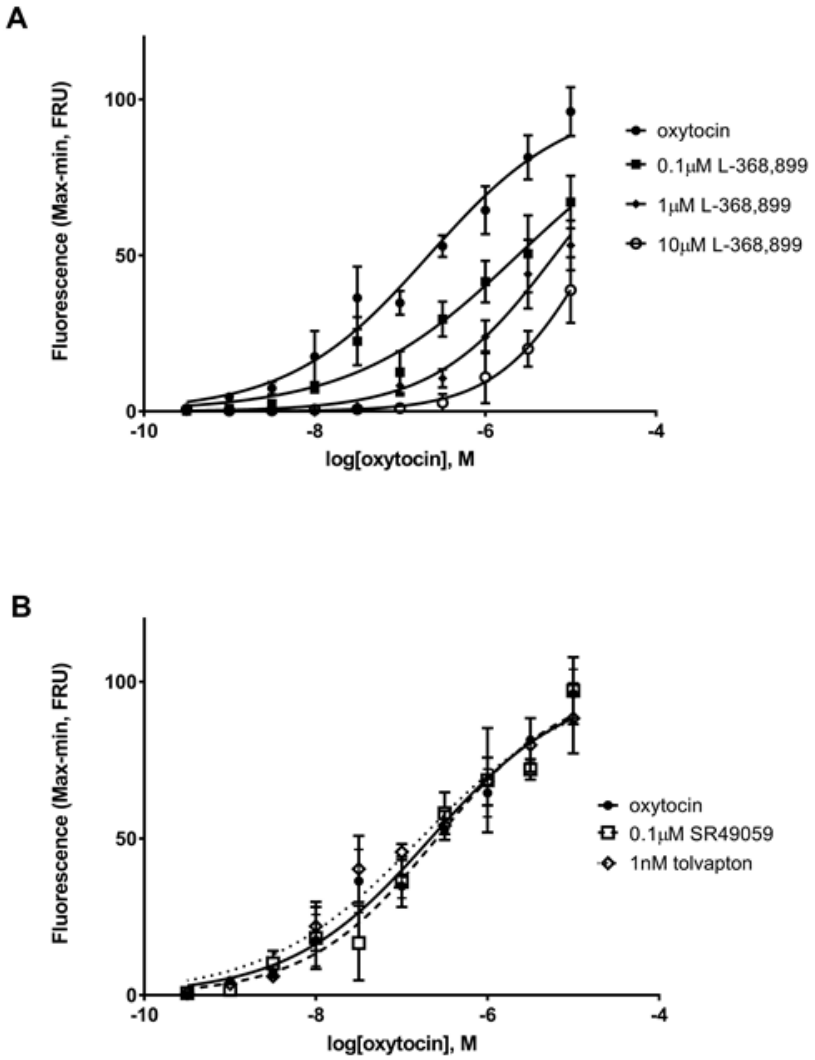


Figure 3.5 Free $[Ca^{2+}]_i$ transients dose-response curves in presence of OTR and AVP receptor antagonists in Hs 578t cells.

Dose-response to 0.32nM - 10μM OT in the absence and presence of (A) 0.1μM - 10μM OTR antagonist L-368,899 (B) 0.1μM V_{1a} receptor antagonist SR49059 and 1nM V_2R antagonist tolvapton in Hs 578t cells was recorded using calcium fluorimetry. Results are mean ± SEM values, n=3.

3.3.2 Bioactivity of OT-GET

In order to determine the bioactivity of OT following use of the OT-GET conjugate, free $[Ca^{2+}]_i$ transients elicited by agonists OT and OT-GET agonists was carried out in Hs 578t cells using calcium fluorimetry. OT/OT-GET stimulated free $[Ca^{2+}]_i$ transients induced by free OT in Hs 578t cells ($F_{(5,26)}$= 27.93, p=0.0001; one-way ANOVA; Figure 3.6) however OT-GET 2 ($p<0.01$) and OT-GET 3 to 5 ($p<0.0001$) caused

significant reduction of OT induced $[Ca^{2+}]_i$ transients compared to 0.1µM OT alone. OT induced $[Ca^{2+}]_i$ transients was also significantly reduced in OT GET 3 -5 compared to OT-GET 1 ($p<0.001$) and OT-GET 2 ($p<0.05$ or $p<0.01$).

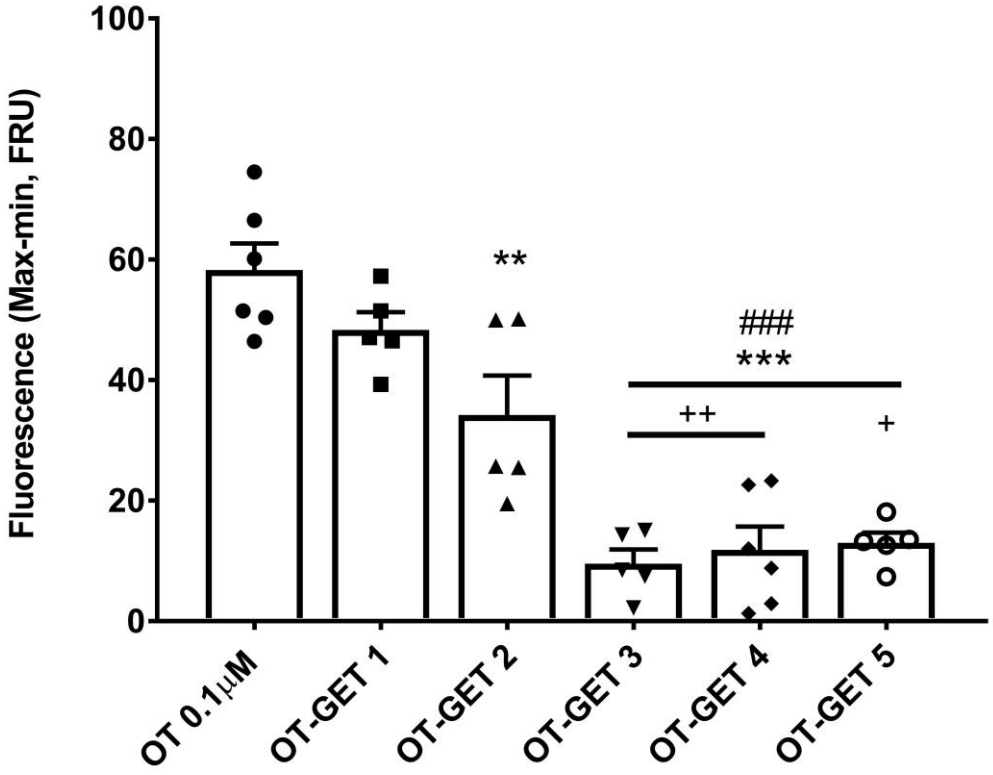


Figure 3.6 Bioactivity of OT-GET.

Free $[Ca^{2+}]_i$ transients in response to 0.1µM OT and OT-GET in Hs 578t cells was recorded using calcium fluorimetry. One-way ANOVA confirmed a significant main effect of OT/OT-GET treatment on free OT induced $[Ca^{2+}]_i$ transients ($F_{(5,21)}=0.2635$, $p=0.0001$). Figure shows individual points and mean ± SEM values, n=5-6; ** ($p<0.01$), *** ($p<0.001$) vs 0.1µM OT; ### ($p<0.001$) vs OT-GET 1; + ($p<0.05$) ++ ($p<0.01$) vs OT-GET 2; Tukey's post-hoc.

GET alone significantly reduced $[Ca^{2+}]_i$ transients ($F_{(5,6)}=374.1$, $p<0.0001$; one-way ANOVA; Figure 3.7) in Hs 578t cells. As none of the concentrations stimulated free $[Ca^{2+}]_i$ transients ($p<0.001$), this indicated responses observed previously was elicited by free OT and not the carrier protein.

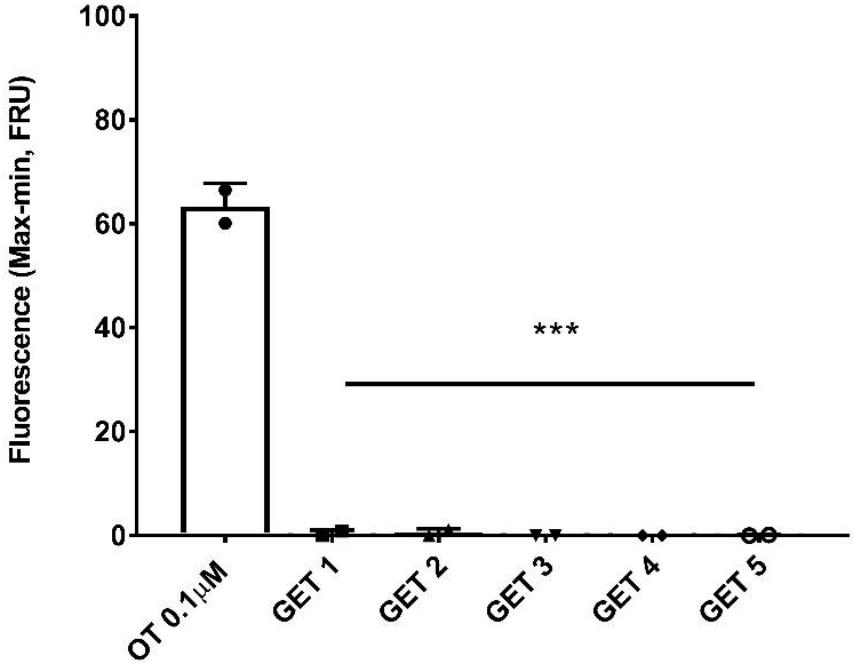


Figure 3.7 Bioactivity of GET.

Free $[Ca^{2+}]_i$ transients in response to 0.1µM OT and GET in Hs 578t cells was recorded using calcium fluorimetry. One-way ANOVA confirmed a significant main effect of treatment ($F_{(5,6)}$=374.1, p<0.0001); Results are individual points and mean ± SEM values, n=2; *** (p<0.001) vs 0.1µM OT; Tukey's post-hoc.

In vivo, the OT-GET conjugate is expected to dissociate after transversing the nasal epithelium, allowing OT to act on its receptors in the brain. 10µg/mL heparin was added to the conjugate to dissociate the OT-GET conjugate as the P21 domain in GET binds to heparin at a higher affinity than it would to OT (Dixon et al., 2016), to mimic this condition.

The presence of heparin ($F_{(5, 36)}$ = 4.886, p=0.0016) and OT/OT-GET treatment ($F_{(1, 36)}$ = 40.66, p=0.0001) significantly affected $[Ca^{2+}]_i$ transients responses in Hs 578t cells (Figure 3.8). A main effect of heparin x OT/OT-GET treatment was also observed ($F_{(5,36)}$=5.42, p=0.0008). In the absence of heparin, $[Ca^{2+}]_i$ transients induced by OT-GET 3 to 5 were significantly lower than 0.1µM OT (p<0.05 or p<0.01) as well as to OT-GET 3 to 5 in the presence of heparin (p<0.01). However,

when heparin was added to the complex, $[Ca^{2+}]_i$ transients were restored to levels similarly to that with 0.1µM OT.

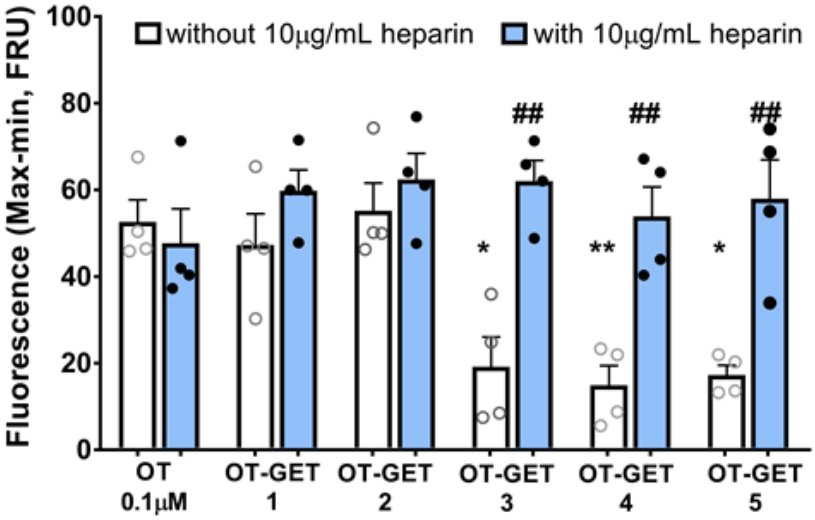


Figure 3.8 Bioactivity of OT-GET in the presence or absence of heparin. Free $[Ca^{2+}]_i$ transients elicited by 0.1µM OT and OT-GET in the presence or absence of 10µg/mL heparin was recorded using calcium fluorimetry. Two-way ANOVA confirmed a significant main effect of heparin ($F_{(5,36)}$=4.89, p=0.0016) and OT-GET ($F_{(1,36)}$=30.66, p=0.0001) treatment as well as heparin x OT-GET interaction ($F_{(5,36)}$=5.42, p=0.0008) on free $[Ca^{2+}]_i$ transients induced. Results are individual points and mean ± SEM values, n=3-4. ; * (p<0.05), ** (p<0.01) OT-GET 3-5 vs 0.1µM OT without heparin; ## (p<0.01), OT-GET 3 – 5 in the presence vs absence of heparin; Tukey's post-hoc.

3.3.3 Air-liquid interface (ALI) culture of RPMI 2650 as an optimal *in vitro* permeation model

3.3.3.1 ALI cultured RPMI 2650 cells produced higher TEER values

As RPMI 2650 cells are reported to grow and differentiate better, producing higher barrier integrity under ALI cultivation (Mercier et al., 2018b), comparison of TEER values of ALI and LCC cultured RPMI 2650 was investigated.

Upon switching culture conditions on day 8, TEER values of ALI cultured RPMI 2650 progressively increased, becoming significantly higher

(p<0.01 on Day 12-16, 20-21, p<0.001 on Day 18; Student's *t*-test) than LCC over the remaining 2 weeks (Figure 3.9).

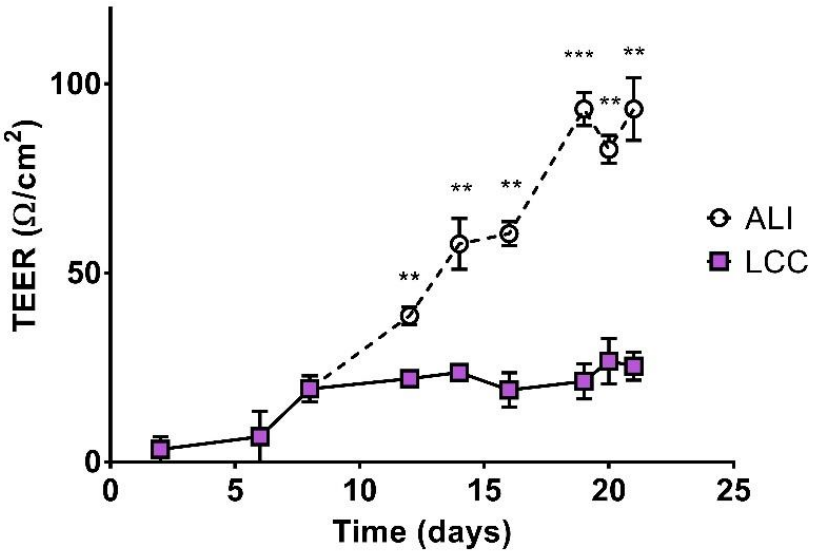


Figure 3.9 TEER values of RPMI 2650 cells.

TEER values of RPMI 2650 cells cultured in ALI or LCC culture conditions (EMEM + 10% heat inactivated FCS) was measured using a voltohmmeter every other day for 21 days. TEER values of cells cultured under ALI cultivation (day 8 onwards) remain significantly higher than that of LCC cultivation (unpaired t-test). Results are mean ± SEM values, n=3. ** (p<0.01), *** (p<0.001) vs baseline.

3.3.3.2 ALI cultured RPMI 2650 produced lower TFD4.4 permeation

In order to further compare and verify barrier integrity of ALI versus LCC cultured RPMI 2650 cells, permeation of TD4.4, a standard high molecular weight paracellular marker, was carried out.

The transport of TD4.4 across RPMI 2650 cell monolayer was assessed over 6h. P_{app} of TD4.4 across ALI cultivated RPMI 2650 (P_{app} = 1.78 ± 0.08 x 10^6 cm/s) was significantly lower (p<0.001; Student's *t*-test) than LCC cultivated cells (P_{app} = 4.06 ± 0.24 x 10^6 cm/s; Figure 3.10). A parallel experiment included filter inserts without a cellular barrier as a positive control was carried out. The presence of a cellular barrier had a main effect on P_{app} (($F_{(2,6)}$=29.12, p=0.0001; one-way ANOVA), where

transport of TD4.4 across filter inserts only showed a significantly higher P_{app} value of 12.49 ± 1.79 x 10^6 cm/s ($p<0.05$) compared to across a LCC and ALI cultivated cell monolayer. This indicated that filter inserts do not affect rate of TD4.4 permeation and any differences seen was due to any differences in cellular barriers formed by ALI versus LCC cultured RPMI 2650.

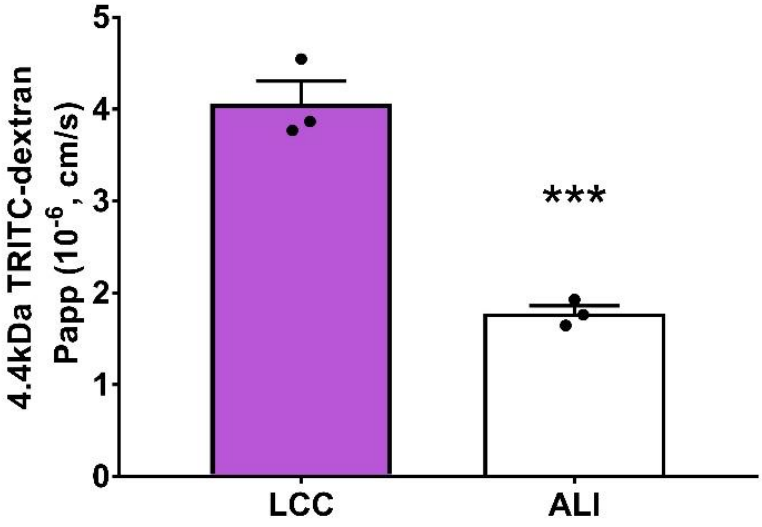


Figure 3.10 TD4.4 permeability.

TD4.4 permeability in RPMI 2650 cells cultured in ALI versus LCC culture conditions (EMEM + 10% heat inactivated FCS) was measured using a fluorescence plate reader. P_{app} of TD4.4 is significantly higher in LCC conditions (unpaired t-test). Results show individual points and mean ± SEM values, n=3; *** ($p<0.001$).

3.3.4 OT-GET improved OT permeation across a RPMI 2650 cell monolayer

ALI cultured RPMI 2650, which exhibited higher TEER and lower TD4.4 P_{app} values indicating a better barrier integrity compared to LCC cultivation, was used to investigate permeation of OT in the presence or absence of GET carrier peptide. As it was unknown as to whether the OT-GET complex would dissociate or remain conjugated post-permeation of the cellular monolayer, heparin was added to samples

collected from the basolateral chamber and final OT concentration calculated after 6h was compared to those without added heparin.

OT/OT-GET significantly increased cumulative OT concentration in the basolateral chamber after 6h ($F_{(3,24)}$=11.47, p=0.0001; Figure 3.11) and this was not affected by the addition of heparin ($F_{(1,24)}$=0.051, p=0.82; Figure 3.11). There was also no interaction between OT/OT-GET x heparin treatment to influence final OT concentrations in the basolateral chamber at 6h ($F_{(3,24)}$=0.074, p=0.97). Overall, this indicates a possibility that the OT-GET complex dissociated and released free OT into the basolateral chamber. However, as addition of heparin clearly produced no fluctuation in readings, all results thereafter are from heparin added samples as an additional precaution to ensure the bioassay detected the full extent of OT permeation.

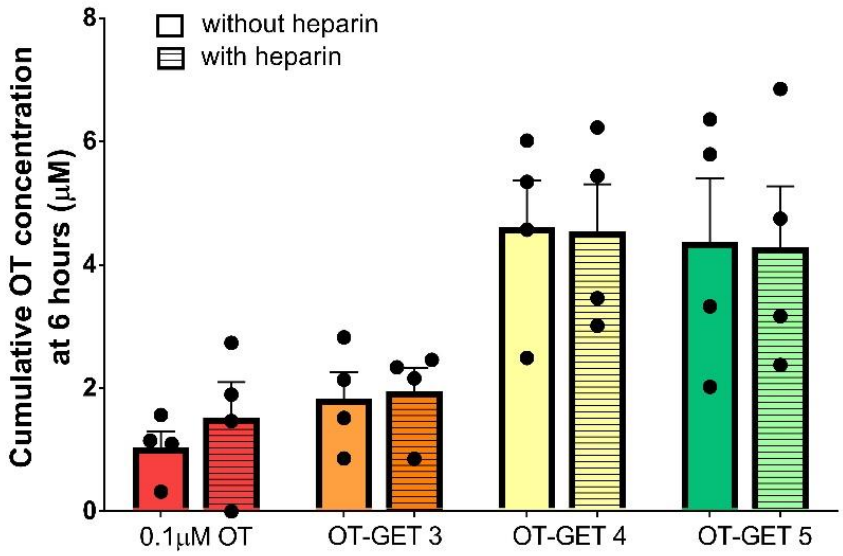


Figure 3.11 Cumulative OT concentration at 6h post-permeation across RPMI 2650 monolayer.

Cumulative OT concentration in the presence or absence of 10μg/mL heparin (added to samples collected from basolateral chamber) after 6h of OT/OT-GET permeation across RPMI 2650 cellular barrier was measured using calcium fluorimetry. Two-way ANOVA revealed that final OT concentration was significantly influenced by OT/OT-GET treatment ($F_{(3,24)}$=11.47, p=0.0001) but not heparin ($F_{(1,24)}$ =0.051, p=0.82), nor was there a heparin x OT-GET

interaction ($F_{(3,24)}$ =0.074, p=0.97). Figure shows individual points and mean ± SEM values, n=4.

Time ($F_{(2,24)}$ =60.39, p=0.0001) and OT/OT-GET treatment ($F_{(3,12)}$ =3.883, p=0.0376) significantly increased free cumulative $[Ca^{2+}]_i$ transients in samples collected from basolateral chamber over 6h (Figure 3.12). There was also a main effect of time x OT/OT-GET treatment ($F_{(6,24)}$ =2.687, p=0.0386). A significant increase in free $[Ca^{2+}]_i$ transients, induced by OT-GET 4 after 6h compared to OT alone (p<0.01) and OT-GET 3 (p<0.05) was noted. OT-GET 5 also significantly increased free $[Ca^{2+}]_i$ transients compared to OT alone at 6h (p<0.01).

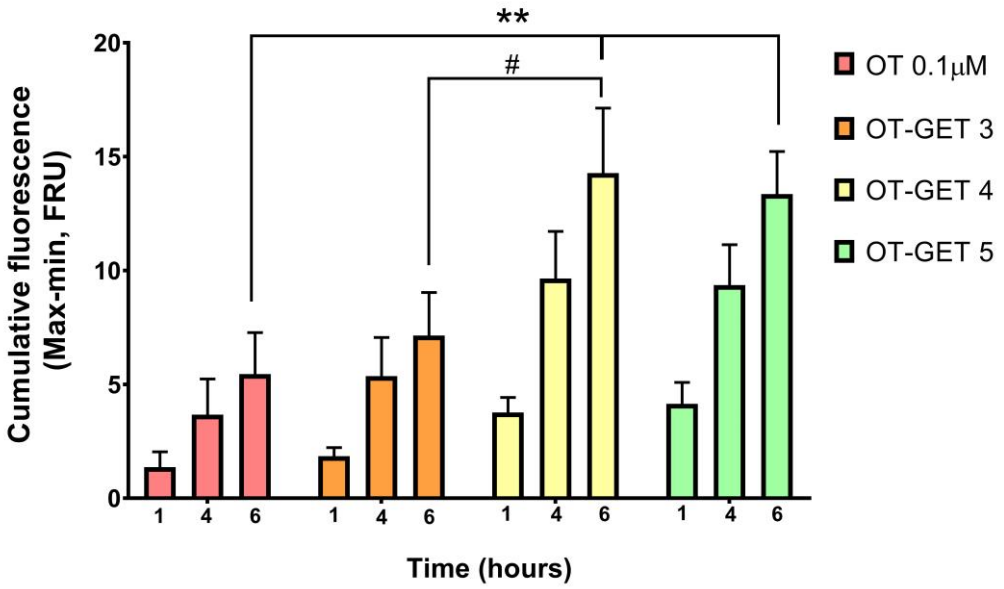


Figure 3.12 Progressive increase in free cumulative $[Ca^{2+}]_i$ transients elicited by 0.1μM OT and OT-GET.

Contents were collected from basolateral chamber of RPMI 2650 transwells over 6h and analysed (no heparin added) using calcium fluorimetry. Two-way ANOVA confirmed main effects of time ($F_{(2,24)}$ =60.39 p=0.0001), treatment ($F_{(3,12)}$ =3.883, p=0.0376) and time x treatment interaction ($F_{(6,24)}$ =2.687, p=0.0386) on $[Ca^{2+}]_i$ transients. Results are mean ± SEM values, n=4; ** (p<0.01) vs 0.1μM OT, # (p<0.05) vs OT-GET 3; Tukey's post-hoc.

OT's P_{app}, which quantifies the rate at which OT crosses the RPMI 2650 cellular barrier was significantly increased by OT/OT-GET treatment ($F_{(3,12)}$ =6.068, p=0.0001; one-way ANOVA; Figure 3.13) such that there was a significant increase in OT's P_{app} by OT-GET 4 and 5 compared to OT alone (p<0.05).

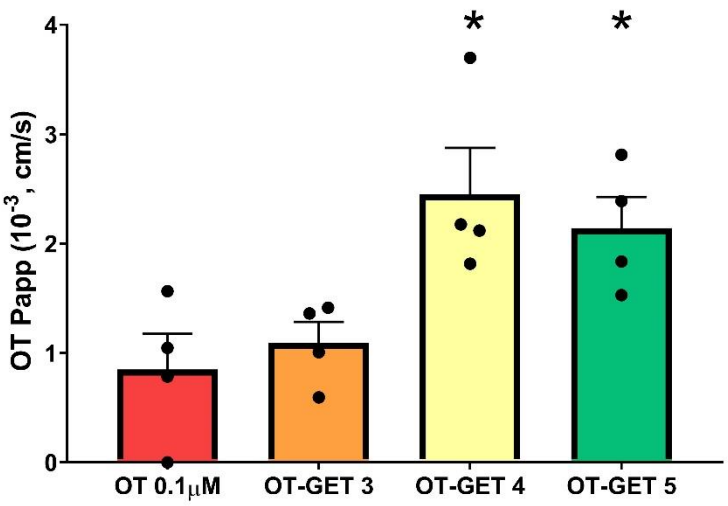


Figure 3.13 Permeability rate of OT and OT-GET.

P_{app} of 0.1μM OT and OT-GET 3 -5 across a RPMI 2650 cellular monolayer was calculated after 6 hours. One-way ANOVA revealed a main effect of OT/OT-GET treatment on OT P_{app} across the nasal epithelial monolayer ($F_{(3,12)}$ =6.068, p=0.0001); *(p<0.05) vs 0.1μM OT; Figure shows individual points and mean ± SEM values n=4; Tukey's post-hoc.

On the other hand, OT/OT-GET treatment ($F_{(3, 12)}$ = 3.883, p=0.0376) increased cumulative OT concentration collected in the basolateral chamber after permeation across RPMI 2650 cellular barrier over 6h ($F_{(2, 24)}$ = 60.39, p=0.0001), such that a time x treatment effect was noted ($F_{(6, 24)}$ = 2.687, p=0.0386). OT-GET 4 and 5 produced the highest cumulative OT concentration in the basolateral chamber after transducing the RPMI 2650 monolayer after 6h, approximately three times more than OT alone (p<0.01) while OT-GET 4 produced approximately two times more cumulative OT concentration compared to OT-GET 3 (p<0.05).

Thus, it seems that the presence of the GET carrier peptide improves rate of OT transduction across the RPMI 2650 barrier, but the amount of OT concentration collected in the basolateral chamber will not increase significantly if the time before collection is 4h and below.

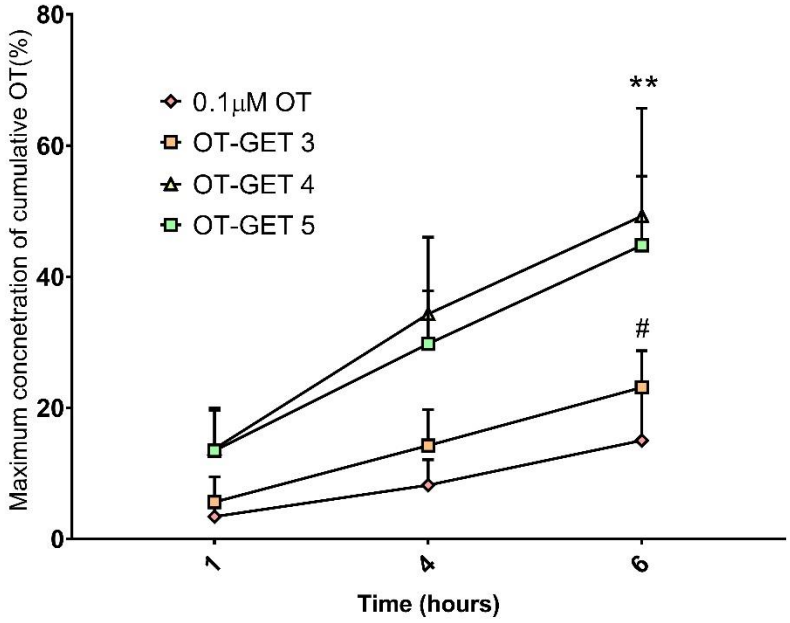


Figure 3.14 Cumulative OT concentration in the basolateral chamber 1, 4 and 6h post OT permeation of a RPMI 2650 cellular barrier in the presence and absence of GET carrier peptide.

Cumulative concentrations were calculated by adding total concentration (determined using an OT standard curve) at each time point with the total concentration of the previous time point and determined using calcium fluorimetry. Two-way repeated measures (RM) ANOVA revealed a main effect of time ($F_{(2, 24)}$ = 60.39, p=0.0001), treatment ($F_{(3, 12)}$ = 3.883, p=0.0376) and time x treatment interaction ($F_{(6, 24)}$ = 2.687, p=0.0386) on cumulative OT concentration collected over 6h. Figure shows mean ± SEM values, n=4; ** (p<0.01) OT-GET 4 and 5 vs 0.1μM OT at 6h; # (p<0.05) OT-GET 3 vs OT-GET 4 at 6h; Tukey's post-hoc.

Incubation of RPMI 2650 cells in serum-free media for 6h caused a significant decrease in TEER values (Table 3.1) such that a three-way ANOVA revealed main effects of time ($F_{(1,40)}$=13.91, p=0.0006), media type ($F_{(1,40)}$=98.17), p=0.0001) and a time x media interaction

($F_{(1,40)}$=13.91, p =0.0006). A significant decrease in TEER values was observed for OT (p<0.01), OT-GET 3 (p<0.01), OT-GET 4 (p<0.001) and OT-GET 5 (p<0.001) incubated in serum free media compared to baseline TEER value.

Serum free media (% of baseline)

	Control		OT		OT-GET 3		OT-GET 4		OT-GET 5	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Base line	100.00		100.00		100.00		100.00		100.00	
6h	82.62	3.87	69.93**	5.66	75.04**	8.39	63.96***	11.19	63.06***	6.72

Growth media (% of baseline)

	Control		OT		OT-GET 3		OT-GET 4		OT-GET 5	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Base line	100.00		100.00		100.00		100.00		100.00	
6h	84.92	0.79	86.35	0.32	97.44	2.56	81.20	9.86	84.23	8.38

Table 3.1 Percentage decrease of RPMI 2650 TEER values incubated in serum free EMEM or serum supplemented EMEM (normal growth media) after 6h compared to baseline.

TEER values were measured using a voltohmeter. Results are mean ± SEM values, n=3. Three-way ANOVA revealed main effects of time ($F_{(1,40)}$ =13.91, p=0.0006), media type ($F_{(1,40)}$ =98.17), p=0.0001) and a time x media interaction ($F_{(1,40)}$ =13.91, p=0.0006) on TEER values. OT/OT-GET treatment ($F_{(4,40)}$ =1.603, p=0.192) , OT/OT-GET x time interaction ($F_{(4,40)}$ =7.064, p=0.5923), OT/OT-GET x media interaction ($F_{(4,40)}$ =1.603, p=0.1924) or OT/OT-GET x time x media interaction ($F_{(4,40)}$ =0.7064, p=0.5923) did not have an effect. ** (p<0.01), ***(p<0.001) compared to baseline TEER value; Dunnett's post-hoc.

However, usage of serum free media for the transwell assay was unavoidable as high fluctuation in Hs 578t cells OT-dose responses was observed when serum supplemented growth media or assay buffer were used (Figure 3.15).

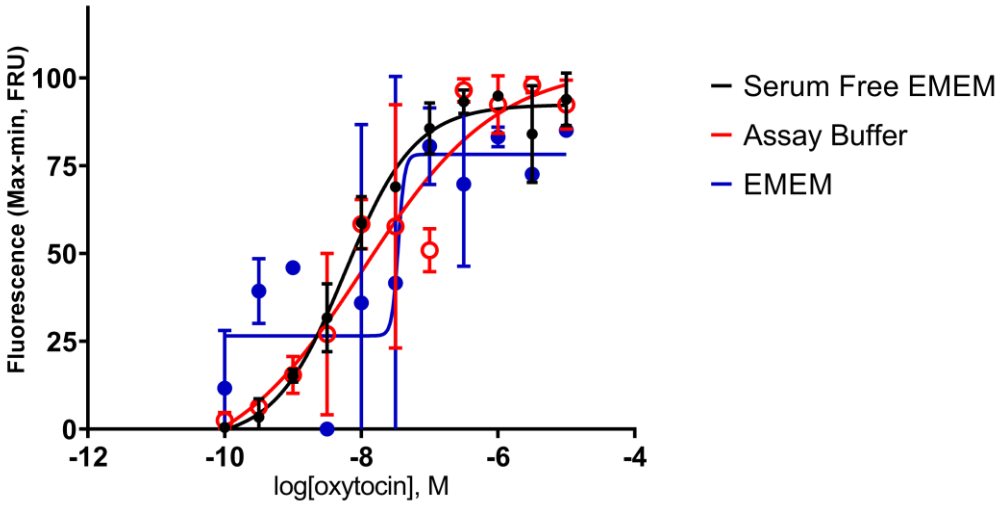


Figure 3.15 OT-dose responses (0.32nM - 10µM OT) in the presence of serum free/supplemented EMEM and assay buffer.

3.3.5 Cell viability

Potential cytotoxicity of OT-GET on the RPMI 2650 cellular barrier was tested using PrestoBlue cell viability assay to ensure barrier integrity was maintained throughout the duration of OT permeability assay. The assay measured fluorescence readout and was directly proportional to the number of metabolically active cells which reduced resazurin to fluorescent resorufin.

A progressive reduction in fluorescence was observed with increasing OT-GET concentrations ($F_{(4,10)}$ =7.511, p=0.0046; one-way ANOVA; Figure 3.16) with OT-GET 5 (0.12µM OT + 120µM GET; to match concentration met by cells during permeability assay prior further dilution for calcium fluorimetry with Hs 578t cells) causing a significant decrease in number of viable RPMI 2650 cells after 6h of incubation compared to media only control ($p<0.01$) and OT ($p<0.05$).

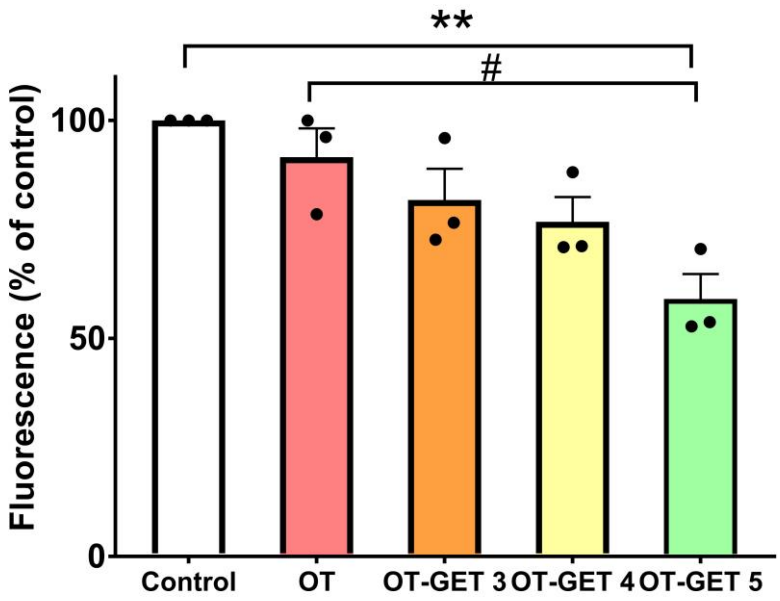


Figure 3.16 RPMI 2650 cell viability after exposure to OT and OT-GET.
Viability of RPMI 2650 cells after 6h exposure to 0.1µM OT and increasing OT-GET concentration (OT-GET 3 – 5) was measured using a PrestoBlue fluorescence assay. One-way ANOVA revealed a main effect of OT/OT-GET on cell viability ($F_{(4,10)}$ =7.511, p<0=0.005) measured using a Presto Blue cytotoxicity assay. Figure shows individual points and mean ± SEM values, n=3; ** (p<0.01) vs control, # (p<0.05) vs OT; Tukey's post-hoc.

3.4 Discussion

3.4.1 Intracellular $[Ca]^{2+}$ transients in HEK 293T, MCF-7 and Hs 578t cells

Functional OTR response was identified in Hs 578t cells as free $[Ca^{2+}]_i$ transients were robustly produced only in Hs 578t cells but not in MCF-7 or transient OTR transfected HEK 293T cells.

Although OT failed to induce a Ca^{2+} response in OTR transfected HEK 293T cells, indicating a lack of OTR expression, they responded to the muscarinic agonist carbachol, suggesting they possess a functional signalling pathway. We proceeded to investigate OT responses in cell lines which endogenously express OTR: MCF-7 (Amico et al., 2002, Cassoni et al., 2006) and Hs 578t (Amico et al., 2002, Copland et al., 1999, Schiffmann and Gimpl, 2018). Hs 578t cells showed a more robust dose dependent Ca^{2+} response to OT with an EC50 of 0.26 ± 1.25µM. This EC50 value is a higher concentration compared to Copland et al., (1999) who obtained a value of 1.9 ± 1.2nM. However, they recorded Ca^{2+} transients in single cells after 50 min incubation at 25^{o}C while the current study recorded Ca^{2+} transients in a cell aggregation after an incubation time of 40 minutes at 37^{o}C. The difference in total accessible receptor sites as well as temperature is likely to contribute to the higher potency observed in this study.

In the presence of increasing L-368,899 concentrations, a parallel rightward shift of the OT dose-response curve in Hs 578t cells was observed, although a similar maximum response for all curves, a typical characterisation for a dose response curve in the presence of a competitive antagonist, was not achieved. This could signify L-368,899 as an irreversible non-competitive antagonist although literature does not support this claim (Pettibone et al., 1993). Instead, the apparent decreased in maximum efficacy is likely because a higher concentration of OT was not used, due to material constraints. No right shift occurred in the presence of SR49059 or tolvapton, indicating OT had no effect on V_{1a} or V_2 receptors, if present.

As OT is able to activate OTR and V_{1a} receptor, both of which are $G\alpha_q$ coupled, this indicates that changes in calcium transients in Hs 578t are due to OTR activation only. Consistent with this, Copland et al., (1999) also reported the absence of V_{1a} receptor mRNA in the Hs 578t cell line.

3.4.2 Bioactivity of OT-GET

Stimulation of Hs 578t cells with OT-GET 2 -5 produced a significant reduction in free $[Ca^{2+}]_i$ transients compared to OT alone, possibly due to GET encapsulated OT being unable to bind to the same affinity or efficacy with OTRs, reducing subsequent $G\alpha_q$ protein coupled downstream reaction. This suggests a certain GET concentration threshold, in this case OT:GET concentration ratio of 1:0.1 and above, must be used to conjugate OT to ensure efficient OT encapsulation.

Addition of heparin unconjugated the complex as the P21 domain in GET binds to heparin at a higher affinity than it would to OT (Dixon et al., 2016). The resultant elevation in free OT would be available to bind to, and activate membrane incorporated OTR expressed in Hs 578t cells, bringing free $[Ca^{2+}]_i$ transients back to levels similar to that elicited by OT alone, as seen in OT-GET 3 - 5. This ability of GET to uncouple from its peptide for subsequent bioactivity is also observed by Dixon et al. (2016) where GET-Cre recombinase, NANOG (a transcription factor involved in pluripotency and self-renewal of embryonic stem cells) and Myoblast determination protein 1 (regulates muscle differentiation) enhanced green fluorescent protein (GFP) expression in NIH3t3 mouse fibroblast cells, rescued mouse embryonic stem cell pluripotency and promoted myogenic differentiation in HUES7 human embryonic stem cells respectively. In this case, addition of heparin to GET-Cre recombinase decreased fluorescence as opposed to the current study. This is due to the different localisation of the target receptor. OTR on Hs 578t cells are expressed extracellularly, thus cleavage of OT-GET allows free OT to act on OTR whereas Cre protein requires GET assistance in order to enter the cell and undergo nuclear translocation to produce resulting expression of GFP.

3.4.3 Validation of RPMI 2650 as an *in vitro* nasal epithelial permeation model

RPMI 2650 cells grown on 0.4µm pore size polyester filter inserts at ALI for 20-21 days produced a confluent monolayer with TEER values comparable to that of previous literature (Mercier et al., 2018b, Wengst and Reichl., 2010). Our maximal average TEER value for ALI culture is 88 ± 4.73 Ω/cm^2 which falls within the 80 - 130 Ω/cm^2 range reported by Wengst and Reichl (2010) and Mercier et al. (2018b) as well as the 60 - 180 Ω/cm^2 range seen in human excised nasal mucosa (Mercier et al., 2018b). To further validate barrier integrity, permeability of a high molecular weight paracellular marker TD4.4 was tested. ALI cultivation achieved a significantly tighter monolayer barrier with a P_{app} of 1.78 ± 0.08 x 10^6 cm/s compared to LCC of 4.06 ± 0.25 x 10^6 cm/s. This ALI P_{app} value matches the 0.70 – 2.48 x 10^6 cm/s value found by Reichl and Becker (2012) and Wengst and Reichl (2010). In both studies, length of cultivation including day of media switch, were identical. The only differences were the material of filters used: both used pore sizes of 3µM, but Reichl and Becker (2012) used polyethylene terephthalate and Wengst and Reichl (2010) used polycarbonate material. This places further emphasis that ALI cultivation is vital to achieve a reproducible RPMI 2650 model.

The RPMI 2650 cell line has been criticised in the past for its suitability in permeability studies. Using traditional LCC methods, De Fraissinette et al. (1995) reported formation of cell clusters when grown on filters with no cell polarisation, microvilli or tight junctions detected. Werner and Kissel (1996) reported a lack of cell polarity, poor differentiation and inability to form a confluent monolayer. However, optimisation studies which emphasise the usage of ALI culture, initial seeding density as well as the pore size and type of coating of cell culture inserts have addressed the above issues (Mercier et al., 2018b), producing well differentiated cells in a tight monolayer as seen in the current study. Many other studies report positive expression of tight junction proteins which include ZO-1 and occuldin (Bai et al., 2008, Kreft et al., 2015, Kurti et al., 2013).

Although this study did not carry out staining to validate tight junction expression, our TEER and TD4.4 values indirectly supports production of a confluent epithelial membrane with natural morphological characteristics. Certainly, criticism of the cell line still remains, mainly due to its different morphology – RPMI 2650 consist of squamous epithelial cells, lack cilia (Ladel et al., 2019) and there are contradictory reports of mucus expression (Ladel et al., 2019, Goncalves et al., 2016, Pozzoli et al., 2016). However, the next alternative is usage of a primary nasal cell line. The limitations of primary cell culture is that passaging are limited to a maximum of three times to maintain desired morphological characteristics (Wu et al., 1985), which means frequent sampling would be required which may affect reproducibility of results due to heterogeneity in differentiation patterns. As such, the immortalized cell line RPMI 2650 remains the best option, provided a standardised cell culture condition be maintained throughout.

3.4.4 OT-GET increased OT transduction across RPMI 2650 without affecting cell viability

Dixon et al. (2016) previously showed that GET is able to successfully deliver its cargo into cells. However, whether the same approach would be successful across a cellular barrier is unknown. In this study, OT-GET 4 and 5 improved OT delivery across the RPMI 2540 monolayer, with significantly higher OT P_{app} and cumulative OT concentration in the basolateral chamber after 6h compared to OT alone.

Absorption of peptides across cellular barriers depends on various factors including physiochemical properties of the applied drug and ABC transporter mediated efflux (Grassin-Delyle et al., 2012). OT, a hydrophilic peptide, may utilise either the paracellular or transcellular route for transepithelial passage, but with its larger molecular size of 1kDa possibly producing physical restriction to bypass the space between adjacent cells, the latter is usually the more important absorption pathway. P_{app} of standard compounds typically fall between 1.0×10^{-7} to 1.0×10^{-5} cm/s (Gao et al., 2001), however OT delivered by GET or alone had a much higher 1.0×10^{-3} cm/s value. No study has determined OT

P_{app} in an RPMI 2650 cell model thus far, although in an *in vitro* BBB bilayer model, which consisted of adult mouse cortex epithelial cells and postnatal mouse cultured astrocytes, P_{app} was approximately 3.0 x 10^{-6} cm/s (Chakraborty, 2017). Compared to a BBB model in which TEER values were around 200Ω/cm^2 (Chakraborty, 2017), RPMI 2650 at 80Ω/cm^2 is inherently 'leakier'. This leaky characteristic may contribute to increased paracellular mediated passage of OT observed. Additionally, Gao et al. (2001) suggests that a high P_{app} indicates the presence of an active transport mechanism (transcellular route). The only identified transporters for OT thus far are RAGE transporters on endothelial cells of the BBB (Yamamoto et al., 2019) which are at most, weakly expressed in the nasal mucosa (Dzaman et al., 2015) and Peptide-transporter system-1 (PEPT1) that transports radiolabelled OT from brain to blood (Durham et al., 1991) and is found in human nasal mucosa (Agu et al., 2011) but weakly expressed in RPMI 2650 (Dolberg and Reichl, 2018). The nasal epithelial (human excised and RPMI 2650) also expresses a range of other solute carrier (SLC) transporters (Anand et al., 2014) and we cannot rule out the possible roles they may play in OT transportation. With OT-GET, GET utilises membrane bound heparin sulphates as well as trypsin-sensitive and detergent soluble molecules to interact with the cellular membrane. Subsequent cell uptake utilises lipid raft micropinocytosis although the majority of the cargo remain trapped within endosomes (Dixon et al., 2016), which would explain the approximately 4x lower free $[Ca^{2+}]_i$ transients observed in our permeability study compared to the bioactivity study.

The improved membrane adherence of the GET complex combined with the protection of OT from drug efflux transporters likely increased the amount of OT-GET entering the cells compared to OT alone. This would result in more free and conjugated intracellular OT for subsequent release into the basolateral chamber. RPMI 2650 expresses several efflux transporters: P-glycoprotein (Anand et al., 2014, Dolberg and Reichl, 2016), multidrug-resistance associated protein (MRP) 1 and 2

and breast cancer resistance protein (BCRP) (Mercier et al., 2018a), although it is unsure if OT is a substrate for any of them.

Overall, the rate-limiting step for OT transport across RPMI 2650 may be due to its reliance on transcellular passage, an energy expedited process involving transporters. On the other hand, the uptake of OT-GET into cells is via an endocytotic pathway. This increases protection of OT from enzymatic degradation, providing higher concentrations for subsequent release. The main advantage of GET is that unlike traditional CPPs which depend on high extracellular concentrations to drive transduction, low concentrations of GET are sufficient to observe biological effects *in vitro* (Dixon et al., 2016) which in turn may reduce any subsequent unwanted side-effects. However, the overall high cationic charge (11+ P21 and 8+ 8R), which has long been a main cytotoxicity concern with CPPs (Saar et al., 2005) will require careful consideration in order to identify an OT to GET ratio that produces efficacy without concomitant adverse side-effects. Efforts to increase endosomal release of GET conjugated peptide also requires optimisation. In another study, GET peptides in poly-lactic-co-glycolic acid (PLGA) microparticles with co-encapsulation of L-histidine showed gradual release of red fluorescent protein cargo over a 10 day period whilst maintaining transduction activity, as tested via flow cytometry in NIH 3t3 cells (Abu-Awwad et al., 2017). The modification of OT-GET in a similar method may be able to increase release of OT from its GET endosomes.

The decrease in RPMI 2650 TEER values over 6h due to incubation in serum free media also requires addressing. Are the observed results due to actual effects of GET or due to the disruption of cell barrier integrity from serum free media incubation? However, the usage of normal growth media or assay buffer (used in the calcium fluorimetry assay) caused huge fluctuations in Ca^{2+} release, and thus incubation of RPMI 2650 in serum free media for permeability testing was unavoidable. However, one would assume if the disruption in cell barrier integrity due to serum free media was a main contributing factor, concentration of free OT

transported across the monolayer by OT-GET 3 would be similar. This was clearly not seen.

A separate observation of TEER values of RPMI 2650 incubated in EMEM growth media showed no significant decrease. This observation was validated further by cytotoxicity testing where only OT-GET 5 caused any apparent reduction suggesting little cytotoxicity with lower ratios. This further supports OT-GET 4 conjugation ratio (1 OT: 10 GET concentration) being the most optimal to improve OT membrane transduction whilst avoiding unwanted side-effects. It is to be noted that the concentrations seen by RPMI 2650 cells in the Presto Blue viability assay are 12 times higher (resulting in GET 3-5 concentrations of 0.12uM, 12uM and 120µM respectively) to match the concentration seen in the permeability assay. Preliminary studies in the Dixon laboratory found that GET concentrations above 20µM increased cytotoxicity steadily in Caco-2 intestinal cells, which corresponds well with results seen in this Chapter.

3.5 Conclusion

Robust $[Ca^{2+}]_i$ transients dose-responses was produced by Hs 578t cells in response to OT, which was blocked by OTR but not V_{1a} or V_2 receptor antagonists. As such, usage of Hs 578t cells in a calcium fluorimetry assay is the most suitable and sensitive to detect biological changes that may be induced by OT-GET.

In order to successfully predict OT absorption across a nasal epithelial *in vivo*, a suitable and robust *in vitro* model was utilised. RPMI 2650 cells grown over 3 weeks, with ALI cultivation for 2 weeks in 0.4µm polyester Transwell filter produced a suitable permeation barrier, with TEER values matching that of normal mucosa and similar permeability values of a standard high weight paracellular molecular marker, TD4.4.

By using the above RPMI 2650 model, OT-GET 4 and 5 resulted in the highest concentration of OT crossing the barrier, indicating GET does improve OT permeability across an *in vitro* nasal epithelial monolayer. Yet, the levels of free $[Ca^{2+}]_i$ transients induced was approximately 4x lower when a RPMI 2650 cell barrier was introduced prior to OTR activation. This indicated a large concentration of trapped OT within GET endosomes. An ELISA test would be able to provide an approximate quantification on the concentration of OT that remained trapped within the RPMI 2650 cell layer. However, OT-GET 5 showed some cytotoxic effects on cell viability in a Presto Blue assay, indicating OT-GET 4 (1 OT to 10 GET) may represent the most suitable OT to GET ratio, for use in *in vivo* studies, as described in Chapter 4.

Overall, five different OT-GET doses were tested for their bioactivity and the most successful three doses examined in detail in their ability to improve OT permeability whilst avoid cytotoxicity. Cell permeability to OT and potential cytotoxicity effects was tested over 6 hours and a single OT to GET conjugation ratio that could enhance permeability without cytotoxicity was identified. Results from this Chapter shows great potential for OT-GET to be tested further *in vivo* as well as provided a guide to subsequent dosage to be used in the rat. Chapter 4

subsequently explores OT-GET's ability to enhance brain penetration and affect behaviour in the rat.

Chapter 4 Acute *in vivo* effects of intranasal oxytocin compared with an oxytocin-peptide conjugate (OT-GET)

4.1 Introduction to Chapter

In Chapter 3, we identified that an OT:GET concentration ratio of 1:10 was able to improve OT permeation across an *in vitro* nasal epithelial barrier without producing cytotoxicity effects. In this Chapter, we utilised the GET system for the first time *in vivo* and investigated if this success translated to improved CNS penetration *in vivo* in rats by investigating intranasal OT/OT-GET's behavioural effects on PCP-induced locomotor activity in an acute PCP rat model for schizophrenia and social interaction in otherwise healthy rats. We also monitored the extent of intranasal OT brain penetration using a fluorescent marker conjugated to GET and by quantifying OT content in the olfactory bulb of the post-mortem brain using ELISA.

4.2 Intranasal OT administration

The intranasal route of administration is a popular alternative to improve delivery of OT to the brain with the preclinical and clinical behavioural effects following OT administration described in detail in Chapter 1. Intranasal OT specifically, increases social behaviour and interaction in NHPs, rodents and prairie voles, with changes in behaviour occurring as soon as 5 minutes post-administration in mice (Bales et al., 2013, Huang et al., 2014) and 30 minutes in rats (Dai et al., 2018) and NHPs (Brooks et al., 2021) (Table 4.1). These changes in social behaviour are also seen with peripheral (systemic) OT administration (Kohli et al., 2019, Ramos et al., 2013), although one clear advantage with intranasal delivery is the absence of side-effects such as hypothermia and bradycardia seen with peripheral delivery (Calcagnoli et al., 2015, Hicks et al., 2014, Kohli et al., 2019).

Species	Author	Subject	Dose regime	Dose	Behaviour observation	Time after administration for behavioural observation
NHPs	Brooks et al., 2021	male and female chimpanzees and bonobos	Acute	40IU	increased eye contact in bonobos	30 - 60 minutes
	Chang et al., 2012	male rhesus macaques	Acute	24IU	promote prosocial choice and attention	2 hours
	Simpson et al., 2014	male and female infant macaques	Acute	25IU	increased positive social behaviour with human caregiver	1-2 hours
	Simpson et al.,2017	male and female infant macaques	Acute	20IU	improved working memory and gaze following in males only	1 hour
Mice	Huang et al., 2014	C57BL/6J adult mice	Acute, chronic (2 weeks)	9.6µg - 19µg	Acute 19µg dose increased ano-genital sniffing and overall social behaviour Chronic 19µg dose decrease in overall male-male mice social interaction duration	acute: 5 minutes chronic: after 2 weeks of treatment
	Kitagawa et al., 2021	POGZ WT/Q1038R mice model of ASD	Acute	200µg	increased social interaction towards intruder mice	30 minutes
	Tanaka et al., 2018	male ddY mice	Acute	250µg	supressed plasma corticosterone elevation in restraint stressed mice	5 minutes

Species	Author	Subject	Dose regime	Dose	Behaviour observation	Time after administration for behavioural observation
Rats	Calcagnoli et al., 2015	male WTG rats	Acute, chronic (7 days)	20µg	acute and chronic doses reduced offensive behaviour, increased social exploration and attention, facilitated partner preference	Acute: 30 minutes Chronic: 7 days after last treatment
	Dai et al., 2018	adolescent VPA-Induced ASD model	acute	20µg	improved social preference	30 minutes
	Joushi et al., 2021	maternally separated adolescent male rats	chronic (7 days)	20µg	improved social interaction and social recognition of a stranger rat	3 days prior
	Kent et al., 2016	male SD rats	acute	5µg, 20µg	increased social interaction with 20µg dose only	40 minutes
	Matsuo et al., 2020	prenatal VPA-exposed rats	chronic (35 days)	12µg/kg	reversed object recognition impairment, improved social interaction	24 hours
	Park et al., 2017	male SD rats	acute	200µg	improved stress-induced impairments of object recognition	1 hour
	Wang et al., 2019	male SD rats	acute	20µg	restored prosocial contact of rats previously exposed to a single prolonged stress	same day (exact time not stated)

Species	Author	Subject	Dose regime	Dose	Behaviour observation	Time after administration for behavioural observation
Prairie Vole	Bales et al., 2013	adult male and female prairie voles	Chronic (21 days)	0.08 - 8IU	Acute behaviour: all doses increased social interaction in males Long term behaviour: 0.08IU and 0.8IU impaired partner preference in males	acute: 5 minutes chronic: after 3 weeks of treatment

Table 4.1 Preclinical animal studies in non-human primates (NHPs), rodents and prairie voles examining intranasal OT effects on social behaviour and/or cognition.

Dose of 1IU equal 2.3µg. ASD (autism spectrum disorder); ddY (Deutschland, Denken, and Yoken); SD: Sprague Dawley; VPA: valproic acid.

However, intranasal OT levels that produce positive behavioural effects are high: 20IU – 40IU (46µg - 93µg) in humans (Goldman et al., 2011, Michalopoulou et al., 2015, Shin et al., 2015b), 24IU – 80IU (56µg – 186µg) in NHPs (Lee et al., 2017, Modi et al., 2014) and 20 µg in rats (Calcagnoli et al., 2015, Kent et al., 2016, Neumann et al., 2013). This is more than the basal pituitary content, estimated at 0.5µg - 1µg in the adult rat and 28µg in humans (Heller and Zaimis, 1949, Leng and Ludwig, 2016). Despite these high concentrations, the amount of OT estimated to penetrate the brain remains low. Tanaka et al. (2018) measured OT concentration in the nasal cavity of mice, administered via a peristaltic pump from the oesophagus, over 180 minutes. Only 10% of drug transversed the nasal epithelium. Coupled with findings of direct transport percentage of 99.5% to the olfactory bulb, 96% to occipital half of brain and 97% to frontal half of the brain, the final percentage of the initial dose that penetrated the brain would be approximately ~3%. The remaining ~97% of drug would have likely been swallowed into the gut or redistributed in the circulation to the rest of the body. Based on this study, the estimated percentage of OT that penetrates the brain is higher than with peripheral administration which is estimated at 0.2% - 0.002% (Mens et al., 1983, Yamamoto et al., 2019) but still low. However, Tanaka et al. (2018) administered a high dose of 200µg OT and their method of delivery differ from the conventional method, which typically pipette aqueous solution to the squamous epithelium of the rhinarium in conscious animals. As such, compared to common doses delivered in behavioural studies as outlined in Table 4.1, the actual amount of OT delivered to the brain is likely to be much lower. Indeed, measuring the increase in CSF OT levels after intranasal administration in NHPs via nasal sprays puts the estimate at a very conservative 0.002% - 0.003% increase (Chang et al., 2012, Dal Monte et al., 2014, Modi et al., 2014). Furthermore, plasma OT levels increase concurrently with intranasal OT administration in humans (Striepens et al., 2013) and rodents (Neumann et al., 2013, Smith et al., 2019b), remaining elevated up to 60 minutes post-application. Bearing in mind that OTRs are also abundantly expressed in the periphery, such as uterus, heart and kidney (Kimura et

al., 1998), any chosen dose has to strike an optimal balance between achieving its intended central effects without eliciting unwanted peripheral side-effects.

Thus, it is of interest to investigate if the novel conjugate peptide OT-GET (evaluated in this thesis) will achieve optimal brain penetration using lower levels of OT to minimise peripheral side-effects.

4.3 Phencyclidine (PCP) model for schizophrenia

In addition to examining brain penetration, it was also important to compare behavioural effects elicited by OT-GET versus OT alone.

PCP is an NMDA receptor antagonist that has been shown to reinstate and aggravate psychosis in patients with schizophrenia and induce cognitive and social dysfunction in normal volunteers (Jones et al., 2011, Jentsch and Roth, 1999). Psychosis represents the significant feature of patients with schizophrenia (but is also a common feature of multiple other psychiatric disorders) and its definition requires the presence of hallucination, delusions or both hallucinations without insight and delusions (Arciniegas, 2015). The first episode of psychosis usually occurs during adolescence and young adulthood with a higher incidence rate in men (McGrath et al., 2004, Jongsma et al., 2019). In line with the hypoglutamatergic theory of schizophrenia, the PCP rodent model exhibits hypofunctioning NMDA receptors on GABA interneurons which increases excitoxicity in the hippocampus and PFC due to decreased inhibition (Jones et al., 2011). Over time, this leads to apoptosis and hypodopaminergic activity in the mesocortical pathway, believed to be responsible for the negative and cognitive symptoms of schizophrenia (Davis et al., 1991). On the other hand, increased non-NMDA receptor glutamate mediated transmission increases dopamine levels in the striatal regions and is believed to be responsible for the observed hyperactivity (Takahata and Moghaddam, 2003), used as an index for positive symptoms. As a consequence of the resemblance of long-term effects of PCP administration and putative changes seen in

schizopohrenia, sub-chronic PCP is often used as a pharmacological model of schizophrenia in rodents, akin to other genetic or neurodevelopmental models.

A valid robust animal model of any disorder should aim to fulfil the triad of criteria: construct, face and predictive (Figure 4.1).

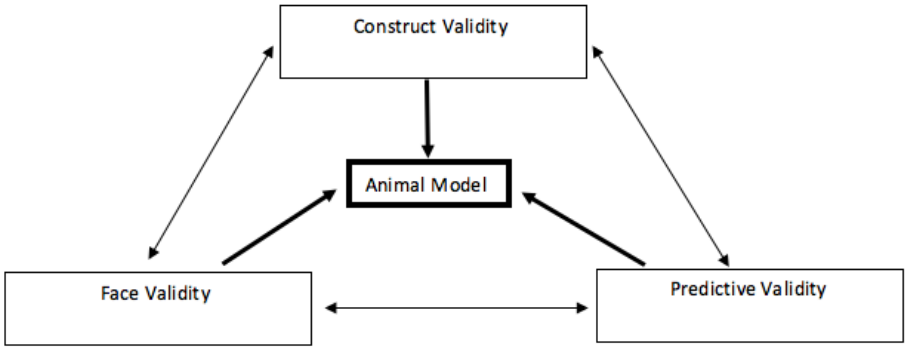


Figure 4.1 A good animal model should fulfil the above triad of validity. In respect to a schizophrenia model: construct validity refers to a reliable aetiology causing the disease in humans to be replicated in the animal model; face validity refers to the core behavioural symptoms encompassing positive, negative and cognitive symptoms which should be reflected in the animal model; predictive validity indicate that the animal model should respond as expected to existing therapy with antipsychotic treatment as seen in humans.

Although it is impossible to recreate a perfect model akin to a human disorder as replication for symptoms of psychosis such as hallucinations and delusions cannot be confirmed in animals, the PCP model is used as it produces positive, negative and cognitive symptoms akin to that of patients, with the chronic model producing more robust cognitive deficits encompassing more cognitive impairments (Amitai et al., 2007) compared to the acute model. The PCP model hold some construct validity in the fact that chronic administration in rodents induces neurochemical changes similar to that of patients: decreased prefrontal cortex dopamine levels (Jentsch and Roth, 1999) , reduced hippocampal parvalbumin-immunoreactive neurones (Jenkins et al., 2010, Wiescholleck and Manahan-Vaughan, 2013) as well as reduction in GABA sysnthesising enzyme GAD67 mRNA and protein (Bullock et al.,

2009, Hasimoto et al., 2003). However, some of the cognitive impairments seen do not persist beyond treatment and hyperactivity as an index of positive symptoms has been critiqued (refer next paragraph) as well as the fact that the administration of PCP to adult rats fail to take into consideration the proposed neurodevelopmental origin of schizophrenia (Jones et al., 2011), As such, attempts have been made to address these concerns such as the dual-hit neonatal PCP and post weaning social isolation rat model which produces a wider range of more severe cognitive impairments (Gaskin et al., 2011). Predictive validity of the model remains controversial as antipsychotics, such as clozapine and haloperidol, show inconsistent ability to reverse PCP-induced cognitive effects (Jentsch and Roth, 1999, Jones et al., 2011).

The work in this chapter examined the effects of intranasal OT and OT-GET on acute PCP-induced hyperactivity as an index for 'antipsychotic-like' activity (Jones et al., 2011). Hyperactivity induced by acute PCP reflect an increase in non-NMDAr glutamatergic neurotransmission and subsequent increase in limbic-striatal DA levels (relevant to the hyperdopaminergic mesolimbic theory) and are reversed by first and second generation antipsychotics in rodents (Freed et al., 1984, Kitaichi et al., 1994). However, hyperactivity as a readout for positive symptoms have been criticised as the relationship between the two is not exclusive seeing that drugs such as delta-9-tetrahydrocannabinol are able to transiently exacerbate positive symptoms in patients with schizophrenia (D'Souza et al., 2005) yet is cataleptic in rodents (Pertwee and Greentree, 1988). Furthermore, hyperactivity as a phenotypic readout is not exclusive only to schizophrenia but also a hallmark of other neuropsychiatric disorders such as Attention Deficit and Hyperactivity Disorder. Yet, considering as it is impossible to completely model or confirm psychosis in animals, the best consideration is to choose a behaviour readout that best conserves the neural circuitry in humans. In this case, the acute PCP-induced hyperactivity was chosen over the chronic PCP model, which displays hypersensitivity to subsequent PCP administration with reduced frontal blood flow, consistent with the

hypofrontality observed in patients (Jentsch and Roth, 1999) as opposed to the increased forebrain dopaminergic transmission seen in acute PCP. The acute-PCP hyperactivity behavioural assay is a quick and straightforward assay to assess for a biological readout of OT/OT-GET's potential behavioural effect. This will allow us to decide if further investigation is justifiable using a longer-term, more expensive model. Furthermore, a robust positive control have been replicated in the lab which reverses the observed hyperactivity (Abrahams et al., 2020, Kohli et al., 2019), allowing for confidence in the study paradigm to be carried out.

As of now, there have not been any studies utilising the PCP rodent model to investigate socio-behavioural changes post-intranasal OT. However, intra-amygdala infusion of 1000ng OT increased social interaction (Lee et al., 2005) in a chronic-PCP rat model of schizophrenia, whereas in an acute-PCP rat model, 0.1mg/kg of s.c. OT decreased PCP-induced hyperactivity and increased social interaction (Abrahams et al., 2020, Kohli et al., 2019).

4.4 Aims of Chapter

In order to evaluate the potential blood brain penetration of OT-GET *in vivo* this chapter aims to examine the acute effects of intranasal administration of oxytocin compared with an oxytocin-peptide conjugate (OT-GET) on:

i. Body temperature

ii. Locomotor activity

iii. PCP-induced hyperactivity

iv. Social interaction

v. Extent of brain penetration of oxytocin

4.5 Materials and methods

4.5.1 Animals

Male adult Lister Hooded rats (150-200g; Charles River UK) were housed in line with ARRIVE guidelines in three-four per cage in individually ventilated cages (56 x 38 x 22cm) lined with sawdust and with cardboard tubes and wooden blocks for environmental enrichment. Rats were maintained in the University animal facility in a controlled environment (21 ± 2ºC and humidity 55 ± 15% on a 12h light-dark cycle, lights on at 0600h). Food (Beekay rat and mouse diet, B& K Universal Ltd, UK) and water were available *ad libtium*. Behavioural experiments were conducted during the light phase between 0800 – 1700h. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act, 1986 with approval from the University of Nottingham Local Ethical Committee.

Adult male rats were used for all behavioural studies as to avoid any sex-related confounding effects as estrogen has been showed to affect social behaviour in female rodents (Garcia et al., 2016) as well as regulate OTR expression (Jurek and Neumann, 2018) and use of juvenile animals, although more relevant to the pathophysiology of schizophrenia, was avoided due to ethical reasons as this was the first use of OT-GET *in vivo* for behavioural testing. All testing was done in the light phase due to practical reasons such as access to facilities.

4.5.2 Intranasal administration

Previous studies have shown intranasal OT increased social interaction/affiliation behaviour in rats at 20µg (Calcagnoli et al., 2015, Dai et al., 2018, Kent et al., 2016). Taking into consideration that conjugation with GET may increase brain penetration of OT, a starting dose of 10µg OT (termed ‘low’ dose hereafter) was chosen for this study as the degree of improved brain penetration when OT was conjugated with cell penetrating peptide GET was unknown.

Rats were handled 3-5 days prior first behavioural test to accustom them to intranasal administration. On the day of experiment, intranasal administration was carried out according to Lukas and Neumann (2012). Briefly, rats were lightly restrained and 10 µL of saline/OT/OT-GET was distributed uniformly to the squamous epithelium of the left and right rhinarium. Oxytocin acetate and GET peptide were provided by Bachem, U.K. and the Dixon lab, Centre for Biomolecular Sciences, University of Nottingham, respectively. The solution was applied over 15 seconds and rats were restrained in the same position for an additional minute before returning to locomotor activity boxes/home cages.

4.5.2.1 Study 1: Low dose OT

First group of rats (n=12/group) received intranasal saline (10µL), OT alone (10µg), or conjugated to GET (10µg GET: low OT-GET 1:1 or 1000µg GET: low OT-GET 1:100) and were used to examine the effect in body temperature, LMA and PCP-induced hyperactivity tasks.

4.5.2.2 Study 2 and 3: High dose OT

As the initial dose of 10µg OT did not produce any behavioural changes between OT or OT-GET, the dose was increased 10-fold to 100µg (termed ‘high’ dose hereafter) in future studies.

First group of rats (n=4/group) in the high dose study received saline (10µL), OT (100µg) alone or conjugated to GET (100µg GET: high OT-GET 1:1 or 1000µg GET: high OT-GET 1:10) or systemic OT (0.1mg/kg, s.c.) and were examined in the PCP-induced LMA task. Due to the COVID-19 lockdown, we were unable to carry out the second half of the experiment to increase n numbers to match that of the low dose group. This was referred to as Study 2.

Instead, a second group of rats (n=9-10/group) received intranasal saline (10µL), OT (100µg) alone or conjugated to 100µg GET (high OT-GET 1:1; dose chosen based on results from first high dose study) or systemic OT (0.1mg/kg, s.c.) and PCP-induced LMA and social interaction tasks examined. This was referred to as Study 3.

4.5.3 Temperature recording

High doses of peripheral OT (0.3mg/kg i.p. and above) causes hypothermia and suppresses locomotor activity in rats (Hicks et al., 2014, Klenerova et al., 2009, Kohli et al., 2019). Consequently, it was important to ensure that the chosen OT/OT-GET doses did not produce confounding effects on body temperature regulation.

Temperature microchips (Bio-Thermo idENTICHIP; AnimalCare Ltd, UK) were implanted subcutaneously 4 days prior to first behavioural test to enable recording of core body temperature using a digital chip reader commencing 30 minutes after habituation. Baseline temperature was established upon administration of intranasal OT/OT-GET and readings measured at 15 minutes intervals during locomotor activity for 90 minutes.

4.5.4 LMA recording

Rats were placed in an unfamiliar Perspex arena (39cm x 23.5cm x 24.5cm) and locomotor activity using a computerised infra-red activity system (Photobeam Activity System-Home Cage, San Diego Instruments, CA, USA) was recorded in low light (40LUX). Spontaneous ambulatory activity, fine movement and rears were recorded for 120 minutes in 5 minutes epochs. Ambulation was recorded when two consecutive adjacent lower beams were interrupted, rears when a separate layer of upper beams were interrupted and fine movement when longitudinal beams were interrupted. Saline/OT/OT-GET were delivered intranasally after 30 minutes of habituation. In the PCP study, saline or PCP (Sigma Aldrich, U.K; 5.6mg/kg) were administered i.p. 30 minutes after first administration of saline/OT/OT-GET. Locomotor activity was recorded for an additional 60 minutes (Figure 4.2).

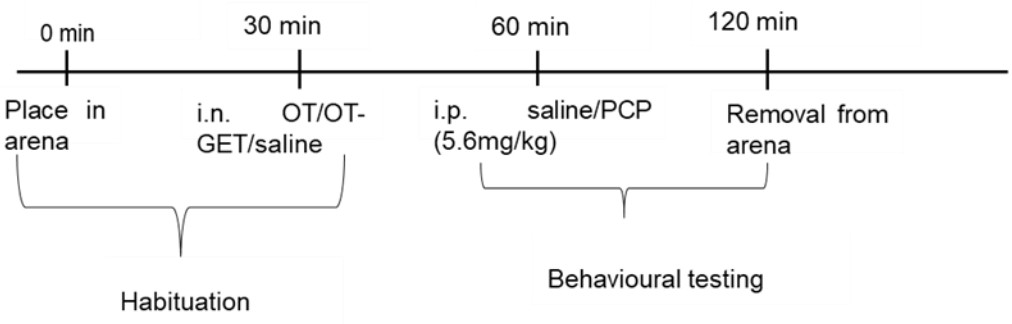


Figure 4.2 Protocol timeline of the PCP-induced LMA task.

Rats were administered intranasal saline/OT/OT-GET after 30 minutes of habituation and saline/PCP 30 minutes later. Behaviour was recorded for an additional hour before removal from arena. I.n.= intranasal; i.p.= intraperitoneal, min=minutes.

In PCP-induced LMA tasks, the combination of saline/OT/OT-GET with saline/PCP resulted in four treatment groups for Study 1 (n=12/group): saline/saline, saline/PCP, OT/PCP, OT-GET (1:10)/PCP, six treatment groups for the Study 2 (n=4/group): saline/saline, saline/PCP, OT/PCP, OT (s.c.)/PCP, OT-GET (1:1)/PCP and OT-GET (1:10)/PCP and five treatment groups: saline/saline, saline/PCP, OT/PCP, OT (s.c.)/PCP, OT-GET (1:1)/PCP for Study 3 (n=9-10/group). Table 4.2 shows the treatment groups used for each study.

Study 1	Study 2	Study 3
Saline/saline	Saline/saline	Saline/saline
Saline/PCP	Saline/PCP	Saline/PCP
OT (i.n.)/PCP	OT (i.n.)/PCP	OT (i.n.)/PCP
OT-GET (1:10)/PCP	OT-GET (1:1)/PCP	OT-GET (1:1)/PCP
	OT-GET (1:10)PCP	OT (s.c.)/PCP
	OT (s.c.)/PCP	

Table 4.2 Treatment groups for Study 1-3.

Study 1 is referred to as ‘low dose study’ where OT concentration used was 10µg. Study 2 and 3 is referred to as ‘high dose study’ where OT concentration used was 100µg. I.n.: intranasal, s.c.: subcutaneous

A full Latin square design for treatment groups was not achieved in this case as the primary aim of the study was to examine OT/OT-GET's ability to reverse PCP-induced LMA. Furthermore, prior examination of OT/OT-GET alone on normal LMA showed no sedation effect in Study 1, thus it it unlikely that the second administration of saline would alter OT/OT-GET's effect on LMA.

4.5.5 Social interaction

Previous literature suggest increased social interaction after intranasal OT administration (Calcagnoli et al., 2015, Huang et al., 2014), therefore a social interaction task was included for rats in Study 3. After a 7-day washout period following PCP-induced LMA task, rats were divided into three treatment group (n=8 pairs/group) receiving: intranasal saline (10µL), OT (100µg) and high OT-GET 1:1 and individually housed overnight to enhance time spent in social interaction (Niesink and van Ree, 1982). On the day of experiment, pairs of rats matched for body weight and drug treatment but from different litters and cages were administered saline/OT/OT-GET and sprayed on the nape with green or red hair dye (Claire's Temporary Hair Colour, U.K.) 10 minutes before task. Forty minutes after drug administration, both rats were placed in an unfamiliar circular arena of 75cm diameter and 45cm wall and drug effect on social interaction (10 minutes duration) determined using Ethovision XT v8.5, Noldus in low light (40 LUX). The time choice of 40 minutes for OT/OT-GET pre-treatment was chosen as high OT-GET 1:10 but not high OT-GET 1:1 showed a significantly higher ambulatory count compared to saline treated rats at this particular time point in Study 2, suggesting this may be the peak timing of which OT-GET exerts its effect.

Resultant behaviour was scored manually via video recording obtained from Ethovision software. The scorer was trained by another experienced member of the lab and competency ensured via practice trial comparisons with the trainer where timings for behavioural measures differ no more than 5 seconds (max). It is to be noted that scorer was not blinded to group treatments as drug preparation was done by the scorer, however treatments were pseudo-labelled immediately upon preparation

and remained as such until completion of data analysis and the long period (weeks) between drug preparation and data analysis further ensured no bias occurred. Behaviour was divided into prosocial behaviour: body sniffing, ano-genital sniffing, following, crawling over and under and lying side-by-side and aggressive behaviour: boxing and biting and pinning.

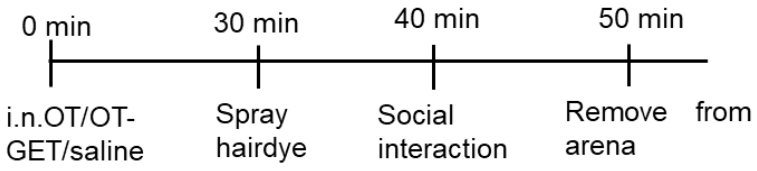


Figure 4.3 Protocol timeline for the social interaction task.
Rats were administered intranasal saline/OT/OT-GET and 30 minutes later, green or red hairspray applied and allowed to settle for an addition 10 minutes before being subjected to task for 10 minutes. I.n.: intranasal.

4.5.6 Collection of brain and nose tissue

Brain tissue was collected on separate days after all behavioural tasks were completed to examine extent of OT-GET brain penetration.

For Study 1, fluorescein isothiocyanate (FITC) was chosen as a fluorescent tracer to conjugate with GET as it is affordable, highly absorptive and has good water solubility. Rats were administered intranasal saline (10µL), OT (10µg), low OT-GET 1:100 (with and without FITC) and 1000µg GET (with and without FITC). Rats were killed by concussion followed by decapitation 60 minutes later. This particular time point was chosen based on Neumann et al. (2013) who found a significant increase and peak in rat's OT levels in amygdala and hippocampus extracellular fluid (ECF) microdialysate samples at 60 minutes after intranasal delivery.

For Study 3, the fluorescent marker was switched to tetramethylrhodamine isothiocyanate (TRITC)-AF488 as auto-fluorescence in paraformaldehyde (PFA) fixed tissues was observed in

FITC brain samples and FITC emission wavelength (488nm) was in close range to 4′,6-diamidino-2-phenylindole (DAPI, 405nm), which increased excitation light carryover. Rats were intranasally administered saline (10µL), OT (100µg i.n. and 0.1mg/kg s.c.), high OT-GET 1:1 (with and without TRITC). Rats were killed by concussion followed by decapitation 40 minutes later, selected as the apparent peak effect of OT/OT-GET on reducing hyperactivity was 10 minutes post-PCP treatment (40 minutes after OT/OT-GET was administered).

Brains and nose tissue were rapidly dissected following concussion and decapitation. The nose was cut perpendicular approximately as shown in Figure 4.4. A mid-sagittal cut was made for the brain with one half dissected to obtain the hypothalamus, hippocampus, frontal cortex and olfactory bulb, which were transferred to liquid nitrogen immediately before subsequent storage at -80^{o}C. Noses and the other sagittal halves of the brains were stored in 4% PFA in PBS and 24h later, transferred to 30% sucrose in PBS solution before snap freezing in isopentane. Samples were stored at -80^{o}C.

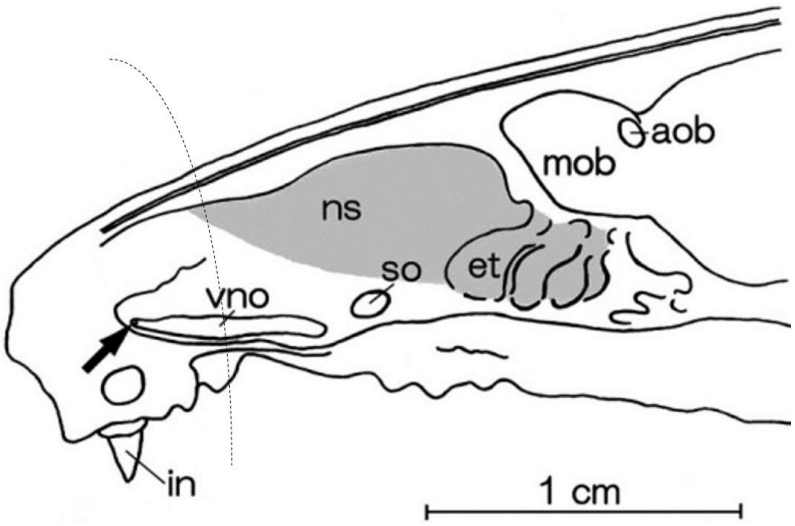


Figure 4.4 Parasagittal section of rat's nasal cavity.

A section of the nose (indicated by the dotted line) was removed from concussed and decapitated rats and immediately stored in 4% PFA and 30% sucrose solution 24h later. Noses were snap freezed in isopentane after an additional 24h. aob:accessory olfactory bulb, et: endoturbinates, in: incisor, mob: main olfactory bulb, ns: nasal septum, so: septal olfactory organ of

Masera, vno: vomeronasal organ. The olfactory epithelium is the shaded region. Figure taken from Taniguchi and Taniguchi (2014).

4.5.7 Confocal scanning microscopy

Confocal scanning microscopy was used to visualise extent of OT-GET brain penetration post 60 minutes of intranasal delivery in rats in Study 1.

Sagittal brain and nose tissue sections were coated with OCT embedding matrix (VWR International Ltd, UK) and sectioned at 60µm (brain) and 40µm (nose) using a freezing microtome (Anglia Scientific, U.K.). Sections were floated out on PBS solution, stained with DAPI 1:2000 and mounted on 3-amino-propylmethoxysaline (APES)-coated slides with DABCO fluorescent mounting medium. Slides were visualised under a Zeiss LSM710 confocal laser scanning microscope (Zeiss LSM710 META, Germany) with images acquired using Zen Black 2012 Imaging Software and processed using Fiji image processing software.

4.5.8 ELISA analysis of OT levels in the brain

In Study 3, the olfactory bulb and hippocampus was chosen as regions for investigation as the olfactory bulb is believed to be the main brain region targeted post intranasal delivery of peptides (Crowe et al., 2018), while OT peak in hippocampus dialysate have been observed in rats (Neumann et al., 2013) and mice (Smith et al., 2019a), 30-60 minutes post-drug administration.

Tissue samples were homogenised at 4^{o}C at 1mL RIPA buffer (Sigma-Aldrich, U.K.) per 100mg tissue and 100µL protease inhibitor cocktail (Sigma-Aldrich, U.K.) per 1mL RIPA buffer before subjected to sonification ~10 seconds (Soniprep 150, MSE U.K.) then vertical disc rotation at 4^{o}C for an hour. Resulting supernatants were subjected to a Lowry assay for protein level estimation before adjusted to a common protein concentration of 8mg/mL for the olfactory bulb and 12mg/mL for the hippocampus.

For quantitative determination of OT concentration, neat samples were used with a commercial OT ELISA kit (Enzo, USA, ADI-900-153A-0001) with a sensitivity range of 15.6 – 1,000 pg/mL, intra-assay precision at low, medium and high concentrations of 12.6, 10.2, and 13.3% respectively and inter-assay precision at low, medium and high concentrations of 20.9, 16.5, and 11.8% respectively. OT levels were determined using a microplate reader (SpectraMax M5e, Molecular Devices) in singular samples in order to accommodate for all samples as there was a possibility that endogenous OT levels may be too low for assay detection in saline controls, which may lead to loss of n numbers for data analysis (funding limitations provided only enough resources for a single 96 well plate to be ran). Furthermore, both areas of the brain were of equal interest to be examined: the olfactory bulb is well documented as the first point of entry and OT increase in hippocampus microdialysate post-intranasal delivery have been noted by Neumann et al. (2013) and Smith et al. (2019b). The coefficient of variant for controls ran in duplicates was 3.8% and final data was analysed using SoftMaxPro 7.0 software against a linear regression standard curve (R^2=0.9573).

4.5.9 Statistical Analysis

Statistical analysis were conducted using GraphPad Prism 7 and differences between groups assessed using one-way or two-way repeated measures (RM) analyses of variance (ANOVA) with Tukey post-hoc where applicable. Data were assessed for normality using D'Agostino-Pearson normality test. All test carried out were two-sided, results presented as mean ± SEM with an alpha criterion of $p<0.05$. Simple randomisation of treatment groups were carried out in Microsoft Excel 2016 using the RAND() function. Final treatment groups were cross-checked to ensure animals in each cage received different treatments and that total weight of animals between groups were approximately similar. Group sizes for treatment groups were determined based on preliminary data from another PhD student (Abigail Abrahams) as well as Kohli et al. (2019) using the NC3R Experimental Design Assistant (https://eda.nc3rs.org.uk/).

4.6 Results

4.6.1 Effect of low dose intranasal OT and OT-GET on general LMA and body temperature in otherwise healthy rats

In order to ensure the selected intranasal OT/OT-GET dose did not cause hypothermia or sedation, effect on core body temperature and LMA was investigated in Study 1.

During LMA, rats' core body temperature progressively decreased over 90 minutes post-intranasal drug administration ($F_{(7,301)}= 34.52$, $p=0.0001$; Figure 4.5). Low dose OT/OT-GET treatment did not influence this hypothermia ($F_{(3, 43)} = 0.9119$, $p=0.4432$) and there were no time x treatment interaction ($F_{(21, 301)} = 0.6271$, $p=0.8980$).

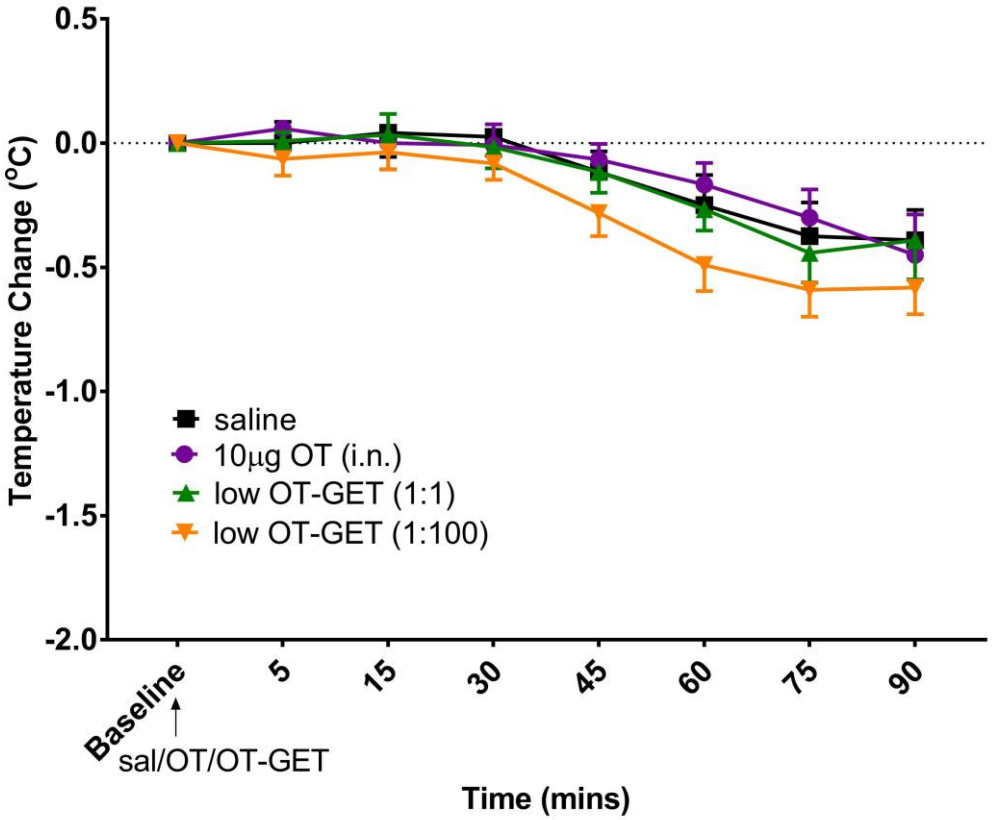


Figure 4.5 Effects of intranasal OT/OT-GET on core body temperature.
A progressive decrease in core body temperature of adult male LH rats' over 90 minutes post intranasal saline/10µg OT/low OT-GET (1:1 and 1:100), measured using radio-telemetry from chips implanted in the nape of the neck, from baseline (°C, mean ± SEM, n=12 per group except (n=11) OT-GET (1:100)) was observed. Two-way RM ANOVA revealed a main effect of time only ($F_{(7, 301)}$ = 34.52, p=0.0001) on core body temperature change, which was not affected by OT/OT-GET treatment ($F_{(3, 43)}$ = 0.9119, p=0.4432) or time x treatment interaction ($F_{(21, 301)}$ = 0.6271, p=0.8980). I.n. = intranasal, sal=saline.

Figure 4.6 shows a representative 2h timeline during which rats underwent LMA with intranasal saline/OT/OT-GET administered after 30 minutes of habituation. The 30 minutes of habituation ensured all rats exhibited a low level of locomotor activity prior to drug treatment and allowed for identification of any drug-induced LMA changes. This period of habituation was excluded from any statistical analysis in subsequent figures.

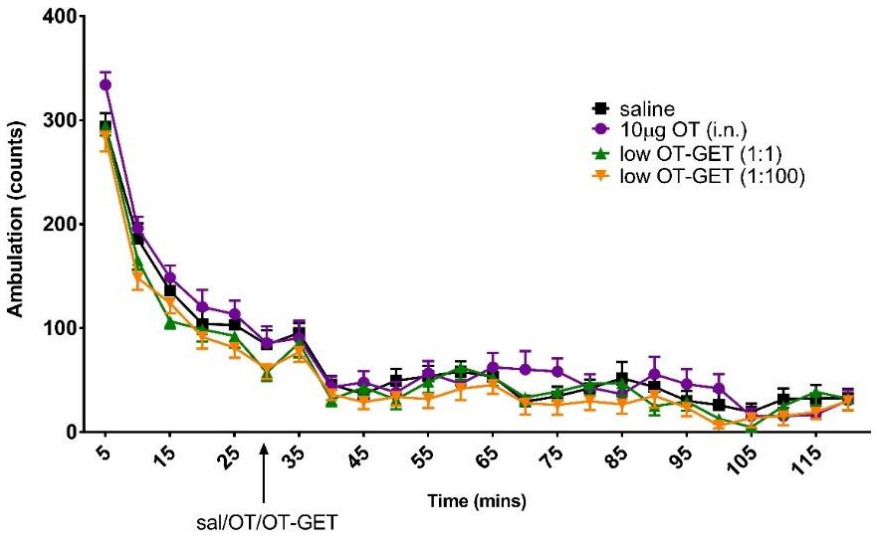


Figure 4.6 Representative 2 hours timeline for LMA task.

Time course change in ambulatory counts of male adult LH rats (mean ± SEM, cumulative counts/5 minutes epoch, n=12 per group) was recorded over two hours. Rats were placed in individual Perspex boxes and allowed to habituate to their new surroundings for 30 minutes before intranasal saline/10µg OT/low OT-GET (1:1) and (1:100) was administered. Locomotor activity was observed for an additional 90 minutes before returning to home cages. I.n. = intranasal, sal=saline.

Figure 4.7A – C shows that the low dose OT/OT-GET did not affect rats' normal ambulatory locomotor activity ($F_{(3, 44)}$ = 1.606, p=0.2020), rears ($F_{(3, 41)}$ = 0.1225, p=0.946) or fine movement ($F_{(3, 44)}$ = 0.197, p=0.2646), all of which progressively reduced with time post treatment (ambulation: $F_{(18, 774)}$ = 13.19, p=0.0001; rears: $F_{(18, 738)}$ = 7.469, p=0.0001; fine: $F_{(18, 792)}$ = 12.04, p=0.0001). There was no time x treatment interaction across all movement types (ambulation: ($F_{(54, 774)}$ = 0.7615, p=0.8955); rears: ($F_{(54, 738)}$ = 0.8574, p=0.7570); and fine movement: ($F_{(54, 792)}$ = 0.767, p=0.8885)).

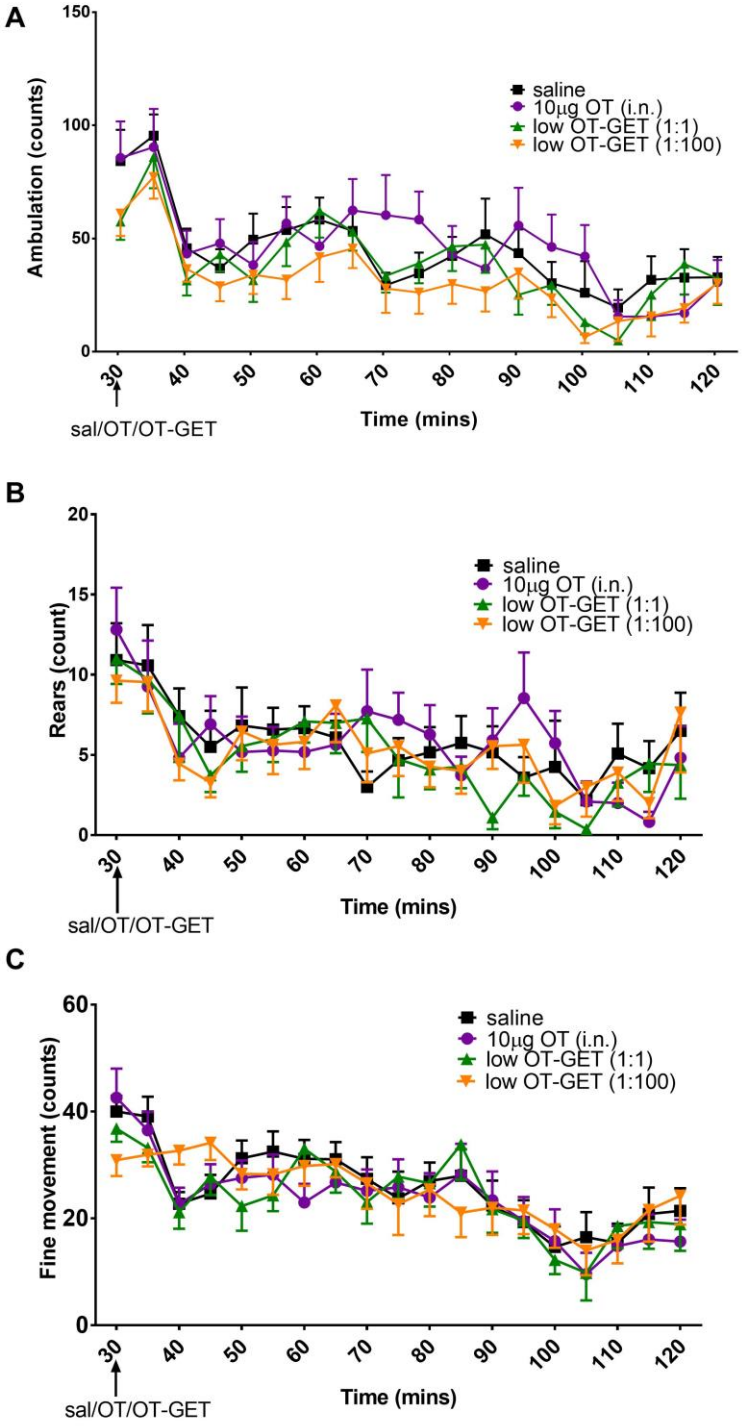


Figure 4.7 Effect of low dose intranasal OT and OT-GET on locomotor activity.

Comparative effect of saline/10µg OT/ low OT-GET (1:1) and (1:100) on (mean ± SEM, cumulative counts/5 minutes epoch, n=11-12 per group) (A) ambulatory, (B) rears and (C) fine movement from drug application, 30 – 120 minutes after placement in the box. Two-way RM ANOVA revealed a main effect of time only

across all LMA parameters (ambulation: $F_{(18, 774)}$ = 13.19, p=0.0001; rears: $F_{(18, 738)}$ = 7.469, p=0.0001; and fine: $F_{(18, 792)}$ = 12.04, p=0.0001) with the first common pre-drug 30 minutes habituation excluded from statistical analysis. There was no main effect of OT/OT-GET treatment (ambulation: $F_{(3, 44)}$ = 1.606, p=0.2020; rears $F_{(3, 41)}$ = 0.1225, p=0.946; and fine movement: $F_{(3, 44)}$ = 0.197, p=0.2646), or time x treatment interaction (ambulation: $F_{(54, 774)}$ = 0.7615, p=0.8955; rears: $_{(54, 738)}$ = 0.8574, p=0.7570; and fine movement: $F_{(54, 792)}$ = 0.767, p=0.8885) on LMA. I.n. = intranasal, sal=saline.

Saline/OT/OT-GET treatment did not significantly reduce cumulative ambulatory, rears and fine movement counts recorded between 30-120 minutes post drug treatment (ambulation: $F_{(3, 43)}$ = 1.606, p=0.2020; rears: $F_{(3, 41)}$ = 0.1225, p=0.9463; and fine movement: $F_{(3, 44)}$ = 0.197, p=0.8979, one-way ANOVA; Figure 4.8A-C).

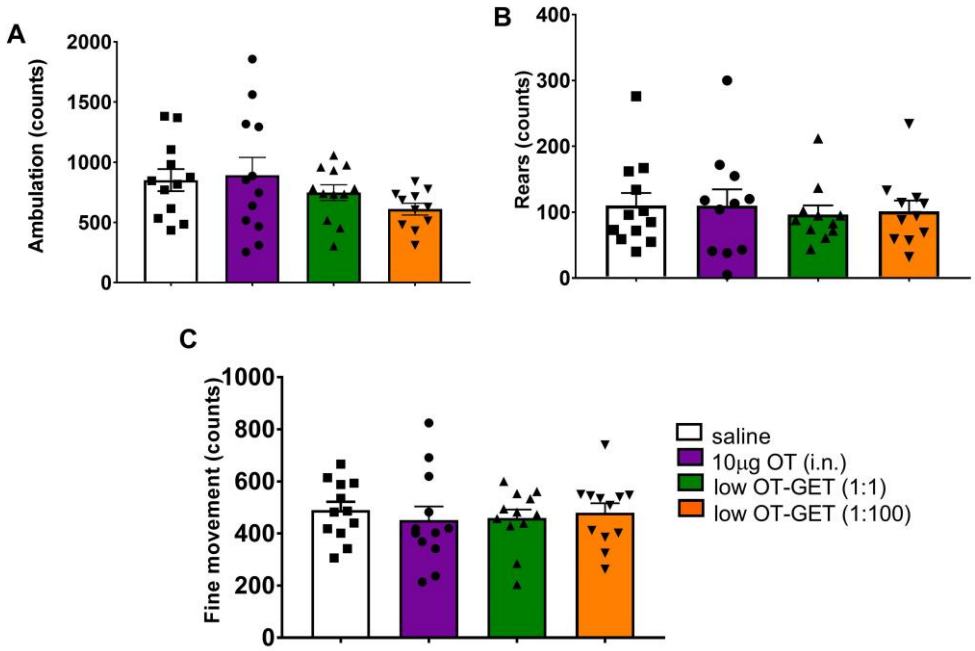


Figure 4.8 Effect of low dose intranasal OT and OT-GET on cumulative locomotor activity counts.

Comparative effect of saline/10µg OT/ low OT-GET (1:1) and (1:100) on cumulative (mean ± SEM, n=11-12 per group) (A) ambulatory (B) rears and (C) fine movement counts in the 90 minutes following drug injection. One-way ANOVA revealed no treatment effect on any locomotor activity counts

(ambulation: $F_{(3, 43)}$ = 1.606, p=0.2020; rears: $F_{(3, 41)}$ = 0.1225, p=0.9463; and fine movement: $F_{(3, 44)}$ = 0.197, p=0.8979).

4.6.2 Effect of intranasal OT and OT-GET on temperature, PCP-induced hyperactivity, social interaction and brain penetration

4.6.2.1 Study 1: Low dose OT treatment

As both doses of OT-GET had no significant impact on core body temperature and LMA, low OT-GET (1:100) was chosen for subsequent temperature, PCP-induced LMA studies with concomitant OT brain penetration analysis to assess if any brain penetration of OT occurred.

4.6.2.1.1 Temperature

During LMA, rats showed a significant and progressive decrease in core body temperature over 90 minutes ($F_{(7, 294)}$ = 148.3; p=0.0001) post saline/OT/OT-GET administration ($F_{(3, 42)}$ = 9.936; p=0.0001), with a main time x treatment interaction ($F_{(21, 294)}$ = 9.3; p=0.0001; Figure 4.9) observed. The maintained drop in temperature at 60 – 90 minutes (30 minutes after the second drug administration of either saline or PCP) was significant for all rats that received PCP compared to saline, regardless of the earlier treatment with saline, OT or OT-GET ($p<0.001$). Rats that received OT-GET followed by PCP had an earlier drop in temperature at 45 minutes compared to saline controls that did not receive PCP ($p<0.01$). Within PCP treated rats, a significant drop in temperature was noted for animals pre-treated with OT-GET ($p<0.05$) compared to those given saline at 60 minutes.

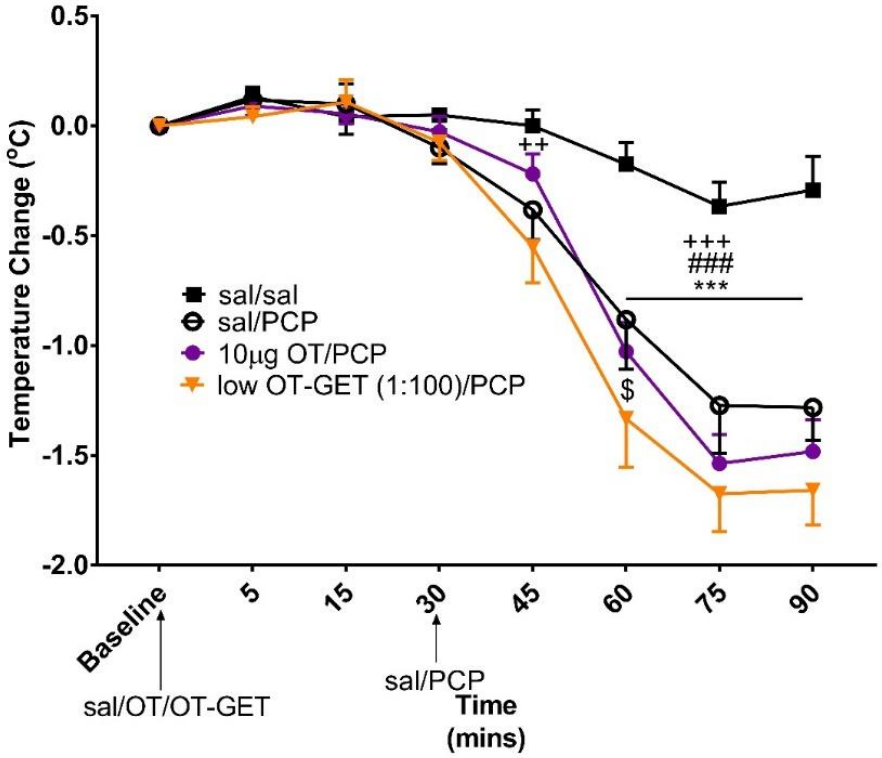


Figure 4.9 Effects of low dose OT and OT-GET on core body temperature in rats administered PCP.

A progressive and significant decrease in core body temperature from baseline (°C, mean ± SEM, n=11-12 per group) occurred following PCP (5.6mg/kg, i.p.) administration in adult male LH rats. Two-way RM ANOVA revealed a main effect of time ($F_{(7, 294)}$ = 148.3; p=0.0001), treatment ($F_{(3, 42)}$ = 9.936; p=0.0001) and time x treatment interaction ($F_{(21, 294)}$ = 9.3; p=0.0001). *** (p<0.001) sal/sal vs sal/PCP, ### (p<0.001) sal/sal vs OT/PCP, ++ (p<0.05), +++ (p<0.001) sal/sal vs OT-GET/PCP, $ (p<0.05) sal/PCP vs OT-GET/PCP; Tukey post-hoc. Sal=saline.

4.6.2.1.2 Locomotor activity

To analyse PCP-induced change in horizontal ambulation, rears and fine movement, locomotor activity was recorded over 120 minutes, where first drug injection of intranasal saline/OT/OT-GET was delivered at 30 minutes and saline or PCP (5.6mg/kg, i.p.) 30 minutes later. The first 60 minutes of activity (30 minutes of habituation and 30 minutes post intranasal OT/OT-GET), where a basal response was acquired to ensure all rats had habituated to the chambers to allow for subsequent

identification of any PCP-induced hyperactivity change, was excluded from statistical analysis and following figures. All three parameters (Figure 4.10A-C) attenuated with time (ambulation: $F_{(12, 492)}$ =67.46, p=0.0001; rears: $F_{(12, 504)}$ = 46.35, p=0.0001; and fine movement: $F_{(12, 528)}$ = 40.85, p=0.0001) and were altered by PCP such that there was a main effect of treatment (ambulation: $F_{(3,41)}$ =8.187, p=0.0002; rears: $F_{(3,42)}$ =6.944, p=0.0007; and fine movement: $F_{(3,44)}$ = 16.21, p=0.0001) as well as a time x treatment interaction (ambulation: $F_{(36,492)}$ =5.031, p=0.0001; rears: $F_{(36,504)}$ = 1.491, p=0.0358; and fine movement: $F_{(36, 528)}$=2.382, p=0.0001). Saline, OT or OT-GET pre-treatment did not reverse the magnitude of LMA responses induced by PCP.

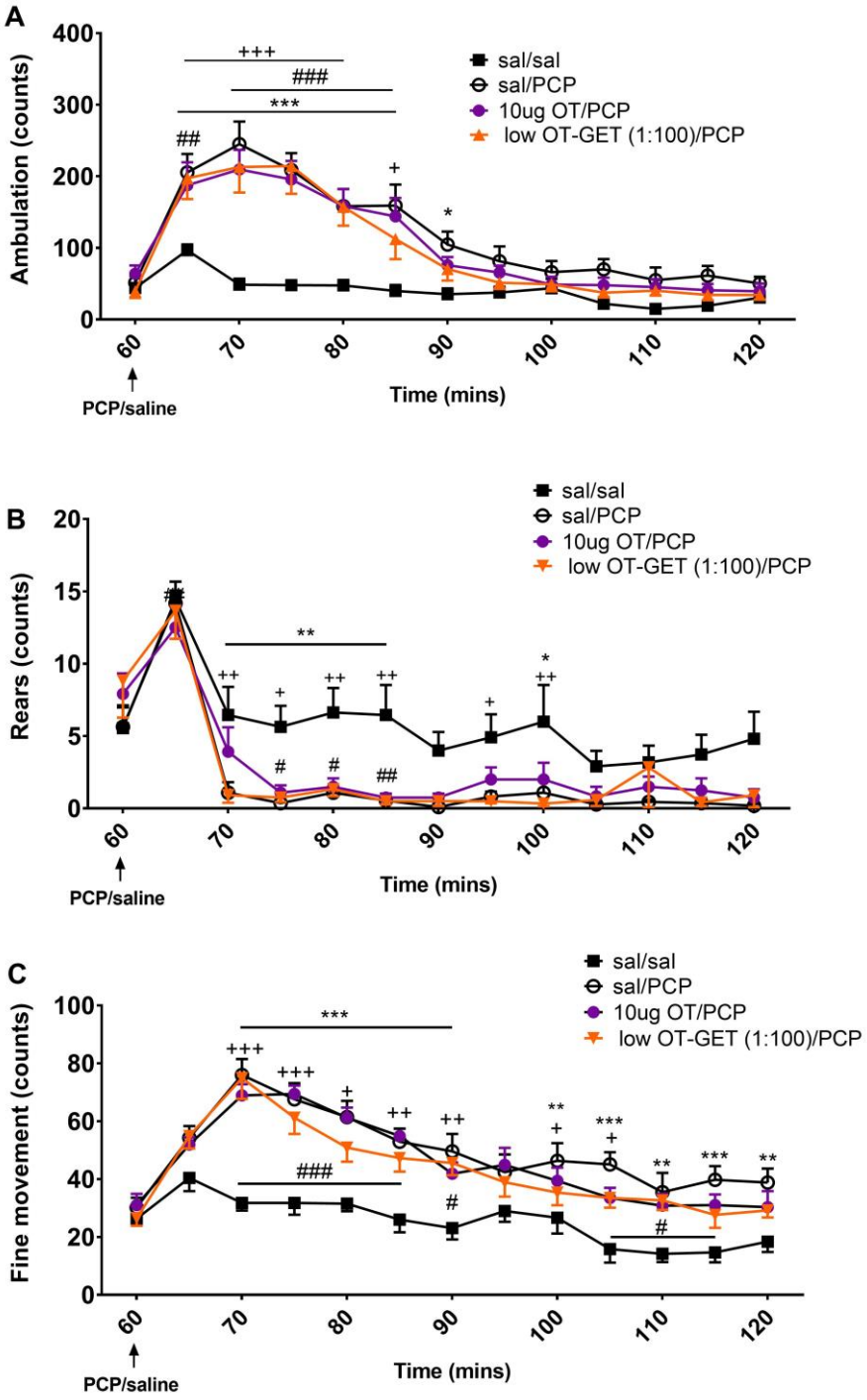


Figure 4.10 Effect of low dose OT and OT-GET on locomotor activity in rats administered PCP.

Comparative effect of intranasal saline/10µg OT/ low OT-GET (1:100) pre-treatment, given 30 minutes prior saline or PCP (5.6mg/kg, i.p.) at 60 minutes, on (mean ± SEM cumulative counts/5 min epoch, n=11-12 per group) (A) ambulatory, (B) rears and (C) fine movement counts, 60 – 120 minutes after placement in box. Two-way RM ANOVA, revealed a main effect of time across

all LMA parameters (ambulation: $F_{(12, 492)}$ =67.46, p=0.0001; rears: $F_{(12, 504)}$ = 46.35, p=0.0001; and fine movement: $F_{(12, 528)}$ = 40.85, p=0.0001) with the first 30 minutes of habituation and subsequent 30 minutes of OT/OT-GET activity excluded from statistical analysis. A main effect of treatment (ambulation: $F_{(3,41)}$ =8.187, p=0.0002; rears: $F_{(3,42)}$ =6.944, p=0.0007; and fine movement: F(3,44) = 16.21, p=0.0001) and time x treatment interaction (ambulation: $F_{(36,492)}$ =5.031, p=0.0001; rears: $F_{(36,504)}$ = 1.491, p=0.0358; and fine movement: $F_{(36, 528)}$=2.382, p=0.0001) was also observed. *(p<0.05), ** (p<0.01), *** (p<0.001) sal/sal vs sal/PCP; # (p<0.05), ## (p<0.01), ### (p<0.001) sal/sal vs OT/PCP; + (p<0.05), ++ (p<0.01), (p<0.001) +++ sal/sal vs OT-GET/PCP; Tukey post-hoc. Sal = saline.

As expected, PCP significantly increased cumulative ambulatory ($F_{(3,41)}$ =10.07, p=0.0001; one-way ANOVA) and fine movement ($F_{(3, 44)}$ =20.3, p=0.0001; one-way ANOVA) counts as well as suppressed rear movement ($F_{(3,42)}$ = 7.22, p=0.0001; one-way ANOVA) counts recorded over a 30 minutes period after PCP injection (Figure 4.11A-C). None of the PCP-induced changes were reversed by intranasal OT (p<0.01 or p<0.001) or OT-GET (p<0.01 or p<0.001) compared to saline controls.

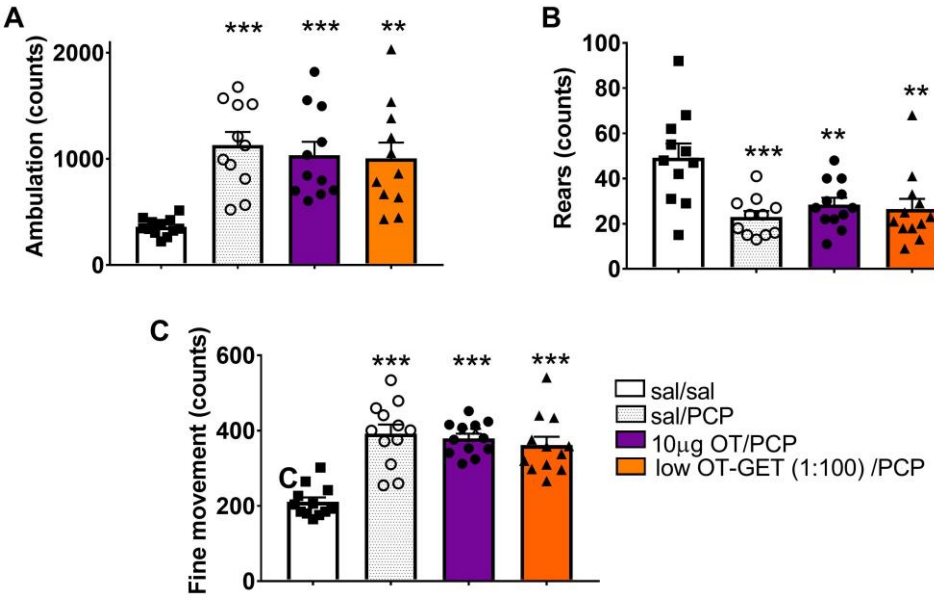


Figure 4.11 Effect of low dose intranasal OT and OT-GET on cumulative locomotor activity counts during peak PCP effect.

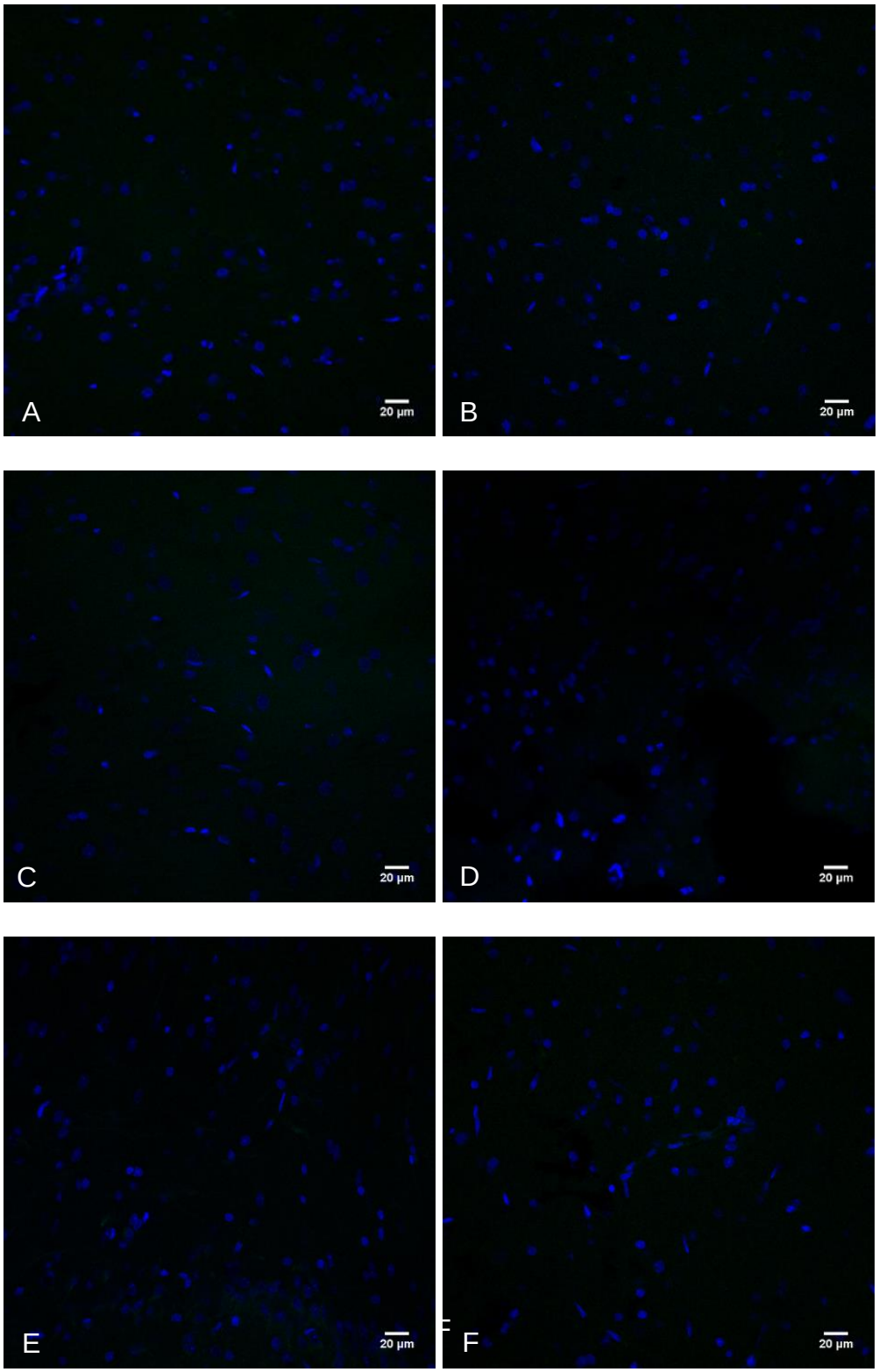
Comparative effect of intranasal saline/10µg OT/ low OT-GET(1:100) on (mean ± SEM, n=11-12 per group) cumulative (A) ambulatory, (B) rears and (C) fine movement counts in 30 minutes post PCP injection. One-way ANOVA revealed a significant main effect of PCP on LMA; ambulation: $F_{(3,41)}$ =10.07, p=0.0001; rears: $F_{(3,42)}$ = 7.22, p=0.0001; and fine movement: $F_{(3, 44)}$ =20.3, p=0.0001). ** (p<0.01), *** (p<0.001) vs sal/sal; Tukey's post-hoc. Sal=saline.

4.6.2.1.3 Uptake of OT into the nose and brain

Visualisation of the respiratory epithelium, olfactory bulb and brainstem using FITC conjugated OT-GET was carried out to examine degree of brain penetration. In order to ensure complete drug wash-out and to minimise any possible carry-over effects, rats were administered FITC conjugated OT-GET to examine degree of brain penetration seven days after the last behavioural task.

Brain

FITC fluorescence was not detected in the 2D images of the olfactory bulb (Figure 4.12A-F) and brainstem (Figure 4.12G-L) using scanning laser confocal microscope (n=1/group).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B
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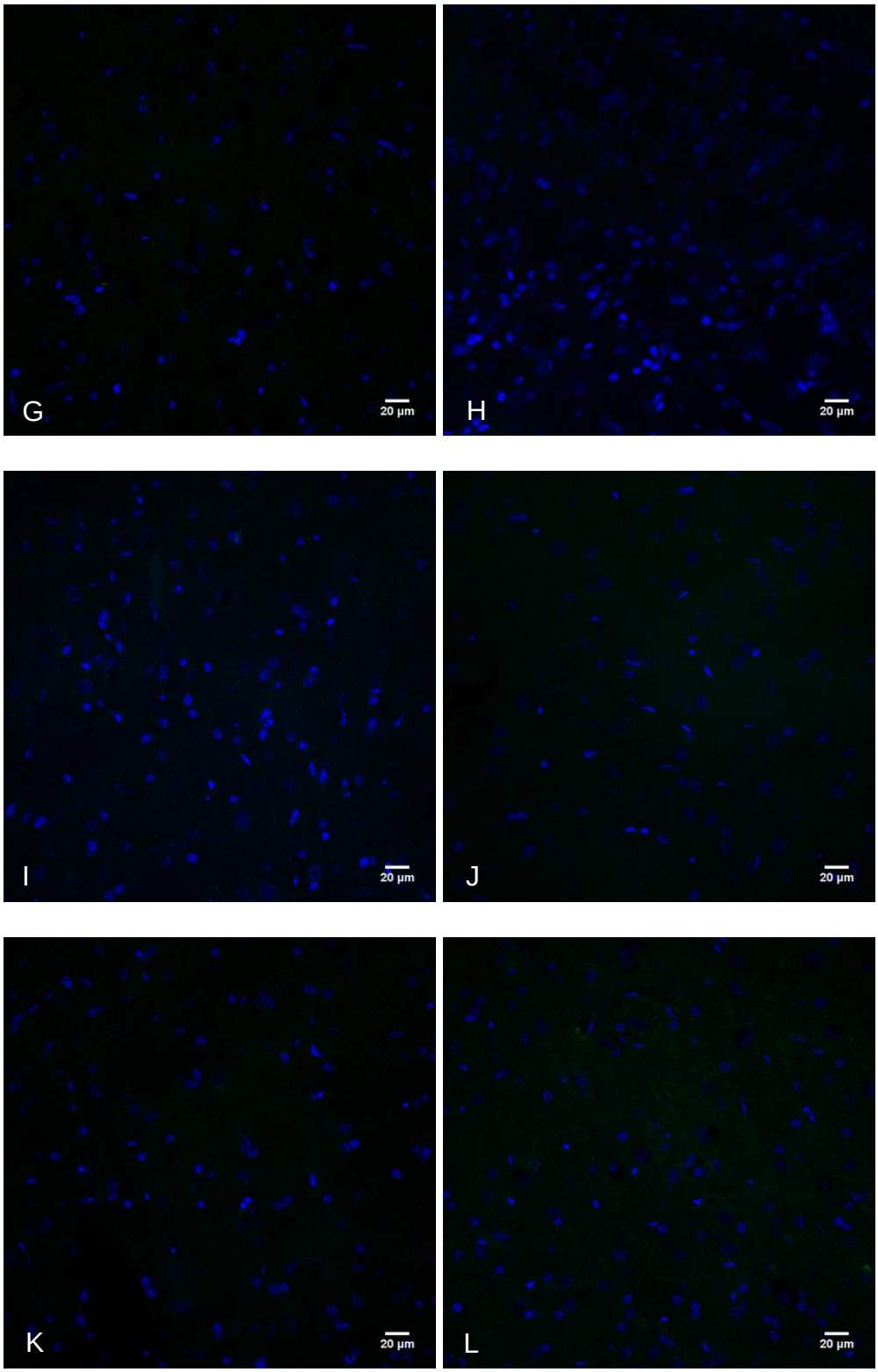

Figure 4.12 2D images of rat olfactory bulb and brainstem.

Images of (A-F) olfactory bulb and (G-L) brainstem in rats pre-treated with saline/OT/OT-GET/GET/FITC-GET/FITC OT-GET 60 minutes prior concussion using a Zeiss LSM710 confocal laser scanning microscope (Zeiss LSM710 META, Germany) and Zen Black 2012 Imaging Software before being

processed using Fiji image processing software. Treatments are as followed: A/G: GET ; B/H: OT ; C/I: OT-GET ; D/J: saline ; E/K: FITC-GET ; F/L: FITC-low OT-GET (1:100). The same rat was used for visualisation in each pair of pictures. Slices were stained with DAPI to indicate presence of cell nuclei. Laser emission of 405nm and 488nm were used for visualization of DAPI and FITC respectively with magnification of 40X oil immersion and numerical aperture (N.A.) of 1.3, n=1.

Nose

As no fluorescence were detected for FITC-OT-GET/GET in the brain, respiratory epithelium slices were examined to investigate if the drug was visible in nasal tissue (Figure 3.13A-F) in the same rat. FITC was present in respiratory epithelium of rats treated with FITC low OT-GET (1:100) but not in FITC GET treated tissue. Rats which did not receive FITC (Fig 4.13A-D) showed slight fluorescence traces which may be due to artefact or cross contamination as rats were placed in the same cage for an hour after receiving respective treatments.

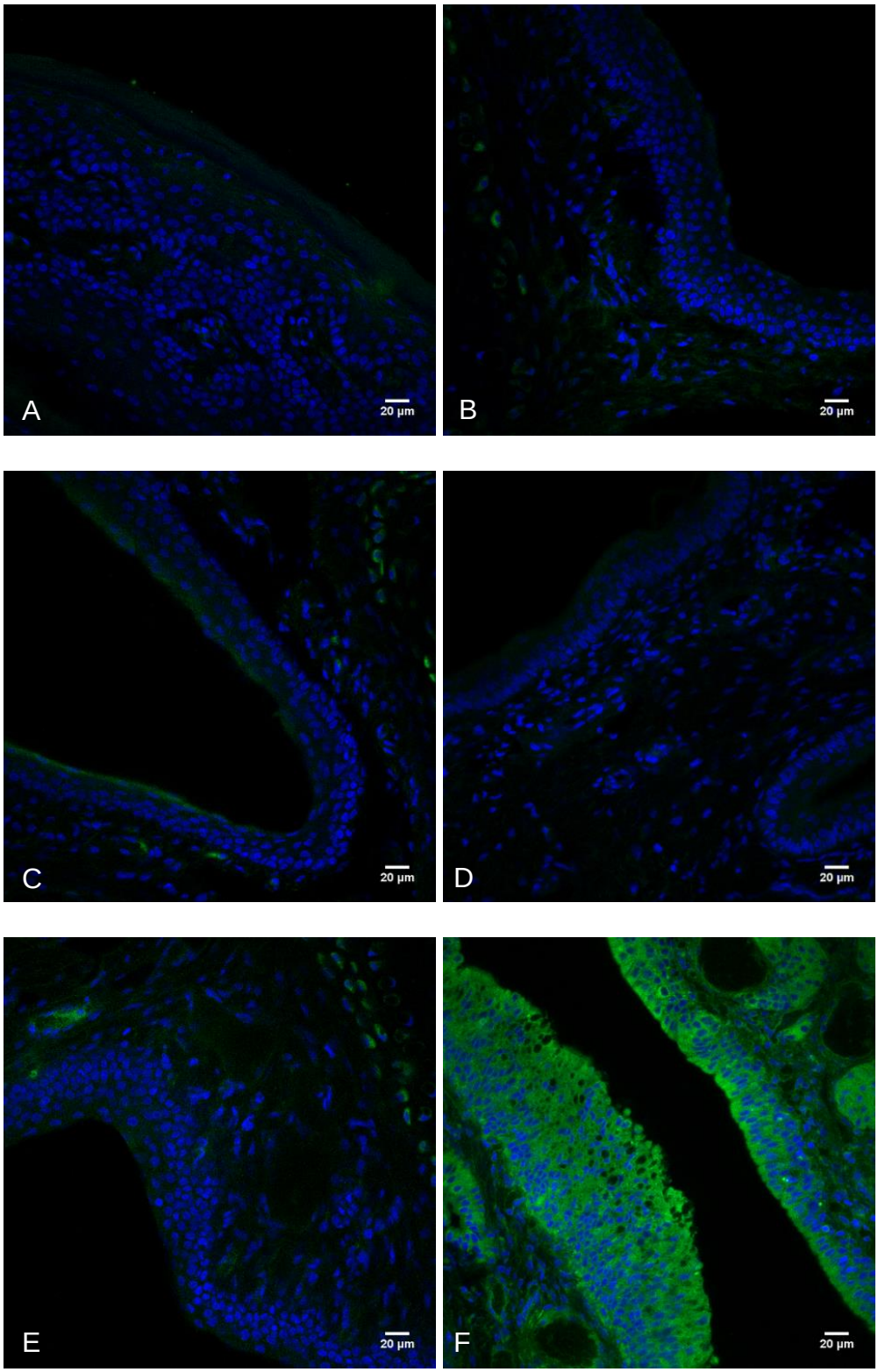

Figure 4.13 2D images of rat respiratory epithelium.

Images acquired from rats pre-treated with saline/OT/OT-GET/GET/FITC-GET/FITC OT-GET 60 minutes prior concussion using a Zeiss LSM710 confocal laser scanning microscope (Zeiss LSM710 META, Germany) and Zen Black 2012 Imaging Software before being processed using Fiji image

processing software. Treatments are as followed: A: GET ; B: OT ; C: OT-GET ; D: saline ; E: FITC-GET ; F: FITC-low OT-GET (1:100). Slices were stained with DAPI to indicate presence of cell nuclei. Laser emission of 405nm and 488nm were used for visualization of DAPI and FITC respectively with magnification of 40X oil immersion and numerical aperture (N.A.) of 1.3, n=1. The same rat was used in visualizing brain and respiratory epithelium tissues.

To confirm the lack of fluorescence observed in brains of FITC-OT GET treated rats, the respiratory epithelium and olfactory bulb of an additional FITC- low OT-GET (1:100) treated rat was examined and the same observation was obtained: fluorescence traces were noted only in the nose but not the brain (Fig 4.14).

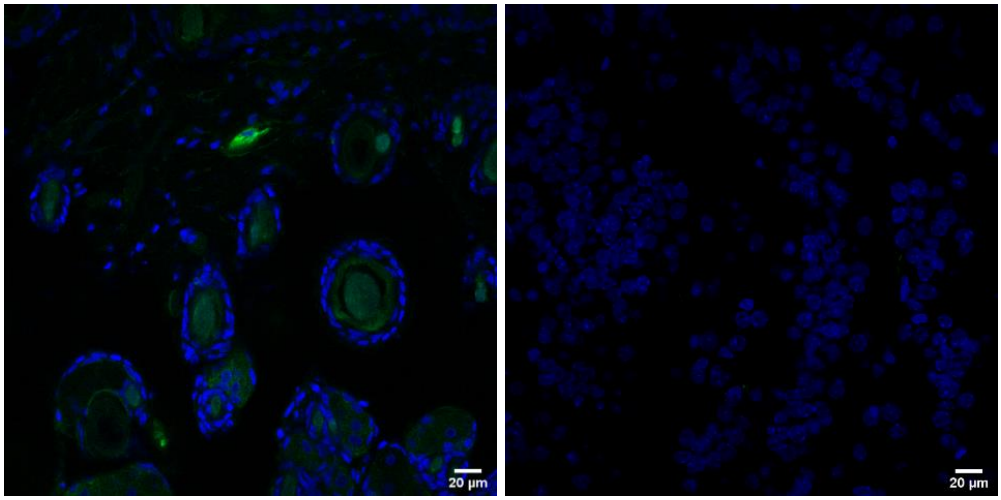

Figure 4.14 2D images of respiratory epithelium (left) and olfactory bulb (right).

Images acquired from a rat pre-treated with FITC OT-GET 60 minutes prior concussion using a Zeiss LSM710 confocal laser scanning microscope (Zeiss LSM710 META, Germany) and Zen Black 2012 Imaging Software before being processed using Fiji image processing software. Laser emission of 405nm and 488nm were used for visualization of DAPI and FITC respectively with magnification of 40X oil immersion and numerical aperture (N.A.) of 1.3, n=1.

4.6.2.2 Study 2 and 3: High dose OT treatment

As there was no behavioural effect nor any evidence of brain penetration of OT (either alone or conjugated to GET) in Study 1, a higher dose of

100µg OT (alone and conjugated to GET) was performed. OT s.c. was also included as an additional positive control as it has previously shown to decrease PCP-induced cumulative ambulatory counts (Kohli et al., 2019).

Adult male LH rats that received high dose treatment were divided into two studies: Study 2 and Study 3. This is because replication of Study 2 to increase n numbers to reflect that of Study 1 was halted due to the COVID-19 lockdown. Upon resuming of research activities, a separate sufficiently powered high-dose study (Study 3) with a similar protocol and drug dosage was carried out, but this time a single high OT-GET dose was chosen, based on the LMA results from Study 2.

4.6.2.2.1 Locomotor activity

For Study 2, a similar protocol to that described in Section 4.5.4 was followed with the first 60 minutes of LMA (30 minutes of habituation and 30 minutes post OT/OT-GET administration) excluded from statistical analysis and following figures. PCP-induced hyperactivity attenuated over time ($F_{(12, 204)}$ = 1.284, p-=0.0001) but neither treatment ($F_{(5, 17)}$ = 1.875, p=0.1508) nor time x treatment interaction ($F_{(60, 204)}$ = 1.284, p=0.1026) had a significant main effect (Figure 4.15).

PCP treated animals had significantly higher ambulatory counts at 65 – 75 minutes ($p<0.01$ or $p<0.001$) post PCP-administration compared to saline treated group, as expected. However, intranasal OT ($p<0.01$ or $p<0.001$) and s.c. OT ($p<0.05$ or $p<0.01$) counts remained significantly higher compared to saline animals at the same time points (excluding at 65 minutes for intranasal OT), indicating a lack of OT effect to reduce PCP-induced hyperactivity. Hyperactivity observed in high OT-GET (1:10) treated animals was also not reversed as there were no significant difference in LMA counts compared to PCP treated animals. Interestingly, there was no significant difference in ambulatory counts between high OT-GET 1:1 treated rats compared to saline at any time point.

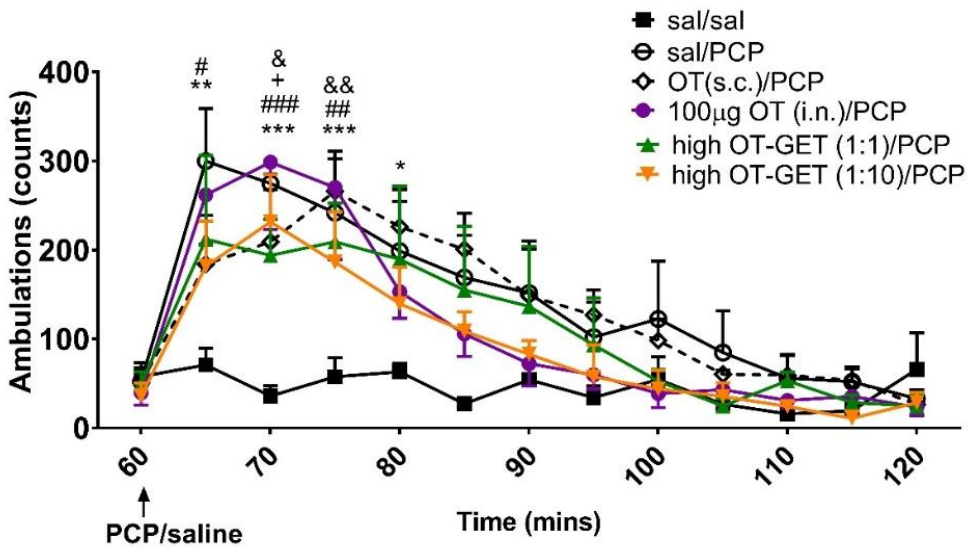

Figure 4.15 Effect of high dose intranasal OT and OT-GET on PCP-induced hyperactivity.

Comparative effect of saline/100µg OT/high OT-GET (1:1) and (1:10) and OT (0.1mg/kg, s.c.) on PCP-induced hyperactivity where OT/OT-GET pre-treatment was given 30 minutes prior saline or PCP at 60 minutes (5.6mg/kg; i.p.). Effects on (mean ± SEM cumulative counts/5 min epoch, n=4 each) ambulatory counts of male adult LH rats observed, 60 – 120 minutes after placement in box. Two-way RM ANOVA, revealed a main effect of time ($F_{(12, 204)}$ = 1.284, p-=0.0001) with the first 30 minutes of habituation and subsequent 30 minutes of common pre-drug activity excluded, but not treatment ($F_{(5, 17)}$ = 1.875, p=0.1508) or time x treatment interaction ($F_{(60, 204)}$ = 1.284, p=0.1026). ** (p<0.01) ***(p<0.001) sal/sal vs sal/PCP; # (p<0.05) #(p<0.01) ### (p<0.001) sal/sal vs i.n. OT; & (p<0.05) sal/sal vs s.c. OT; + (p<0.05) sal/sal vs high OT-GET 1:10, Tukey's post hoc test. S.c=subcutaneous, i.n.=intranasal, sal=saline

None of the drug treatment produced significant changes in cumulative ambulatory counts recorded over a 30 minutes period after PCP injection ($F_{(5, 17)}$ = 2.034, p=0.1239; one-way ANOVA; Figure 4.16).

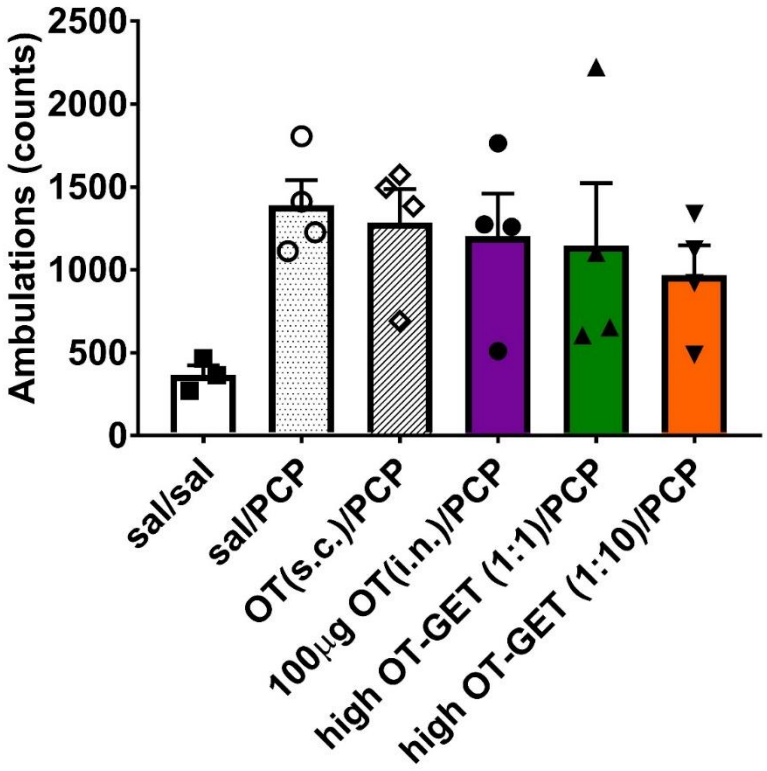

Figure 4.16 Effect of high dose intranasal OT and OT-GET on cumulative ambulatory count during peak PCP effect.

Comparative effect of intranasal saline/100µg OT/high OT-GET (1:1) and (1:10) and OT (0.1mg/kg, s.c.) on (mean ± SEM, n=4 per group) cumulative ambulatory counts in 30 minutes post PCP injection. One-way ANOVA revealed no changes in cumulative ambulatory counts by any treatment group ($F_{(5, 17)}$ = 2.034, p=0.1239). S.c.=subcutaneous, i.n.=intranasal, sal=saline.

For Study 3, the high dose OT-GET (1:1) was analysed in more detail. The OT to GET conjugation ratio of 1:1 was chosen as it showed no significant difference to saline control across all time points post PCP administration in Study 2. Furthermore, preliminary studies in mice from the Dixon laboratory (unpublished) saw a statistically significant decrease in blood glucose level with a lower dose of an insulin-GET complex, further justifying the usage of a lower OT-GET conjugation ratio.

Over the 60 minutes post-PCP injection, PCP increased ambulatory and fine movement counts and decreased rear counts such that there was a main effect of time (ambulation: $F_{(12, 504)}$ =28.96, p=0.0001; rears: $F_{(12, 516)}$

= 20.41, p=0.0001; and fine movement: $F_{(12, 504)}$ = 32.18, p=0.0001), treatment (ambulation: F(4, 42) =10.37, p=0.0001; rears: $F_{(4, 43)}$ = 18.86, p=0.0001; and fine movement: $F_{(4, 42)}$ = 9.409, p=0.0001) and there was a time x treatment interaction (ambulation: $F_{(48, 504)}$ =3.057, p=0.0001; rears: $F_{(48, 516)}$ = 2.766, p=0.0001; and fine movement: $F_{(48, 504)}$ = 1.917, p=0.0004; Figure 4.17).

OT (0.1mg/kg, s.c.) significantly attenuated the PCP-induced hyperactivity seen for ambulatory movements between 70-85 minutes ($p<0.05$ or $p<0.01$) compared to rats pre-treated with saline. Interestingly, ambulatory counts for OT-GET treated animals showed a trend to remain the highest amongst all treatment groups, and this increase compared to intranasal OT alone, was significant ($p<0.05$) at 80 minutes. OT s.c. also decreased rear movement counts compared to saline controls between 60-70 minutes ($p<0.01$ or $p<0.05$), intranasal OT at 60-65 minutes ($p<0.05$ or $p<0.01$) and OT-GET at 65 minutes ($p<0.01$). The magnitude of fine movement changes induced by PCP was not reversed by OT/OT-GET treatment groups.

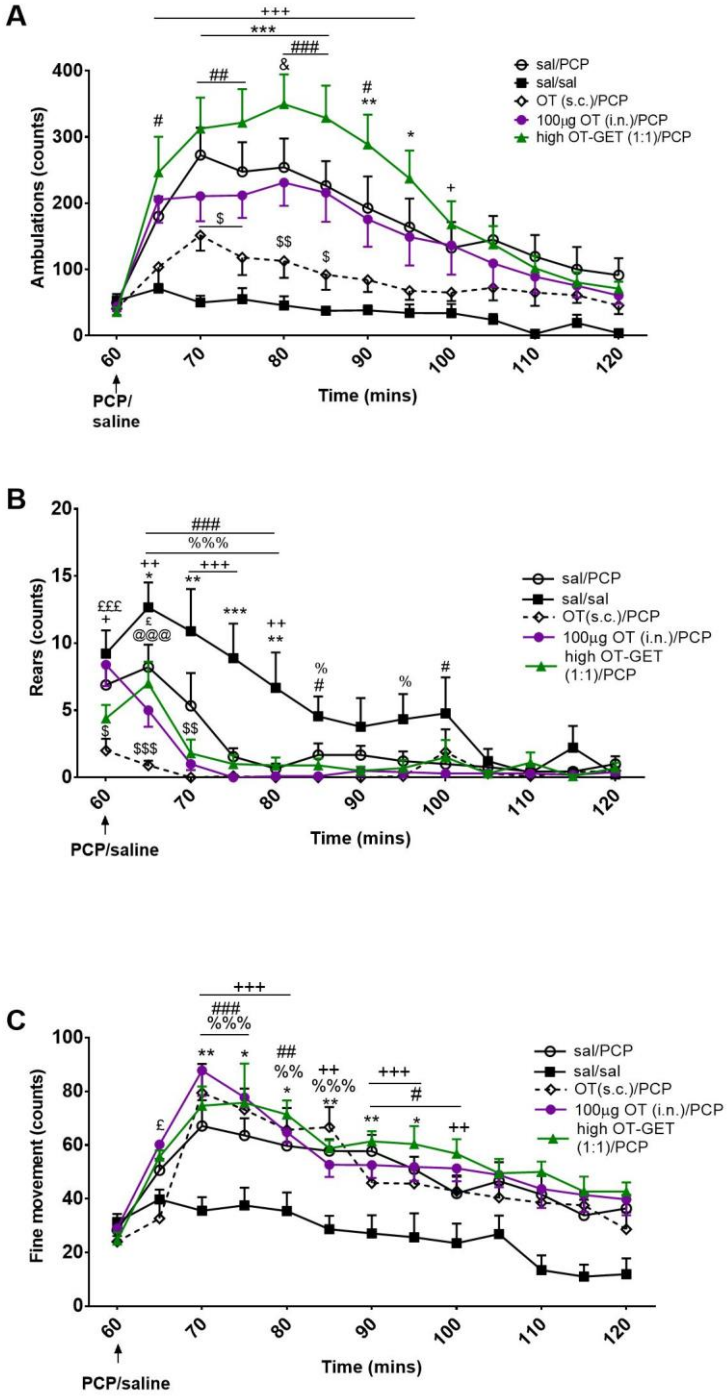

Figure 4.17 Effect of high dose intranasal OT and OT-GET on locomotor activity in rats administered PCP.

Comparative effect of intranasal saline/100µg OT/high OT-GET (1:1) and OT (0.1mg/kg, s.c.) given 30 minutes prior saline or PCP (5.6mg/kg; i.p.) at 60 minutes on (mean ± SEM cumulative counts/5 min epoch, n=9-10 each) (A) ambulatory, (B) rears and (C) fine movement observed, 60-120 minutes after placement in box. Two-way RM ANOVA showed a main effect of time

(ambulation: $F_{(12, 504)}$ =28.96, p=0.0001; rears: $F_{(12, 516)}$ = 20.41, p=0.0001; and fine movement: F(12, 504) = 32.18, p=0.0001), treatment (ambulation: $F_{(4, 42)}$ =10.37, p=0.0001; rears: $F_{(4, 43)}$ = 18.86, p=0.0001; and fine movement: $F_{(4, 42)}$ = 9.409, p=0.0001) and a time x treatment interaction (ambulation: $F_{(48, 504)}$ =3.057, p=0.0001; rears: $F_{(48, 516)}$ = 2.766, p=0.0001; and fine movement: $F_{(48, 504)}$ = 1.917, p=0.0004). *(p<0.05) ** (p<0.01) ***(p<0.001) sal/sal vs sal/PCP; # (p<0.05) ## (p<0.01) ### (p<0.001) sal/sal vs OT (i.n.)/PCP; + (p<0.05) ++ (p<0.01) +++ (p<0.001) sal/sal vs OT-GET/PCP; % (p<0.05) %% (p<0.01) %%% (p<0.001) sal/sal vs OT(s.c.)/PCP; & (p<0.05) OT(i.n.)/PCP vs OT-GET/PCP; $ (p<0.05) $$ (p<0.01) sal/PCP vs OT(s.c.)/PCP; £ (p<0.05) ££ (p<0.01) OT (i.n.)/PCP vs s.c./PCP; @@ (p<0.01) OT-GET/PCP vs s.c./PCP; Tukey's post-hoc. S.c.=subcutaneous, i.n. = intranasal, sal=saline.

As expected, PCP significantly increased cumulative ambulatory ($F_{(4, 43)}$ = 12.89, p=0.001) and fine movement $F_{(4, 38)}$ = 7.513, p=0.001) counts as well decreased cumulative rear movement ($F_{(4, 43)}$ = 23.17, p=0.001) counts (Figure 4.18A – C) in the 30 minutes following PCP injection.

Overall, intranasal OT and OT-GET cumulative ambulatory (p<01 or p<0.001), rears (p<0.001) and fine movement counts (p<0.01 or p<0.001) remained significantly higher (ambulation and fine movement) or lower (rears) compared to saline controls. Consistent with the time course data, OT (0.1mg/kg, s.c.) reversed PCP-induced hyperactivity such that cumulative ambulatory and rear movement counts were significantly lower than PCP treated animals (p<0.05 or p<0.01). Interestingly, OT-GET, but not intranasal OT alone, caused a significant increase in cumulative ambulatory counts compared to s.c. OT (p<0.001).

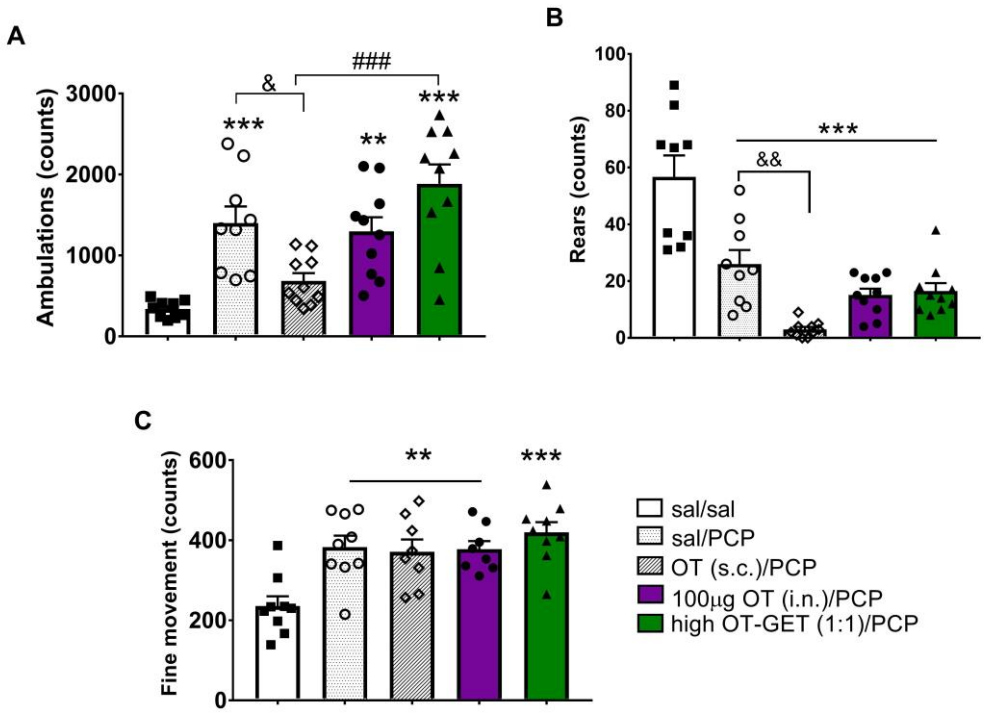

Figure 4.18 Effect of high dose intranasal OT and OT-GET on cumulative locomotor activity counts during peak PCP effect.

Comparative effect of intranasal saline/100µg OT/ high OT-GET (1:1) and OT (0.1mg/kg, s.c.) on (mean ± SEM, n=9-10 per group) cumulative (A) ambulatory, (B) rears and (C) fine movement counts in 30 minutes post-PCP injection. One-way ANOVA confirmed a main effect of treatment (ambulation: $F_{(4, 43)} = 12.89$, p=0.001; rears: ($F_{(4, 43)} = 23.17$, p=0.001) ; and fine movement $F_{(4, 38)} = 7.513$, p=0.001)). ** (p<0.01) *** (p<0.001) vs sal/sal; & (p<0.05) && (p<0.01) sal/PCP vs OT (s.c.)/PCP; ### (p<0.001) OT(s.c.)/PCP vs high OT-GET(1:1)/PCP; Tukey post-hoc. S.c.=subcutaneous, i.n.=intranasal, sal=saline.

4.6.2.2.2 Social interaction

Pairs of male adult LH rats were pre-treated with intranasal saline/100µg OT/high OT-GET (1:1) 40 minutes prior to a social interaction task, which lasted 10 minutes each round.

Prosocial (ano-genital and body sniffing, following, lying side-by-side, crawling over and under; Figure 4.19A) and aggressive behaviours (boxing and biting, pinning; Figure 4.19B) were analysed separately. For prosocial behaviour, intranasal OT and OT-GET treatment did not show

a significant main effect ($F_{(2,126)}$ = 0.8809, p=0.4253) or treatment x behaviour interaction ($F_{(10,126)}$ = 1.197, p=0.2992) but there was a main effect of behavioural components scored ($F_{(5,126)}$ = 1.996, p=0.0001). Rats that received intranasal OT spent more time body sniffing its counterpart compared to rats that received saline (p<0.05). For aggressive behaviour however, there were no main effects of treatment ($F_{(2,42)}$ = 1.233, p=0.3016), behaviour components ($F_{(1,42)}$ =1.094, p=0.3015) or treatment x behaviour interaction ($F_{(2,42)}$ = 0.1932, p=0.8250).

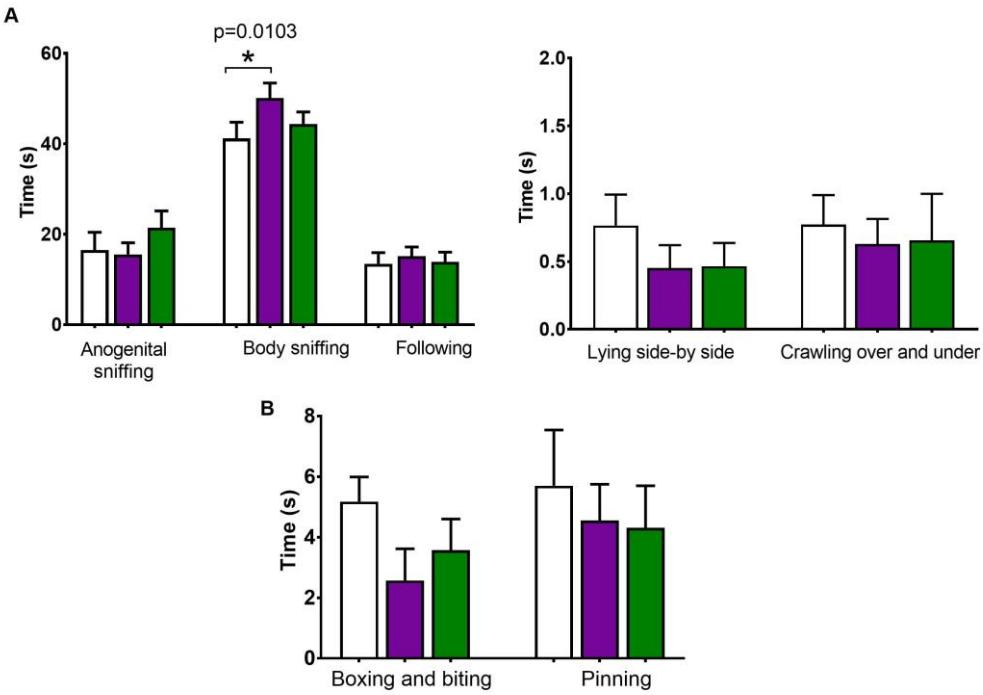

Figure 4.19 Effect of high dose intranasal OT and OT-GET on social behaviour.

Comparative effect of intranasal saline, 100µg OT and high OT-GET (1:1) on (A) prosocial and (B) aggressive behaviour between two similar weight male Lister-hooded rats from different litters. Drugs were administered 40 minutes prior and behaviour recorded over 10 minutes. Lying side-by-side and crawling over and under is depicted using a separate time scale as these prosocial behaviour occurred less frequently. All data are presented as mean ± SEM, n=8pairs per group. Two-way ANOVA showed a main effect of behaviour components ($F_{(5,126)}$ = 1.996, p=0.0001) but not treatment ($F_{(2,126)}$ = 0.8809, p=0.4253) or treatment x behaviour interaction ($F_{(10,126)}$ = 1.197, p=0.2992) for

prosocial behaviours. No main effects of treatment ($F_{(2,42)}$ = 1.233, p=0.3016), behaviour ($F_{(1,42)}$) =1.094, p=0.3015) or treatment x behaviour interaction ($F_{(2,42)}$ = 0.1932, p=0.8250) were noted for aggressive behaviour.* (p<0.05) saline vs OT (i.n.); Tukey post-hoc. I.n.=intranasal.

A further examination of total time spent in prosocial ($F_{(2, 21)}$ = 0.582, p=0.5674) and aggressive ($F_{(2, 21)}$ = 0.8362,p=0., 4473) behaviour confirmed there was no significant difference between OT or OT-GET treated animals (Figure 4.20).

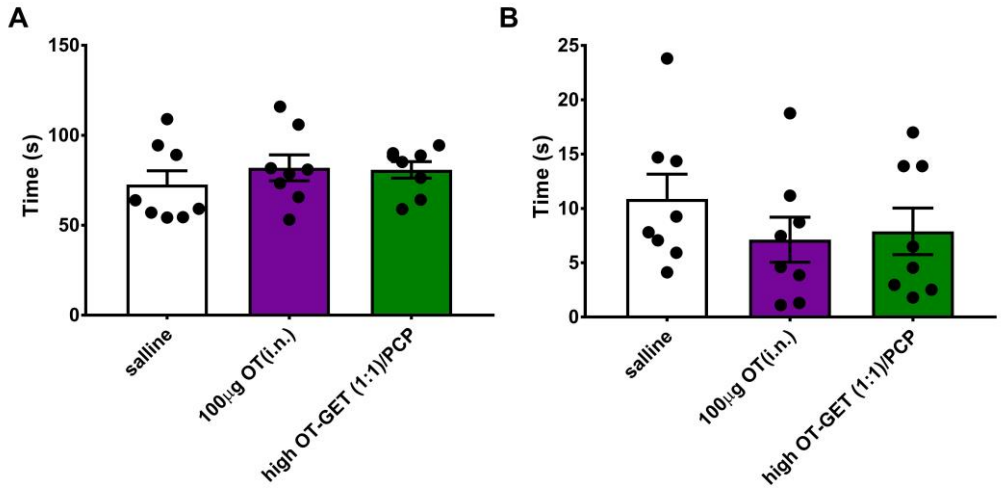

Figure 4.20 Effect of high dose intranasal OT and OT-GET on total social interaction time.

Comparative effect of intranasal saline, 100µg OT and high OT-GET (1:1) on total time spent in (A) prosocial and (B) aggressive behaviour between two weight-matched male Lister-hooded rats from different litters. Drugs were administered 40 minutes prior and behaviour recorded over 10 minutes. All data are presented as mean ± SEM, n= 8 pairs per group. One-way ANOVA revealed no effect of OT/OT-GET treatment on total time spent in prosocial ($F_{(2, 21)}$ = 0.582, p=0.5674) or aggressive ($F_{(2, 21)}$ = 0.8362,p=0., 4473) behaviour. I.n.=intranasal.

4.6.2.2.3 Quantification of OT in the brain

The lack of fluorescence in the brain noted in Study 1 alongside *in vitro* data (Chapter 3.3.4) suggests a possible unconjugation of the OT-GET complex upon transversing the epithelial barrier. As the chosen fluorescent marker was conjugated to GET and not OT, the visualisation of OT penetration into the brain via confocal microscopy may not be accurate. As such, quantification of OT levels was performed using an ELISA instead of confocal microscopy for Study 3. In order to ensure complete drug wash out, rats were administered intranasal saline, 100µg OT or high OT-GET (1:1) as well as s.c. OT (0.1mg/kg) 3 days after the last behavioural task to examine degree of brain penetration.

OT levels in the olfactory bulb was significantly altered after drug treatment ($F_{(3, 27)}$ = 4.042, p=0.017, ANOVA; Figure 4.21A) where rats administered high OT-GET had a significantly higher OT concentration than those that received intranasal OT alone (p<0.05). No significant changes were found in the hippocampus ($F_{(3, 23)}$ = 0.9331, p=0.4407, one-way ANOVA; Figure 3.21B).

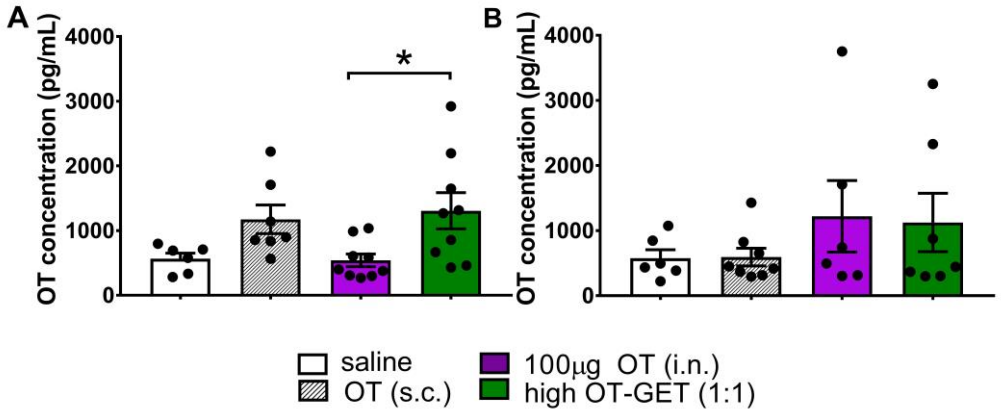

Figure 4.21 OT concentration in the olfactory bulb and hippocampus.
Comparative effect of intranasal saline/100µg OT/high OT-GET (1:1) and OT (0.1mg/kg, s.c.) on total OT concentration (pg/mL) in the rat (A) olfactory bulb and (B) hippocampus 40 minutes post drug treatment. All data are presented as mean ± SEM, n= 6-9 per group. ANOVA showed a main effect of OT/OT-GET treatment on OT concentrations in the olfactory bulb ($F_{(3, 27)}$ = 4.042, p=0.017 but not the hippocampus ($F_{(3, 23)}$ = 0.9331, p=0.4407). * (p<0.05) 100µg

OT (i.n.) vs high OT-GET (1:1); Tukey post-hoc. I.n=intranasal; s.c.=subcutaneous

4.7 Discussion

4.7.1 Temperature

A common side-effect seen with peripheral OT administration include hypothermia and sedation (Calcagnoli et al., 2015, Hicks et al., 2014, Kohli et al., 2019) and thus it was important to confirm our chosen intranasal doses of peptides would not cause similar unwanted side-effects. In Study 1, neither 10µg OT nor low OT-GET (1:1 and 1:10) affected core body temperature and normal locomotor activity in otherwise healthy male adult LH rats. However, a trend of decreasing core body temperature was observed, which may be a result of acclimatising to room temperature.

In PCP-treated animals, a significant decrease in core body temperature occurred regardless of first drug administration group. (Pechnick and George, 1989) showed that acute administration of PCP causes significant hypothermia in the rat although at 20mg/kg, four times the amount administered in our study. They also observed a non-significant drop in body temperature at 10mg/kg and suggested that the hypothermia is unlikely to be caused by the parent compound but may be due to subsequent metabolites produced. Rats treated with OT-GET/PCP also showed a significant ($p<0.05$) drop in temperature compared to saline/PCP group. However, this difference in temperature was not significant at subsequent time points.

4.7.2 Locomotor activity

We next examined intranasal OT/OT-GET's ability to attenuate PCP-induced hyperactivity, commonly used as an index of antipsychotic-like behaviour (Jones et al., 2011).

In Study 1 and 2, neither OT nor OT-GET reversed PCP-induced hyperactivity. The lack of effect in Study 2 is likely due to an insufficient

power as only 4 rats per group was included and systemic OT (0.1mg/kg, s.c.), an additional positive control group which attenuates PCP-induced hyperactivity (Kohli et al., 2019), failed to do so. In Study 3, 0.1mg/kg s.c. OT but not intranasal OT or OT-GET attenuated PCP-induced hyperactivity, although cumulative ambulatory counts during the peak PCP effect was significantly increased in OT-GET treated rats compared to that in those given s.c. OT.

OT s.c. has been robustly replicated several times in our group to reverse PCP-induced hyperactivity by Kohli et al. (2019) and Abrahams et al. (2020) and now with this current study. Yet, the mechanisms of how this may work remains relatively uncertain as only one study has examined the effect of systemic OT on PCP-induced hyperactivity (Kohli et al., 2019). PCP increases microdialysate dopamine in the frontal cortex and NAc post-PCP injection (Li et al., 2010, Millan et al., 1999) yet Kohli et al. (2019) saw a significant and persistent increase in microdialysate dopamine (but not its metabolites) overflow in the NAc of male adult LH rats accompanied by a decrease in PCP-induced hyperactivity after systemic OT treatment. Thus, it appears contradictory as to how OT may reverse PCP-induced hyperactivity when both PCP and OT increases DA release. However, multiple studies have dismissed striatal DA's role in hyperactivity resulting from NMDA receptor antagonism, suggesting the observed hyperactivity after acute PCP administration is due to increased non-NMDA receptor glutamate mediated transmission in the PFC leading to increased dopamine levels in the PFC and striatal regions. For example, application of an AMPA receptor antagonist failed to reduce DA levels in the NAc yet reversed PCP-induced hyperactivity (Adams and Moghaddam, 1998) and injection of an NMDA receptor antagonist into the NAc and ventral tegmental area (VTA) prevented PCP-induced hyperactivity but not the resulting increase in DA (Takahata and Moghaddam, 2003). Furthermore, application of an mGlu 2/3 agonist LY379268, blocked PCP-induced hyperactivity in genetically modified dopamine-deficient mice, implicating a role for glutamate transmission instead (Chartoff et al., 2005).

Thus, OT may play a role in attenuating PCP-induced hyperactivity via glutamate and GABA neurotransmission modulation instead. Qi et al. (2012) observed that OT inhibited MAP induced glutamate release and increased GABA release in the medial PFC as well as decreased NMDA receptor subunit NR1 expression, which was enhanced by MAP administration. Furthermore, increased extracellular glutamate in the PFC and NaC have been observed after PCP administration (Adams and Moghaddam, 1998) and decreased GABA interneuron activity and increased pyramidal firing occur following inhibition of NMDA receptor by NMDA receptor antagonist dizocilpine maleate in awake rats (Homayoun and Moghaddam, 2007). Separate *in vitro* studies examining OT's interaction with other neurotransmitters have found a relationship between OT and glutamate: stimulation of the mPFC in mice brain sections by OT significantly suppresses glutamatergic neurotransmission (Ninan, 2011) as well as between OT and GABA: OT stimulates fast spiking parvalbumin interneuron activity, modulating circuit signal-to-noise ratio and improving information processing in rat hippocampal slices (Owen et al., 2013).

No previous studies have examined intranasal OT's effect on PCP-induced hyperactivity but results in the current study suggest a lack of effect. Considering that only 0.002% of 5µg OT administered subcutaneously is thought to pass the BBB to reach the CNS after 10 minutes in rats (Mens et al., 1983) and an OT bioavailability of approximately 0.2% in the CSF was found after s.c. injection of 30ng in mice (Yamamoto et al., 2019), a higher concentration of OT via intranasal delivery is expected to reach the brain directly. Yet, intranasal OT/OT-GET failed to reverse hyperactivity while administration via the subcutaneous route succeeded. Might there be an important role for peripheral OTR or even V_{1a} receptor, which OT is known to have an affinity for, in mediating locomotor activity?

In otherwise healthy adult male rats, administration of peripheral OT is known to cause a reduction in locomotor activity (Angioni et al., 2016, Hicks et al., 2016, Wolfe et al., 2018). Yet, attempts to elucidate whether

peripheral/central OTR/V_{1a} receptors are responsible for this effect have shown mixed results. Angioni et al. (2016) showed a role of central OTR in the substantia nigra when the selective OTR antagonist d(CH_2)$_5$Tyr(Me)$_2$-Orn$_8$-vasotocin was given centrally and that destruction of OTR expressing neurones by microinjection of oxytocin-saporin, a neurotoxin that selectively destroys OTR expressing neurones (Baskin et al., 2010), reversed the locomotor inhibition seen in male Sprague-Dawley rats. Yet, in healthy adult male Wistar rats, peripheral pre-treatment with selective V_{1a} receptor antagonist, SR-49059, which crosses the BBB, also blocked OT-induced decrease in locomotor activity (Hicks et al., 2016). Similarly, Wolfe et al. (2018) saw a reverse in locomotor inhibition caused by s.c. OT (1mg/kg) in an open field test with peripheral and not central administration of mixed OTR/V_{1A} receptor antagonist [Pmp$_1$,Tyr(Me)$_2$]AVP in rats. In contrast, neither peripheral or central administration of the OTR antagonist barusiban reversed the locomotor inhibition.

Collectively, it may indicate a role for peripheral V_{1a} receptor in locomotor activity mechanisms modulated by OT. However, these experiments were done in otherwise healthy animals, and not in the presence of NMDA receptor antagonists and there exists the possibility that OT's effect in PCP treated animals may utilise an entirely different pathway altogether.

4.7.3 Social interaction

Intranasal administration of 100µg OT but not high OT-GET (1:1) significantly increased the time adult male LH rats spent in prosocial body sniffing. The prosocial effects observed in this study are in keeping with that found with systemic or central OT administration (Lukas et al., 2011, Ramos et al., 2013, Kohli et al., 2019) as well as intranasal OT administration (Calcagnoli et al., 2015, Huang et al., 2014, Kent at al., 2016) in rodents (mice and rats).

Rodents primarily utilise olfactory cues for social recognition and memory as lesions in the olfactory bulb or chemically induced anosmia affects

social recognition in rodents (Dantzer et al., 1990, Matochik, 1988, Popik et al., 1991). Mitral cells in the olfactory bulb form excitatory contacts with local granule cells, which in turn send inhibitory inputs back (Stoop, 2012). OTR mRNA and OT-immunoreactive fibres have been found in both mitral and granule cell layer (Knobloch et al., 2012, Yoshimura et al., 1993) and application of OT to olfactory bulb slices obtained from female mice induced formation of long-term potentiation (LTP), which was blocked by pre-treatment with an OT and NMDA receptor antagonist (Fang et al., 2008). This suggests the initial drive to engage in prosocial behaviour may involve NMDA receptor dependent LTP, which is enhanced upon OT treatment. Indeed, application of OT to mitral cells of rats decreased the frequency of spontaneous GABAergic inhibitory postsynaptic currents (Osako et al., 2000) while application to granule cells increased frequency and amplitude of spontaneous glutamatergic excitatory postysynaptic currents (Osako et al., 2001), overall suggesting OT works by enhancing signal to noise ratio in olfactory signalling.

The main projections from the olfactory bulb converge onto the medial amygdala (MeA), an area rich in OTRs (Adan et al., 1995, Dumais et al., 2013, Uhl-Bronner et al., 2005). The amygdala extensively interconnects with the mPFC and the circuit is known to be involved in social decision-making (Gangopadhyay et al., 2021). In clinical studies, intranasal OT modulated amygdala-mPFC activity, where in healthy subjects, amygdala activity was attenuated upon receiving social stimuli, whereas in patients with generalised anxiety disorder, the resting state functional connectivity was enhanced and ‘normalised’ instead, supporting the social salience hypothesis of OT (Dodhia et al., 2014, Kirsch et al., 2005, Petrovic et al., 2008). In OT knock-out mice, social amnesia towards a female conspecific is observed alongside a reduction in neural activation in the MeA, which is rescued upon OT injection into the site (Ferguson et al., 2000, Ferguson et al., 2001).

The NAc, which holds an established role in reward and goal-directed behaviour, may also play a role in increasing social behaviour (Wei et al., 2021). Optogenetic activation of NAc projecting VTA DA neurones

facilitated social interaction in mice with stranger mice introduced into their home cage (Gunaydin et al., 2014) and NAc DA overflow has been noted upon systemic OT administration with an increase in positive social behaviour (Kohli et al., 2019). A plausible reason is that OT reinforces the sense of reward achieved upon social approach and investigation, thus driving longer bouts of interaction.

Overall, these observations suggest OT may modulate social behaviour by exerting its action on these brain areas although much remains to be understood.

4.7.4 OT brain penetration

In Study 1, a failure to observe any fluorescence from administering FITC OT-GET was noted for the olfactory bulb and brainstem region examined, although fluorescence was present in the respiratory epithelium. Overall, this suggests successful application of drug to the nasal cavity albeit a lack of OT brain penetration. Behavioural results supported this claim as intranasal OT/OT-GET failed to reverse PCP-induced hyperactivity in a separate task.

Peptides administered to the nasal cavity, would not only have to transverse the respiratory epithelial, but also subsequently the olfactory epithelium (main point of drug absorption) successfully, in order to reach the brain: primarily the olfactory bulb and the brainstem (Bender et al., 2015, Falcone et al., 2014, Thorne et al., 2004, Johnson et al., 1993, Oviedo et al., 2017). As we were unable to obtain the olfactory epithelium as none of the experimenters were technically trained (furthermore in this case, the brain was the primary area of interest), we are thus unable to confirm if the olfactory epithelium was indeed an impermeable barrier for OT (alone or conjugated to GET) preventing it from reaching its intended target area. Conversely, if OT-GET did successfully increase brain penetration of OT, the lack of fluorescence observed could be due to the un-conjugation of the OT and FITC-GET complex as *in vitro* data (Chapter 3.3.4) hinted at this possibility. As such, in Study 3, the method

of quantifying OT levels in the brain post intranasal administration was carried out via an ELISA assay.

In Study 3, a significant increase in OT occurred in the olfactory bulb but only for intranasal OT-GET compared to OT alone. Although unable to determine whether the increase in OT concentration is due to exogenous peptide or the positive feedback loop triggering endogenous OT production, this observation is in line with literature: Direct Transport Percentage (DTP) calculated using OT concentration obtained via liquid chromatography-mass spectrometry and plasma OT concentrations obtained via area under the curve values saw the olfactory bulb achieve a DTP value at 99.5% in mice (Tanaka et al., 2018). On the other hand, the lack of increase in the hippocampus may represent insufficient time for OT to diffuse into the midbrain. Although Neumann et al. (2013) noted an increase in microdialysate OT in the hippocampus, this may be due to OT travelling via bulk flow in the perineural spaces adjacent to the olfactory and trigeminal nerves. Transport between brain regions on the other hand, may rely on axonal transport and studies utilising wheat germ agglutinin-horseradish peroxidase as a tracer observed endosomes/vesicles containing said tracer in olfactory axons, days after peptide administration (Broadwell and Balin, 1985, Shipley, 1985).

The results of higher OT levels achieved with OT-GET in the olfactory bulb seems contradictory compared to the absence of effect on behavioural tasks, where intranasal OT-GET did not reverse PCP-induced hyperactivity and it was OT alone that increased pro-social body sniffing. Here, it is to be noted that analysis of OT brain penetration was done on a separate day, and thus results are unable to be directly comparable to observed locomotor and social interaction behaviour.

Rodents rely on airborne volatile chemicals released by conspecifics, which are detected via olfactory receptors, primarily in the olfactory epithelium (Trinh and Storm, 2003). These cues then allow them to decide whether to approach the conspecific, where further non-volatile cues are obtained via nose-flank/ano-genital sniffing (Arakawa et al.,

2011). From the olfactory epithelium, axons of olfactory receptors neurones project to the main olfactory bulb, which is rich in OTRs (Grinevich et al., 2016), to mitral cells and subsequently local granule cells, both areas of which OTR mRNA are found (Knobloch et al., 2012, Yoshimura et al., 1993), suggesting the olfactory bulb is a main area of integrating social cues and recognition of a new conspecific. As a possible explanation to the lack of increased prosocial behaviour in OT-GET treated animals despite the seemingly increased OT levels in the olfactory bulb, it may be possible that a certain threshold of OT in the brain was crossed and the induced production of endogenous OT upon the stimulation of social interaction led to continuous stimulation and subsequently, desensitisation and downregulation of OTRs as OT operates on a positive feedback loop (Ludwig and Leng, 2006). Although no data are available regarding changes in brain OTR expression after acute OT administration, continuous OTR stimulation in myometrial and HEK293 embryonic kidney cells causes receptor internalisation (Brighton et al., 2011, Smith et al., 2006). *In vivo*, 2 weeks of intranasal OT (Huang et al., 2014) and 2 weeks of i.c.v. administration (Peters et al., 2014) in mice reduced OTR binding sites in several brain regions, detected via receptor autoradiography. In particular, Huang et al. (2014) noted a significant decrease in OTR binding site in the anterior olfactory nucleus and hippocampus, which may explain for OT-GET's lack of behavioural effect.

An alternative explanation is the possibility of an inhibitory response caused by the high OT concentration. OTR are known to couple to both $G_{q/11}$ and $G_{i/o}$ G proteins and a dose-response study by Busnelli et al. (2012) showed that G_q signaling is activated with an EC50 value of 2.16nM whereas $G_{i/o}$ signaling is activated with EC50 values ranging from 11.5nM to 91.8nM in HE K293 cells. A possibility is that $G_{i/o}$ proteins are activated at a higher concentration than that needed for G_q protein activation and the final concentration of OT in the brain may have crossed this threshold. However, a more plausible reason may be that the olfactory bulb is only responsible for driving the initial engagement, but

the maintenance of social behaviour and interaction is mediated by other parts of the brain such as the VTA where DA flux have been observed post systemic OT alongside an increase in prosocial behaviour (Kohli et al., 2019), where OT concentration was not examined.

4.7.5 Conclusion

Intranasal administration of 10µg OT and OT-GET (1:1; 10µg OT conjugated to 10ug GET and 1:100; 10ug OT conjugated to 1000µg GET) did not produce hypothermia or sedation in normal male adult LH rats nor (excluding OT-GET 1:1) did it reduce PCP-induced hyperactivity.

As such, a higher dose of intranasal OT was administered in the next group of rats: 100µgOT and OT-GET (1:1; 100µg OT conjugated to 100µg and 1:10; 100µg OT conjugated to GET), which again showed no effect on reversing PCP-induced hyperactivity. However, the results from this study was underpowered as the study was unable to be completed due to the COVID-19 pandemic, which halted research for 7 months. Upon resuming of research, a separate group of rats was administered intranasal 100µg OT and high OT-GET 1:1. This conjugation ratio being selected over OT-GET (1:10) as in the previous LMA task, ambulatory counts for OT-GET (1:1) rats did not differ significantly from saline treated animals.

Once again, total cumulative ambulatory counts during peak PCP effect in intranasal 100µg OT and OT-GET (1:1) were significantly higher to that of saline treated group. Although interestingly, total ambulatory counts of OT treated rats showed no significant difference to that of subcutaneous OT treated group, suggesting a mild attenuation in PCP-induced hyperactivity. On the other hand, subcutaneous OT, which was given as a positive control, significantly attenuated PCP-induced hyperactivity, an effect that has been robustly replicated in previous studies within the Fone group. In a social interaction task, intranasal OT (100µg) increased time spent by rats in body sniffing, a prosocial behaviour. Conversely, a separate quantification of OT levels in the brain, administered independently of any behavioural task, showed a significantly higher

concentration of OT in the olfactory bulb in high OT-GET (1:1) treated animals in comparison to 100µg OT alone. This finding seems to contradict the results found for LMA and social interaction, and may be due to OT levels exceeding that of a 'typical' oxytocinergic system leading to OTRs desensitisation, or alternatively activation of $G_{i/11}$ coupled G proteins.

In the absence of ability to track OT-GET diffusion from nose to brain or to determine the concentration level threshold leading to potential OTR desensitisation, it is difficult to decide whether to dismiss OT-GET's ability to enhance intranasal OT brain penetration as *in vitro* and ELISA results show OT-GET increased OT concentration levels across a nasal cell monolayer and olfactory bulb compared to OT alone, despite the lack of behavioural effect. Other attempts at enhancing OT brain delivery have been carried out in the meantime and is discussed in Chapter 5 General Discussion.

Chapter 5 General Discussion

For CNS diseases that display socio-behaviour deficits, such as ASD and schizophrenia, there remains a critical lack of progress in available effective medicines to treat these symptom domains.

OT, an endogenous hormone and neuropeptide with an established physiological role in childbirth and lactation, first gained attention for its ability to modulate social behaviour in the 1970s, where its role in modulating maternal behaviour was established. Other preclinical studies investigating OT's effect on social interaction and cognition followed and positive behavioural effects were demonstrated. Together with a prominent clinical study in 2005 showing intranasal OT increased trust (Kosfeld et al., 2005), this led to a flurry of clinical trials focused on utilising OT as a potential treatment for socio-behavioural deficits in man.

OT's high molecular weight however, hinders it from crossing the blood brain barrier efficiently when administered systemically, leading to the increasingly popular usage of intranasal administration. Intranasal delivery is believed to allow small peptides to access the brain more directly, bypassing the BBB and thus requiring a lower concentration of drug to be administrated to patients providing the potential to reduced risk of systemic side-effects. Yet, the degree of OT brain penetration remains controversial and requires further improvement. As such, the aim of this thesis was to investigate modulation of the CNS OT penetration by intranasal administration of it and a novel conjugation peptide, monitoring OT's ability to affect locomotor activity and social behaviour in rats.

5.1 Summary of findings

The studies reported in this thesis first examined the bioactivity of OT versus the novel cell penetrating peptide conjugate OT-GET and subsequently, GET's ability to improve OT delivery across a nasal epithelial cell monolayer *in vitro* (Chapter 3).

Using the breast tumour cell-line Hs 578t, an OT:GET conjugation ratio of 0.1µM OT to 0.1-10µM GET significantly decreased OTR-induced free $[Ca^{2+}]_i$ transients which was reversed by addition of heparin, which unconjugated the complex. This data suggests a successful and reversible conjugation of OT to GET as an OT-GET complex that holds promise to improve trans-epithelial migration worthy of further evaluation for successful translation into a biological system. Subsequently, conjugation of 1µM and 10µM GET to 0.1µM OT improved the rate of OT delivery as well as cumulative concentration of OT successfully transported *in vitro* across an immortalised human nasal epithelium cell line RPMI 2650. However, a decrease in cell monolayer integrity was observed over the 6h experimental timeline and a further cytotoxicity assay revealed that toxic effects (on cell viability) occurred only in the presence of the highest GET concentration. Overall, this suggested that GET improves OT permeability across an *in vitro* cell monolayer and highlights the importance of choosing an optimal OT to GET conjugation ratio in order to achieve desired effects without concomitant toxicity.

In order to examine whether OT-GET's ability to improve OT permeation could occur *in vivo*, subsequent studies investigated intranasal delivery of OT versus OT-GET on locomotor activity and social behaviour in rats in addition to undesirable systemic side-effects (hypothermia and sedation)(Chapter 4).

Two doses of OT were used for *in vivo* studies: low 10µg and high 100µg. Although the low dose of 10µg was half the concentration typically used in previous rodent studies, it was chosen to ensure sub-maximal behavioural effects, which could be enhanced, and by GET which had been shown to double OT cumulative concentration across a cell

monolayer. 10µg OT and corresponding OT-GET doses (10µg OT conjugated to 10µg GET and 1000µg GET) did not produce hypothermia or sedation, systemic side-effects commonly seen with peripheral OT administration (Calcagnoli et al., 2015, Hicks et al., 2014, Kohli et al., 2019). However, the same dose of OT and OT-GET did not reverse PCP-induced hyperactivity in an acute PCP rat model for the positive symptoms akin to those seen in schizophrenia. A separate administration of fluorescent OT-GET of similar doses to examine OT brain penetration supported the lack behavioural effect, as no evidence of OT penetration to relevant post-mortem brain regions was observed by confocal microscopy.

In rats receiving high dose OT and corresponding OT-GET doses (100µg OT conjugated to 100µg GET), cumulative ambulatory counts of OT treated animals during the peak PCP effect, although significantly higher to the saline treated control group, was not significantly higher than rats administered subcutaneous OT. Of note, OT s.c. was used as an additional positive control, since Kohli et al. (2019) and Abrahams et al. (2020) observed successful reversal of PCP-induced hyperactivity by pre-treatment with 0.1mg/kg OT. In both of these previous studies, s.c. OT also altered social interaction in rats, and in this study intranasal OT alone increased the time rats spent in prosocial body sniffing, suggesting a subtle enhancement of OT on social behaviour. Conversely, OT-GET failed to reverse PCP-induced hyperactivity and did not increase prosocial behaviour during the social interaction task. Interestingly, a separate ELISA analysis of the OT level in the olfactory bulb (chosen as it is a likely first point of brain penetration following intranasal delivery (Crowe et al., 2018), saw a significant increase of OT in OT-GET treated rats compared to those receiving OT alone. This result seemingly contradicts the apparent absence of behavioural alterations following OT-GET. A few possible explanation for this includes possible involvement of other brain regions in mediating social interaction, desensitisation of OTRs in the brain or the inhibition of cAMP dependent signalling pathways due to activation of $G_{i/o}$ G protein instead of $G_{q/11}$, induced by

the high level of OT penetrating the brain which may subsequently have triggered a positive feedback loop of endogenous OT production.

5.2 Intranasal delivery of oxytocin

Delivering peptide drugs to the brain is a recognised challenge, often carried out via three alternative approaches. The first is to administer inside the BBB by intracerebral injection, which is expensive and invasive for patients. A second method would be giving high doses by systemic administration, although this encounters the problem of unwanted systemic side-effects to enable sufficient compound to cross the BBB. A third strategy is intranasal delivery, which is purported to allow drugs to bypass the BBB, by providing higher CNS penetration and increased activity at central corresponding receptors and thus represents an increasingly popular route of administration for many therapeutic compounds intending to target corresponding central receptors.

Despite the utilisation of intranasal OT in many preclinical and clinical studies, the degree of actual drug penetration remains controversial as the pharmacodynamics of intranasal OT delivery remains poorly understood. Many studies still administer OT in doses much higher than endogenous levels to enable production of behavioural effects. Furthermore, intranasal delivery although non-invasive and convenient, poses its own problems too. The nasal cavity, although ‘leakier’ than the BBB, possesses limited permeability dependent on drug structure. The rule of thumb is that molecules larger than 1000Da (OT has a molecular weight of 1007Da) are not well absorbed, but permeability across nasal epithelium is also dependent on combined effects of molecular shape and size as well as cellular interactions (Al Bakri et al., 2018). Enzymatic degradation of the drug by the nasal mucosa also contributes to decreased bioactivity (Meredith et al., 2015).

This thesis explored the usage of a cell-penetrating peptide nanoparticle GET, where results from Chapter 3 and 4 support an increased transduction of OT across a nasal cell monolayer and into the brain

(olfactory bulb). Other nanoparticle formulations encapsulating OT with the intention of improved brain penetration have also been attempted. Zaman et al. (2018) looked at four different nanoparticle formulation for receptor-mediated transport of OT to the brain and found rabies virus glycoprotein (RVG)-conjugated to bovine serum albumin (BSA) the most favourable formulation to delivery OT to the brain *in vitro*, with an initial burst of 35% OT release over 6h followed by sustained release up to over 80% for 30 days. Using a similar formulation, Oppong-Damoah et al. (2019) found higher OT concentrations in CSF of mice after 2h and increased time spent in social interaction time when OT was delivered intranasally in RVG-conjugated BSA nanoparticles while in a mouse model of Sn1a-derived epilepsy, significantly increased and sustained resistance to seizures and improved social behaviour (Wong et al., 2021). The rationale behind using BSA as a polymer material was due to it being biocompatible and biodegradable (Ma et al., 2016) while RVG, which binds to n-acetylcholine on the BBB, has been used as a brain targeting ligand to deliver small interfering RNA to the brain (Kumar et al., 2007). Another study using OT encapsulated in a Phospholipid Magnesome, designed to provide a better drug entrapment and prolonged drug contact with the nasal membrane, showed antinociceptive effects 5 minutes after intranasal delivery in mice (Natsheh and Touitou, 2018).

Nanoparticles aside, a powder formulation of OT may provide advantages over the conventional aqueous solution. Powder formulations may slow down mucociliary clearance, extending the mean residential time of drug in the nasal cavity before it is swept to the nasopharynx where it is swallowed (Al Bakri et al., 2018, Davis and Illum, 2003). The powder form is also more stable, chemically and physically. This is especially useful for OT, which is heat-sensitive and requires constant refrigeration in its aqueous form (Milewski et al., 2016). Fabio et al. (2015) explored the usage of metal cations to stabilise a powder OT formulation, and succeeded in producing particle sizes of less than 2µm and allowed for storage up to 32 weeks at 40^{o}C, with only over 10% of its bioactivity being lost. In a separate study, Milewski et al. (2016)

administered 10 - 80IU powdered OT intranasally to NHPs which resulted in rapid rise of plasma OT and a dose-dependent increase in half-life and relative bioavailability compared to intramuscular injection. The main criticism however, was the measurement of plasma OT as an index brain levels, which is highly controversial to estimate pharmacokinetics (Christensen et al., 2014, Leng and Ludwig, 2016, Martins et al., 2020a).

Alternatively, the usage of nasal sprays versus nebulisers in clinical trials should be examined as nebulisers are purported to deposit smaller aerosolised particles that would allow for drug deposition in the upper and posterior nasal cavity where transport to the brain occurs (Frank et al., 2012). Indeed, aerosolised OT but not intranasal OT (delivered via spray) significantly increased peptide concentrations in CSF of anesthetised rhesus monkeys (Modi et al., 2014). More recently, Martins et al. (2020b) saw different patterns of increased or decreased resting cerebral blood flow (an indirect measurement of OT-induced brain activation) across brain regions post spray and nebulised OT delivery, with changes post nebuliser delivery being induced in brain regions more posterior and distant from point of entry. A subsequent study in male subjects by the same group backed up this initial observation showing changes in the pattern of regional connectivity, noted using graph-based network analysis and allowing for a better understanding of possible neural circuits engaged by OT, were significantly different with nasal versus nebuliser delivery of intranasal OT (Martins et al., 2021a).

5.3 Social salience theory of oxytocin

The focus on OT's ability to modulate social behaviour has strongly focused on its prosocial effects, yet accumulating evidence from clinical trials reveals that OT's effect are not always positive. Shamay-Tsoory et al. (2009) reported increased gloating and feelings of envy in participants who received uneven monetary gains in a reward task, while DeWall et al. (2014) reported OT increased aggressive behaviour towards their partner, but only in people with high aggression traits. As such, OT may not exist exclusively as a prosocial hormone. This gave rise to the social salience hypothesis: OT's effects appear to be context-dependent, and may alter according to the genetic and personality make up of individuals (Shamay-Tsoory and Abu-Akel, 2016). A possible mechanism behind this salience theory is the modulation of tonic dopamine activity in the mesolimbic system by OT (Shamay-Tsoory and Abu-Akel, 2016).

A recent attempt to replicate the infamous OT increases trust study by Kosfeld et al. (2005) further bolsters the social salience theory. In his study, Kosfeld et al. (2005) seated participants together for 5 minutes allowing for brief social contact but prior to their knowledge that they were to participate in a trust based game, and found that subjects administered intranasal oxytocin were willing to trust a stranger and took more risks. However several groups have failed to replicate these findings, based on a meta-analysis of six studies (Nave et al., 2015). The main flaws of these studies were that they were severely underpowered and none of them recreated the minimal social contact situation that Kosfeld et al. (2005) used. As such, Declerck et al. (2020) ran a sufficiently high-powered study, implementing the minimal social contact environment as Kosfeld et al. (2005) did, but also included a separate no prior contact environment to examine the importance of social cues on social behaviour. Interestingly, they found that intranasal OT did not increase trust in participants who had prior social contact and subjects felt less

connected to each other. Conversely, in participants with no prior contact, OT increased trust but only in subjects with a low trust disposition, measured using a validated trust scale, in relative to placebo treated subjects. Overall, it is apparent that any general statement of 'OT increases trust' or any other positive social behaviour is not as straightforward as it seems. Instead, we have to start taking into account the varying effect OT may have in a heterogeneous population surrounded by different positive and negative social cues and how this may potentially affect treatment for patients with socio-behavioural deficits.

Author	Dose	Observation
Declerck et al., 2020	24IU 50 mins prior	Participants had no social contact or allowed to interact 5 mins prior task. In participants with prior contact, OT did not increase trust. In participants without prior contact, OT increased trust in participants with low trust disposition
DeWall et al., 2014	24IU 45 mins prior	OT increased aggression towards their partners, but only in participants prone to physical aggression
Kosfeld et al., 2005	24IU 50 mins prior	Participants allowed to interact 5 mins prior task. Increased trust of participants playing an 'investor' role towards others during a monetary game
Shamay-Tsoory et al.,2009	24IU 45 mins prior	OT increased envy ratings in a relative monetary loss condition and increased gloating related emotions in a relative monetary gain condition

Table 5.1 Positive and negative social behavioural effects elicited by intranasal OT in healthy human volunteers.

In Chapter 4, an increase in prosocial behaviour after pre-treatment with intranasal OT was seen in otherwise healthy adult male rats. There was no changes in aggressive behaviour. The rat is a classical model in social neuroscience, chosen on the basis that they are social animals displaying behaviours ranging from huddling to social recognition (Kondrakiewicz et al., 2019). Neuropeptides, in particular the nonapeptides OT and AVP, has an established role in social behaviour with their importance in maternal behaviour and pair bonding first discovered in the rat and prairie vole respectively (Insel, 2010). As such, the ability of OT to produce similar pro-social behaviour in both rodents and humans (discussed in Chapter 1.5) led to the examination of intranasal OT/OT-GET's social behaviour in the rat in this thesis. However, discrepancies exist: in the rat, OT primarily affects social interaction and recognition of conspecifics via the olfactory pathway while in NHPs, OT modulates visual attention, suggesting visual cues hold dominance for subsequent interpretation and communication in higher order mammals including humans (Althammer et al., 2018).

The examination of complex social behaviour in a simplified environment as described in this thesis cannot accurately represent the diverse repertoire of social behaviour. For example, despite great care to ensure weight-matched rat pairs were from different littermates to ensure no prior recognition which may affect the extent of social interaction, social hierarchy was not taken into consideration in this case, where the more dominant rodent is known to sniff and explore its subordinate counterpart at a higher frequency (Wesson, 2013). Lastly, this thesis examined the effect of intranasal OT/OT-GET on social interaction in otherwise healthy rats, and it remains to be confirmed the effects they might have in a model of impaired social interaction, although intranasal OT has been observed to attenuate social interaction deficits in mouse models of ASD (Hara et al., 2017, Kitagawa et al., 2021).

5.4 General limitations and future studies

Several limitations have been touched on throughout this thesis.

To summarise, Chapter 3.3.4 suggested that the OT-GET complex dissociated before/upon transversing the nasal epithelial monolayer as OT concentration in basolateral chamber samples did not differ between heparin-added and non heparin-added samples. An ELISA to examine OT concentrations in the cellular layer would allow a better understanding of how much free OT is actually released across the membrane in relation to the amount trapped. This will allow future studies regarding GET to focus on areas that require improvement, such as improving peptide release from endosomes, which has been a long-standing issue with CPP technology (Fan et al., 2018). However, the ELISA was unable to be carried out due to financial limitations. In Chapter 4.5.6, the inability to successfully obtain olfactory epithelium due to technical inexperience for microscopy examination for Study 1 meant that it remains undetermined as to whether the lack of OT-GET fluorescent in the brain was due to the nasal cavity being a barrier (indicating the method of intranasal administration/dosage would have to be reviewed) or if the complex dissociated, leaving non-fluorescently labelled OT to penetrate the brain. Future experiments examining the olfactory epithelium in addition to the olfactory bulb would be useful.

In Chapter 4, an understandable question is why test the effect of OT-GET on behavioural assays prior to performing a robust dose-response testing in otherwise normal animals? A detailed dose-response study was originally planned to identify suitable dosage and pre-treatment time. However, due to the pandemic (the University of Nottingham was closed from March 2021 to July 2021 due to COVID-19 and approval of COVID safe risk assessments were required before animal experiments were able to resume in September 2021), the resulting time constraint allowed for only a single final behavioural assay. As such, the study aim was altered to utilise the PCP-induced hyperactivity task, robustly replicated several times in our lab to be reversed by systemic OT, to examine if it

would be a similar suitable phenotypic readout to examine intranasal OT's efficacy. This also explains the high dose of OT (100µg) used in Study 2 and 3 compared to literature (typically 20µg). Understandably, this was a high-risk approach, but if the assay had been successful, would justify the high cost and ethics required for a subsequent detailed dose-response and brain level assessment experiment This would also justify the high number of rats and multiple ELISAs required.

Testing in healthy animals also seemed a logical step before moving on to behavioural assays using the PCP model. However, considering the social salience theory (Chapter 5.3) and that response to OT have been proposed to occur in an inverted U-shaped dose-response curve in rats and humans (Bielsky and Young, 2004, Rilling et al., 2014), this suggests beneficial effects may only be seen in individuals with sub-optimal functioning. Conversely, deleterious effects may occur if the typical 'oxycinergic' system is exceeded. Bearing this in mind, would the examination of OT/OT-GET's effect in healthy animals be the most insightful? Perhaps careful selection of a suitable, well-validated behavioural task in an animal model would be more ideal. PCP was utilised in this Chapter as an acute pharmacological manipulation, and if hyperactivity successfully reversed, a dual hit model such as the neonatal PCP-isolation rearing rat model which represents a more robust animal model for schizophrenia (Gaskin et al., 2014), would be used.

The PCP-induced LMA task used in this thesis was clearly robust and well controlled, as systemic OT successfully reversed the hyperactivity seen. However, neither dose (10µg and 100µg) of intranasal OT/OT-GET had any effect and systemic OT's reversal of hyperactivity may involve peripheral mechanisms (discussed in Chapter 4.7.2). Thus, this assay may not be the most ideal moving forward. Of course, another issue to be mindful of is whether hyperactivity is a suitable index to examine OT/OT-GET's ability in an animal model for schizophrenia, as discussed previously. But again, this particular assay was used mainly to determine if a behaviourally active OT/OT-GET dose could be identified. If it had been successful, a more suitable model, for example the chronic-PCP

model or a dual hit neonatal PCP and post weaning social isolation, to address the fact that environmental insults during gestation or perinatal period play a crucial role in the disease development of heterogeneous disorders such as schizophrenia (Jones et al., 2011, Karimi et al., 2017), would have been used.

Overall, potential future studies to carry out include a full range dose response study in healthy rats to identify suitable OT-GET conjugation dose ratios. This will allow for efficacious doses and pre-treatment times that significantly raises OT levels in the brain to be determined. A parallel examination of OT plasma levels should be carried out to ensure no increase is noted that may cause systemic side-effects. Doses that show potential may be brought forward to be tested in a more robust animal model for schizophrenia such as the neonatal PCP and social isolation model. With current results in mind, it is quite clear that the PCP-induced hyperactivity task is not a fair assessment for the effects of intranasal OT. The social interaction task in animal models would perhaps be more suitable as it has been shown to enhance social interaction over multiple occasions (Calcagnoli et al., 2015, Huang et al., 2014, Kent et al., 2016) and systemic OT has been shown to enhance prosocial behaviour (Kohli et al., 2019 and unpublished work from a PhD student in the Fone lab), which could be used as a positive control.

Looking at a bigger picture of the field, another limitation in this study and much of existing literature is the lack of understanding of the pharmacokinetics and pharmacodynamics of intranasal OT administration. In Chapter 4, an ELISA successfully quantified OT concentrations in the brain after OT delivery, but it is unknown as to whether the increase in OT levels are due to exogenous material or resultant modification of endogenous OT synthesis, storage and/or release, although a study in NHPs contributes the rise in OT to exogenous peptide (Lee et al., 2020). Having this knowledge would allow us to better decipher which social behaviour changes are truly elicited by intranasal OT delivery, as Martins et al. (2020b) found activation of the amygdala by both intravenous and intranasal OT, suggesting a cautious

approach to attributing all behavioural changes to intranasal delivery. This understanding will also better allow trials to decide appropriate doses to obtain desired behavioural effects. As such, being able to track exogenously administered OT would be highly desirable. Beard et al. (2018) has been the only group to attempt an OTR tracer for this purpose: they designed an OTR selective tracer which core was synthesised based on the architecture of a OT analogue dLVT and incorporated fluoride labelling (^{18}F) to enable detection *in vivo*. When administered intranasally in the rat, significantly higher biodistribution in the olfactory bulb and cerebellum was observed after 30 minutes, compared to intravenous delivery. The main drawback of their study however, was the lack of tracer penetration to deeper brain tissue regions, although this may be due to the timeframe used.

5.5 Conclusion

This thesis set out to examine if intranasal delivery as well as the usage of a novel cell penetrating OT conjugate would improve CNS OT penetration and affect social behaviour and cognition in rats while attenuating unwanted peripheral side-effects. We successfully showed that GET does improve OT permeation across a cell monolayer *in vitro* and into the olfactory bulb *in vivo*, although this did not translate to enhanced behaviour effect following intranasal administration in the conscious rat. Further studies regarding receptor desensitisation may be required to understand this contradiction.

OT is an increasingly popular therapeutic for potential treatment of CNS disorders that display socio-behavioural deficits. However, future studies should be designed to accommodate for the social salience theory. Experimental findings may hold less value if studies continue in a traditional way of approaching complex social behaviour via simple one-track tasks. Despite many preclinical animal studies showing a prosocial effect of OT, mixed results are seen when evaluated in clinical trials. Indeed, clinical trials may suffer from a multitude of methodological flaws

including insufficient statistical power and poor replication designs (Quintana et al., 2019), however it may also be an impact of applying OT to a highly heterogenous population. As such, more preclinical animal studies have now considered this, for example examining social behaviour in group-living mice in a semi-natural environment (Anpilov et al., 2020, Kim et al., 2019).

Overall, intranasal OT administration for treatment of social behaviour and cognitive deficits remains promising. However, work on improving brain penetration, understanding its pharmacodynamics and the need to move away from a rigid way of testing complex social behaviour should be a focus for current research to provide robust translational outcomes in the clinic.

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