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Department of Physiology

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Functional characterisation of gene variants of oxytocin and vasopressin receptors implicated in psychological disorders

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I, Michelle Anne Steven, declare that the dissertation, which I hereby submit for the degree MSc Human Physiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Executive Summary

Oxytocin (OXT) and arginine vasopressin (AVP) are well known for their classical roles in initiation of myoepithelial contractions during labour and lactation, and regulation of blood pressure and osmolality, respectively. However, there is also substantial evidence supporting central roles of these neuropeptides in neuronal and cognitive functioning. Indeed, their altered signalling has been implicated in several psychological disorders, social impairments, and behavioural traits.

OXT and AVP elicit their effects through interaction with cognate G protein-coupled receptors (GPCRs). The OXT receptor (OXTR) and two AVP receptor subtypes (AVPR1a and AVPR1b) are expressed in many brain regions. Several studies have highlighted the potential association of genetic variants of these receptors with behavioural/social disorders. Yet, the impacts of these variants on receptor function are often unknown.

Through literature research, a selection of naturally occurring OXTR and AVPR1a/1b variants (11 and 7 variants, respectively) associated with a variety of psychological disorders, were identified. In the case of the OXTR, a range of variants linked to birth disorders (a group of disorders in which OXTR variants/ disrupted OXT function has a very well characterised role) were included for comparison. The variants were first analysed using *in silico* methods to predict their effects on receptor function. This was followed by *in vitro* characterisation of receptor expression (by receptor ELISA assay), receptor signalling (by inositol phosphate accumulation assay) and ligand binding (by radiolabelled ligand binding assay). This *in vitro* analysis demonstrated that OXTR variants V45L, A63V, M133V, H173R, W203R, G221S, A238T, I266V, T273R, T273M and V281M, AVPR1a variant F136L and AVPR1b variants K65N, G191R, R364H and R364L, result in severe or partial reduction in receptor function and, therefore, these variants may contribute to the pathophysiologies of psychological/birth disorders that have been indicated in genetic association studies. The nature of dysfunction for each receptor was then further characterised. For the OXTR the majority are believed to be Class II and IV variants, while Class IV variants seem to predominate for AVPR1a/b.

From comparison of the *in silico* prediction outcomes and *in vitro* analyses, it was

apparent that the ability of variant effect prediction (VEP) programs to successfully predict the functional consequences of GPCR variants was variable and, in some cases, unreliable. The VEP program SIFT appeared most reliable for future *in silico* analysis of OXTR, while LRT, Mutation Assessor and Mutation test, have the highest predictive power for future *in silico* analysis of AVPR1a but none of the VEP programs appear to be accurate for the *in silico* analysis of AVPR1b variants.

Keywords:

G protein-coupled receptors, Oxytocin receptor, Arginine vasopressin receptor 1a and 1b, Psychological disorders, Birth disorders, Gene variants, Variant effect prediction.

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List of Abbreviations

Abbreviation	Definition
OXT	Oxytocin
AVP	Arginine vasopressin
OXTR	Oxytocin receptor
AVPR1a	Arginine vasopressin receptor 1a
AVPR1b	Arginine vasopressin receptor 1b
AVPR2	Arginine vasopressin receptor 2
PVN	Paraventricular nucleus
SON	Supraoptic nucleus
HPA axis	Hypothalamo-pituitary-adrenal axis
CRH	Corticotropin-releasing hormone
ACTH	Adrenocorticotropic hormone
ASD	Autism spectrum disorder
PTB	Pre-term birth
mRNA	Mitochondrial ribonucleic acid
NMDA	N-methyl-D-aspartate
GABA	Gamma-aminobutyric acid
GPCR	G-protein coupled receptor
TM	Transmembrane
ICL	Intracellular loop

ECL	Extracellular loop
DAG	Diacylglycerol
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
IP3	Inositol triphosphate
Ca2+	Calcium ions
PKC	Protein kinase C
SNP	Single nucleotide polymorphism
PC	Pharmacological chaperone
ELISA	Enzyme-linked immunosorbent assay
IP assay	Inositol phosphate accumulation assay
LB	Luria Bertani
FBS	Foetal bovine serum
XTG	X-treme gene
Cpm	Counts per minute
Dpm	Decays per minute
SEM	Standard error of mean
ANOVA	Analysis of variance

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1. Introduction

1.1 Neuroendocrinology

The study of neuroendocrinology describes neuronal circuits as the control centre for organs involved in the endocrine (hormone) system.[1] This interaction between the nervous and endocrine system enables regulation of homeostasis and other physiological processes.[1] The hypothalamus is the integration centre that receives and processes internal and external cues, and is therefore a key structure in neuroendocrinology and plays a vital role in the regulation of downstream endocrine systems.[1] Together with the pituitary gland, the hypothalamus forms part of a hypothalamic-pituitary complex that controls a wide range of physiological processes, which include growth, metabolism, responses to stress, sexual maturation, reproduction, feeding behaviours and social interactions.[2] Defective action of these neurohormonal pathways are a major causal factor for several physiological and psychological abnormalities.[1]

1.1.1 The Hypothalamic-Pituitary Axes

The hypothalamus is a small structure located in the ventral brain, which coordinates the neuroendocrine system. Structurally, the hypothalamus includes grey matter conglomeration of neurons arranged in distinct nuclei, as well as white matter formed by myelinated nerve fibres. The diverse hypothalamic nuclei have distinct functions. These nuclei and their main functions are: the preoptic (thermoregulation), medial preoptic (release of gonadotropic hormones), supraoptic (release of vasopressin and oxytocin), paraventricular (release of thyrotropin-releasing hormone, corticotropin-releasing hormone, oxytocin and vasopressin), anterior hypothalamic (regulation of thermogenesis and sweating), superchiasmatic (regulate circadian rhythms), lateral (primary source of orexin neurons projecting throughout the brain and the spinal cord), dorsomedial hypothalamic (regulate blood pressure and heart rate), ventromedial (satiety control), arcuate (regulate growth hormone-releasing hormone and feeding) and posterior (pupillary dilation and shivering) nuclei.[3] The hypothalamus is anatomically connected to the pituitary gland (also known as the hypophysis) via the

pituitary stalk (or infundibulum). The pituitary gland consists of two lobes developed from distinct parts of embryonic tissue: the posterior pituitary (or neurohypophysis) is comprised of neural tissue, whereas the anterior pituitary (adenohypophysis) is comprised of glandular tissue, originating from the primitive digestive tract.[4]

The different hypothalamic nuclei receive and integrate specific afferent signals emanating from the brainstem, thalamus, basal ganglia, cerebral cortex, and olfactory areas.[5] In response, the hypothalamus stimulates/inhibits the secretion of various hormones from the pituitary into the blood stream, which, in turn, act on various target organs to elicit specific physiological responses. The hypothalamus can affect pituitary hormone release in two distinct ways. The first is through the release of specific releasing/inhibiting neurohormones (such as thyrotropin-releasing hormone, gonadotropin-releasing hormone, growth hormone-releasing hormone, corticotropin-releasing hormone, somatostatin, and dopamine). These neurohormones enter a specialised capillary system known as the hypophysial portal blood system, from where they travel to the anterior pituitary and simulate/inhibit the release of other hormones (such as thyroid stimulating hormone, follicle-stimulating hormone, luteinizing hormone, growth hormone, adrenocorticotropic hormone, and melanocyte-stimulating hormone) into the general circulation.

The second involves release of neurohormones directly into the general circulation from neurons that project directly from the hypothalamus to the posterior pituitary. Arginine vasopressin (AVP) and oxytocin (OXT) are examples of hypothalamic neurohormones released in this manner. Efferent neural pathways from the para-ventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus connect the hypothalamus to the posterior pituitary gland via the hypothalamic-hypophyseal tract, within the pituitary stalk. The cell bodies of these magnocellular neurons originate in the hypothalamus, while their axons descend via the tract, and terminate in the posterior pituitary (Illustrated in

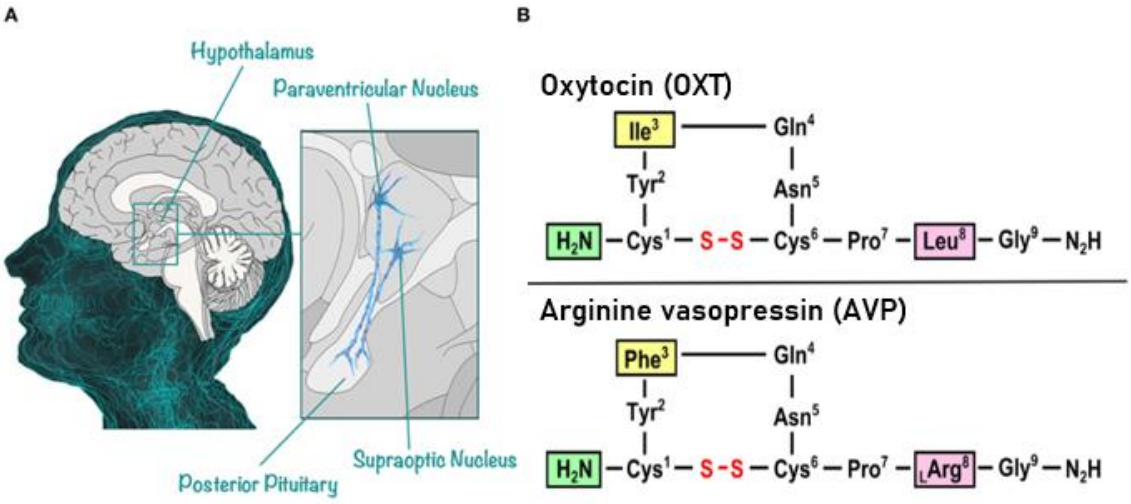


Figure 1A). [5] OXT and AVP are synthesised in these neurons (PVN neurons predominantly synthesise OXT, while SON neurons predominantly synthesise AVP). Following their synthesis, the neurohormones are transported along the axons to the posterior pituitary where they are stored in secretory structures, known as Herring bodies at the axon terminals, with each not stored simultaneously in the same Herring body.[6] The activation (firing) of these neurons is under the control of various stimuli integrated by the hypothalamus and results in release of the stored neurohormones into the general circulation from where they can travel to act on peripheral targets.[7–9]

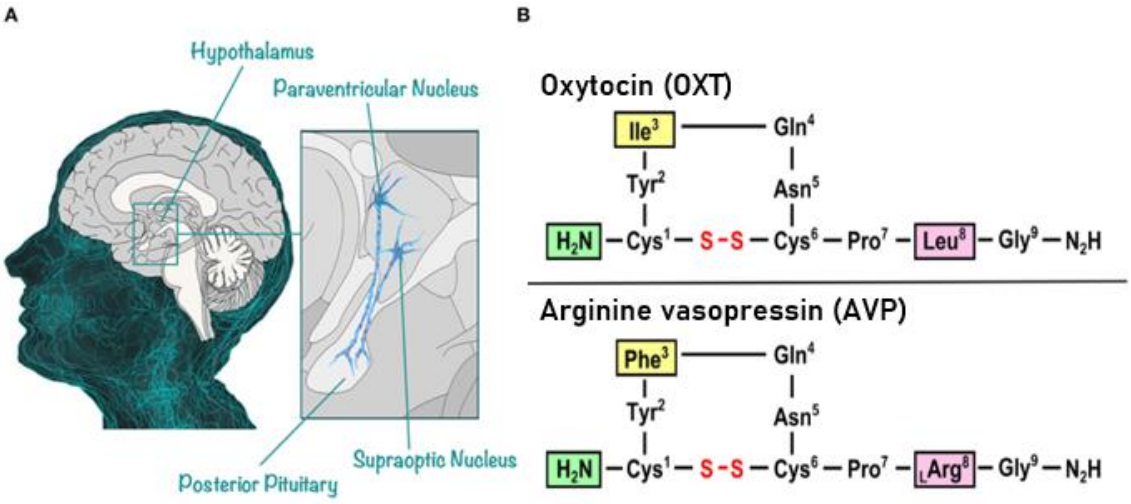


Figure 1: Oxytocin (OXT) and arginine vasopressin (AVP) are two closely related nonapeptides that exert their action on central and peripheral targets.

OXT and AVP are synthesised in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus. Neurons from these nuclei project axons to the posterior pituitary, from where the

peptides are released into the circulation and act on peripheral targets. In addition, dendrites of these PVN and the SON neurons release the peptides directly into the brain, where they act to regulate complex social cognition and behaviours. ***(B)*** *OXT and AVP are structurally very similar and differ by only two amino acids: the amino acid sequence of OXT (top) has an isoleucine (Ile) at position 3 and a leucine (Leu) at position 8, while AVP (bottom) has a phenylalanine (Phe) at position 3 and an arginine (Arg) at position 8. Both peptides contain a cyclic six amino acid ring as a result of a disulfide bond formed by two cysteine residues at positions 1 and 6 (seen in red). (Figure adapted from Aspé-Sánchez et al.*[19] *under the Creative Commons Attribution Licence (CC BY))*

1.1.2 Psychoneuroendocrinology

Psychoneuroendocrinology is the study of the relationship between the endocrine system, the nervous system and psychology. The primary purpose of psychoneuroendocrinology as a science is to shed light on the complexity of how endocrine pathways affect and/or determine behaviours, and thus play a role in psychological disorders and symptoms. Goals of this branch of science include, but are not limited to, understanding the intricacy of psychological disorders by studying the effects of psychological stress, the role of the gut-liver-brain axis, the role of endocrine pathways that control feeding, appetite, sleep and sexual desire, the endocrine correlates of sex differences in human behaviour, and the role of the neuroimmune system and neuroinflammatory pathways. As such, psychoneuroendocrinology includes basic, clinical, and translational knowledge on neuroscience, psychiatry, psychology, neurology, mental health, endocrinology, gastroenterology, human development, genetics, biochemistry, behavioural medicine, and general medicine.[10]

1.2 Psychological Disorders

Psychological disorders (also referred to as mental disorders or mental illnesses) represent a major global health issue. Indeed, it is estimated that more than 50% of people will suffer from a psychological disorder in their lifetime,[11] and that almost 1 in 5 adults in the US will suffer from a mental disorder each year.[12] In South Africa, it is estimated that as many as one in six South Africans suffer from anxiety, depression or substance-use problems (and this does not include more severe psychological disorders such as bipolar disorder or schizophrenia).[13] Furthermore, co-morbidities,

such as human immunodeficiency virus (HIV), which has high incidence in South Africa, can exacerbate mental health disorders. Indeed, it is estimated that over 40% of people living with HIV in South Africa suffer from psychological disorders.[13]

Psychological disorders are broadly defined as the deviant, distressful and dysfunctional patterns of thoughts, feeling and behaviours. The current model used to study these disorders is known as the biopsychological model, which provides a more holistic approach of evaluating the biological, psychological, and social-cultural influences for an individual's psychological disorder.[14] However, these disorders are wide ranging in symptoms and severity, and cover a spectrum of categories which is defined in the American's Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders 5th Edition (DSM-V).[15] With successive editions over the past 60 years, this has become a standard reference for clinical practice in the mental health field and is designed to facilitate more reliable diagnoses of these disorders. The various categories include, but are not limited to, neurodevelopmental disorders (such as autism spectrum disorder; ASD), schizophrenic and other psychotic disorders, bipolar and related disorders, depressive disorders (such as postpartum depression and seasonal defective disorder), anxiety disorders (such as post-traumatic stress disorder, panic disorder and social phobia), trauma- and stressor-related disorders (including abnormal aggressive behaviour), feeding and eating disorders and substance-related/addictive disorders.[15]

1.3 Oxytocin and Arginine Vasopressin

The neuropeptide hormones OXT and AVP are phylogenetically conserved in structure and function. Related peptides are detectable in all vertebrate species and are thought to have evolved from similar ancestral molecules.[16] The nine amino acid sequence of OXT (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly), differs from AVP, by only two amino acids, namely phenylalanine in position 3 and arginine in position 8. Both peptides contain two cysteine residues that form a disulfide bond, creating a cyclic six amino acid ring structure (

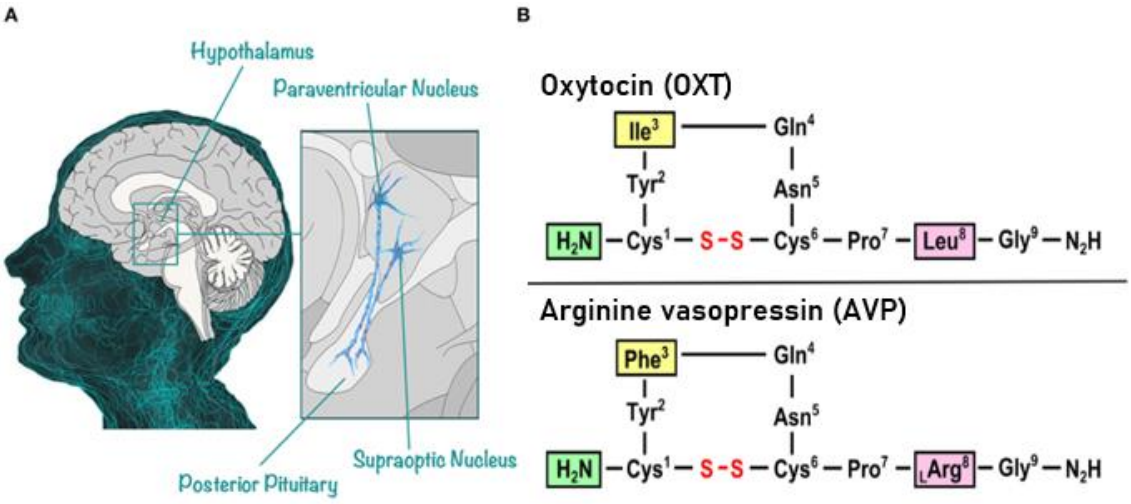


Figure 1B).[17] Oxytocin elicits its effects through a single receptor (the OXTR), while AVP has three known receptors, namely AVPR1a, AVPR1b and AVPR2,[18] which mediate the effects of their respective hormones.

1.3.1 Physiological Roles of OXT and AVP

Activation of their cognate receptors by OXT or AVP stimulates cellular responses that are important for the physiological roles of these hormones within the body.

1.3.1.1 Peripheral roles of OXT and AVP

The OXTR is expressed peripherally at high levels in the uterus and the mammary glands where it is responsible for transducing the well-documented actions of OXT of stimulation of uterine and mammary gland contractions to induce labour onset and milk ejection (see Section 1.3.1.1.1). Peripherally, OXTR expression has also been detected in the ovary, osteoclast cells of the bone and pituitary lactotrophe cells (the cells responsible for prolactin secretion).[9]

The AVPR2 receptor subtype is expressed at high levels on the basolateral membrane of the collecting duct in the medullary portion of the kidney (where it plays a vital role in the body's osmoregulation system (see Section 1.3.1.1.2).[20] While, the AVPR1a and AVPR1b receptors are expressed in several peripheral tissues and organs including platelets, adrenal cortex, kidney, spleen, smooth muscle, endothelium and adipocytes.[20–22]

1.3.1.1.1 The roles of OXT in Labour and Lactation

In females, OXT and its receptor have important roles in foetal expulsion during labour. To prevent pre-term birth (PTB) and ensure optimal survival of the infant, the responsiveness of OXT neurons is restrained by neuroactive steroid metabolites of progesterone during pregnancy, which potentiate gamma-aminobutyric acid (GABA) inhibitory mechanisms that block impulses between neurons. However, while oxytocin neuron activity is inhibited, OXT peptide continues to be produced and accumulate in the neurons projecting to the posterior pituitary. A large increase of OXTR expression in uterine epithelium is also seen in late pregnancy.[23] Consequently, it appears that it is not changes in hormone concentration that are critical to parturition but an increase in uterine sensitivity to OXT. This increase in responsiveness is achieved through a specifically timed up-regulation of OXTRs. The initiation of parturition is induced by decreased progesterone secretion, which releases the GABA-ergic inhibition and thus results in increased in OXT neuron activity, allowing the release of the large amount of stored OXT. [24] Following initiation of labour, OXT is secreted in pulses (throughout the labour processes) and, through its interaction with OXTRs located in the uterine epithelium, acts to stimulate myometrial contraction. This aids in moving the foetus along the birth canal which results in mechanical distention of the cervix and stimulates stretch receptors creating a positive feedback mechanism to the PVN and SON, resulting in the secretion of further pulses of OXT, and concludes with foetal expulsion.[23] OXT also stimulates the release of prostaglandins in the uterus, which also stimulate uterine contraction. Due to its role in labour, exogenous OXT analogues are commonly administered to woman in order to stimulate delivery in stalled labour or to initiate placental delivery.[25,26]

OXT is also important for milk ejection during lactation. The process of lactation is controlled by two neurohormones, prolactin and OXT, which are responsible for the synthesis and ejection of milk, respectively.[27] Prolactin triggers milk synthesis in the mammary gland and OXT stimulates the contraction of myoepithelial cells in the mammary gland, which promotes milk delivery to the young in a pulsatile manner via suckling of the nipple.[28] Similar to labour, a positive feedback mechanism is seen in breast feeding. When a baby suckles at the breast, sensory impulses pass from the

nipple to the hypothalamus, which stimulates synthesis and release of more OXT and thus more milk ejection, which is only terminated by the cessation of suckling.[29] These OXT positive feedback mechanisms are very different from the homeostatic control of the other neuroendocrine reflexes which are typically controlled by negative feedback loops.

1.3.1.1.2 The role of AVP in Water Homeostasis

The primary peripheral role of AVP is in maintaining water balance through vasoconstriction and the regulation of water absorption in renal tubules, which is why AVP is also sometimes referred to as antidiuretic hormone.[30] When the body experiences a state of hypotension (i.e. dehydration), hypothalamic osmoreceptors sense an increase in extracellular osmolarity above a certain threshold (typically around 292 mOsm/kg of water) and stimulate the release of AVP from the posterior pituitary, which is enhanced by angiotensin II through the renin/angiotensin system.[31] AVP binds to AVPR2 receptors found on the basolateral membrane of the collecting tubules of the kidney. This initiates aquaporin-2 water channel synthesis and insertion into the apical membrane of the collecting duct. As a result, AVP increases water permeability of the tubules and reabsorption of water from the urine into the blood. Active AVPR2 receptors are also found on the Loop of Henle in the kidneys where AVP stimulates the reabsorption of sodium ions (which, in turn, stimulates further water reabsorption).[18] Through interaction with AVPR1a receptors on vascular smooth muscle cells, AVP also stimulates vasoconstriction and thus an increase in arterial blood pressure.

1.3.1.2 Central roles of OXT and AVP

In addition to their major peripheral roles, OXT and AVP peptides also have central effects in the brain, which is the focus of the present study. Dendrites from the OXT/AVP neurons of the PVN and SON project to many brain regions where they secrete these neuropeptides via dendritic release.[32–34] Centrally, both OXT and AVP have been implicated in the regulation of complex social cognition and behaviour,

including attachment, anxiety and aggression.[35,36] Indisputably, there is a growing body of evidence showing that the actions of these neuropeptides are impaired in psychological disorders associated with social deficits (see Section 1.3.1.2.1 and 1.3.1.2.2).[8] The complex nature of emotional and cognitive behaviour is regulated by several neurotransmitters and neuromodulators. Indeed, it is hypothesised that the actions of OXT and AVP can be mediated by, or coupled to, the actions of other neurotransmitters such as GABA, norepinephrine and acetylcholine,[37] or neuropeptides such as serotonin, reelin and dopamine.[8] Centrally released AVP and OXT are also involved in modulation of neuroendocrine stress responses (see Section 1.3.1.3), which can also impact physiological functioning.[38]

The central distribution of OXTR is highly species specific, which may contribute to striking differences in social affiliative behaviour between different species.[39] In the human brain, OXTRs are widely expressed in the central and basolateral amygdala, medial preoptic area, anterior and ventromedial hypothalamus, olfactory nucleus, vertical limb of the diagonal band of Broca, ventrolateral septum, anterior cingulate, hypoglossal, and solitary nuclei.[19]

AVPR1a and AVPR1b are also expressed centrally and are found in the lateral septum, thalamus, basal amygdaloid nucleus and brainstem, but not in the cortex.[7] There is no evidence that AVPR2 is located in the brain/central nervous system or that it is associated with psychological disorders.[17]

1.3.1.2.1 OXTs role in Cognitive Functions and Social Interactions

OXT has been associated with interpretation of social cues, pair bonding and maternal-foetal bonding in many species. For example, cerebrospinal fluid levels of OXT in human neonates positively correlates with the degree of social interaction.[40] This is thought to be due to its central release into the amygdala and nucleus accumbens where OXT is believed to act as a social reinforcement signal, enhancing

the response to social cues.[41] OXT is involved primarily with parasympathetic actions of the autonomic nervous system,[42] and has been shown to facilitate prosocial and "approaching" behaviour, social memory and recognition, [43,44] and impedes callous and unemotional traits in children, as well as a reduction in anxiety and reaction to stressors.[45–49] Both association and animal knockout studies have linked the dysfunctional central OXT/OXTR signalling as a possible pathogenesis of social dysfunction in psychological disorders such as ASD (a psychological spectrum disorder characterised by impaired speech and language, impaired reciprocal social interaction and repetitive, stereotyped motor and behavioural activity).[47,50–52] Further evidence of the role of OXT/OXTR in psychological disorders also comes from research examining the expression of the OXTR in post-mortem brain tissue, which report a different distribution and expression levels when comparing both cases and controls.[53] By way of illustration, in individuals with ASD, OXTR binding has been found to be increased in cortical and decreased in subcortical structures compared to the control individuals,[54] while individuals who suffer from major depression show increased OXTR expression in the prefrontal cortex,[55] and schizophrenic individuals showed reduced OXTR mRNA in several brain areas.[53] For these reasons, there is growing interest in the OXT system and its distinct roles in regulating an array of social behaviours.[56] Indeed, recent discoveries suggest a positive effect between the use of OXT analogues and the treatment of neurological cognitive and social disorders (such as ASD and depression)[35,57,58] and both preclinical and clinical studies have indicated that stimulating the OXT system may provoke therapeutic effects to provide relief from symptoms (paranoid delusions and psychosis) expressed for those suffering with schizophrenia.[59] Both acute and chronic OXT administration has also been shown to improve learning, working memory as well as enhancing cognitive benefits.[41] However, there is some debate regarding the effectiveness of administration of OXT (and AVP or their peptide analogues) to enable their access to central regions, which is usually achieved through intranasal administration.[60] Furthermore, several studies examining the effects of OXT in neurological conditions have shown varied degrees of success, highlighting the need for further research to fully understand the functionality of OXT/OXTR in the central nervous system, particularly in pathogenic states. Further evidence of OXT/OXTR roles in psychological functioning/psychological disorders have also come from population genetics studies

concentrating on gene-disease associations through the study of variation/identification of OXT/OXTR variants within certain populations. In such studies OXT/OXTR gene variants have been identified in individuals suffering from various psychological disorders (see Section 1.5 and Chapter 4 for details).

1.3.1.2.2 AVPs role in Cognitive Functions and Social Interactions

Like OXT, AVP also has a complex association with the central nervous system and behaviour. The AVPR1a and AVPR1b receptors are expressed in many brain areas (see Section 1.3), while association studies have linked AVP to a number of behavioural functions including defensive behaviour in animals,[61] sexual cues in human males,[62] hierarchy behaviour and aggression.[63,64] Indeed, a recent study has demonstrated that AVPR1b knockout mice exhibit markedly aggressive behaviour.[64] Clinical studies have also suggested an increase in AVP may be linked to psychological disorders such as anxiety, depression and post-traumatic stress disorder.[19,65,66] Studies show that administration of a selective AVPR1b antagonist produced anxiolytic- and antidepressant-like effects in rodents,[67,68] and reversed stress-induced suppression of neurogenesis in a mouse model of depression.[69] In addition, AVP has been shown to have central roles in circadian rhythm regulation [70], control of body temperature [71], social behaviour, cognition and emotion.[64] Evidence that AVP may play an important role in the regulation of social interaction comes from phencyclidine-induced models of schizophrenia, in which rats display impaired social interaction, and a reduced density of AVPR1a has been observed in several brain regions.[7,64,70] Moreover, NC-1900, an AVP analogue, ameliorates social behaviour deficits induced by an N-methyl-D-aspartate (NMDA)-receptor antagonist.[67] Hence, AVP-related drugs have become the focus of intense interest for the treatment of psychological disorders and memory disturbance. Again, further evidence of AVP/AVPR roles in psychological functioning/psychological disorders have also come from population genetics, in which AVP/AVRR gene variants have been identified in individuals suffering from various psychological disorders (see Section 1.5 and Chapter 6 for details).

1.3.1.3 Roles of OXT and AVP in regulation of the hypothalamic-pituitary-adrenal

axis and stress

The response to chronic stressors is predominantly regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Hypothalamic corticotropin-releasing hormone (CRH) is released into the hypophysial-portal blood system that connects the hypothalamus and anterior pituitary. Here it triggers adrenocorticotropic hormone (ACTH) release from corticotrope cells of the anterior pituitary into the general circulation where it can travel to, and act on, the adrenal glands to stimulate release of a variety of steroid hormones, including the glucocorticoid stress hormone, cortisol.[72] The HPA-axis, and thus cortisol levels, are significantly suppressed by OXT and stimulated by AVP, therefore these two neuropeptides provide contradictory feedback to the stress response.[8]

Acting through AVPR1b receptors expressed on pituitary corticotrope cells, AVP enhances ACTH release by CRH. Indeed, administration of exogenous AVP can result in an increase in cortisol levels.[8] During acute stress, AVP expression and secretion rapidly increases similar to CRH and ACTH,[73,74] and knock-out experiments have demonstrated that AVPR1b receptors are required for maximum ACTH response.[38,74] Studies have also shown that, during chronic stress, pituitary AVPR1b receptors become upregulated as HPA-axis regulation switches from CRH to AVP,[75] suggesting that AVP has a primary role in the HPA-axis adaption to long-term stress stimulation. There is less research studying the AVPR1a-stress relationship, however, a recent study demonstrated the enhanced expression of AVPR1a in woman with post-natal chronic stress.[76]

The role of OXT in the stress response is also less clear. However, there is evidence that it can act to reduce the stress response from the HPA axis.[72] Both physiological and psychological stressors have been associated with increased plasma OXT levels,[57] while other studies have shown intranasal OXT administration induces anti-stress-like effects with a decrease in the activity of the HPA axis.[41,77] The mechanism behind these effects is still unclear, but is believed to be through interaction of OXT with centrally expressed OXTRs in various areas of the brain.[8]

Various studies have shown the impact of chronic stress exposure on the human brain and its' association with neurotoxicity as well as increased vulnerability effects on the

developing brain.[78–80] These neurotoxic environments may cause irreversible damage to the structure of neurons (i.e., neurohormone receptors) and may impact or elevate the imbalance related to psychological disorders and its symptoms.

1.4 OXTR and AVPRs

The OXTR and AVPRs belong to the Rhodopsin-like subfamily of the seven transmembrane domain G protein-coupled receptor (GPCR) super-family.

1.4.1 G Protein-Coupled Receptors

The GPCR family are cell surface proteins responsible for transducing extracellular stimuli to intracellular signalling responses through a complex cascade of intracellular proteins. They regulate the majority of cell surface signal transduction in the body and are the largest family of 'druggable' therapeutic targets.[81] Indeed, GPCRs are targeted by approximately one third of drugs in clinical use.[82]

All GPCRs consist of seven transmembrane (TM) alpha helical structures (TMs1-7), joined by three intracellular loops (ICLs 1-3) and three extracellular loops (ECLs 1-3), and bordered by an extracellular N-terminus and intracellular C-terminus.[83] They can be sub-categorised into five subfamilies (Glutamate-like, Rhodopsin-like, Adhesion, Frizzled and Secretin-like) based on other sequence and structural similarities.[84]

Intracellular signalling by GPCRs is conveyed by their interaction with intracellular guanine nucleotide-binding proteins (G proteins). This is enabled by a conformational change prompted by ligand binding to the GPCR which involves a transition between an inactive and active conformational state that unmasks a G protein binding site on the intracellular surface.[81] This conformational change principally involves movements of TMs 3 and 6, with the cytoplasmic end of TM6 moving outwards to expose the G protein binding site, also known as the 'polar pocket site'.[85]

G proteins are comprised of three subunits: G alpha (Gα), G beta (Gβ) and G gamma (Gγ), where Gβ and Gγ form a strongly associated Gβγ heterodimer. Binding of G proteins to activated GPCRs catalyses the exchange of GDP for GTP on the G protein

Gα subunit. This results in dissociation of the GTP-bound Gα subunit from the Gβγ heterodimer, allowing each subunit to activate to specific downstream effectors. The Gα subunit has intrinsic GTP-ase activity that catalyses the conversion of the bound GTP to GDP, which allows re-association of the Gα and Gβγ subunits, and thus a return of the G protein to its inactive state. There are many subtypes of each subunit, which can come together to form different G protein complexes. However, these can be divided into four major families (categorised by their constituent Gα subunits), namely $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11,}$ and $G\alpha_{12/13}$. Different GPCRs favour interaction with different G protein families, and the activation each of the families results in different cellular responses via activation of distinct intracellular signalling pathways.[86]

Regulation of GPCR activation can be mediated through processes of receptor desensitisation, internalisation, and downregulation to prevent over-stimulation and excess signal generation. Activated GPCRs are recognised and phosphorylated within their intracellular regions by GPCR kinases (GRKs). Other kinases, such as protein kinase A (PKA) and protein kinase C (PKC), that are activated via GPCR-mediated signalling pathways can also phosphorylate GPCRs in an activation-independent manner. Receptor phosphorylation promotes interaction with β-arrestin proteins, which occlude further G protein interaction (desensitisation) and facilitate clathrin-mediated endocytosis of the receptors (internalisation). Depending on the specific context/requirements of the cell, internalised GPCRs can either be recycled back to the cell membrane, where they will be available to undergo an additional cycle of activation or undergo lysosomal degradation (downregulation).[87]

However, a more unfamiliar mechanism is the independent signalling of GPCR's via the interaction with cytoplasmic scaffold proteins.[88] Studies have shown to activate this pathway with the influx of intracellular GRKs and β-arrestin.[88] However, the physiologic consequences of this unconventional signalling, particularly *in-vivo*, have not been explored.

1.4.2 OXTR and AVPR1a/b Synthesis and Processing

The life cycle of GPCRs, such as the OXTR and AVPRs, begins at their translation on

ribosomes of the rough endoplasmic reticulum (ER). Here the proteins are synthesised and fed into the ER lumen, where they are folded and assembled (a process that involves several ER-resident molecular chaperones and enzymes). Once correctly folded, the receptors are then be packaged into vesicles, which transport the receptor to the Golgi apparatus. During the migration through the ER and Golgi, post-translational modifications (e.g. glycosylation) are completed on the receptor to accomplish a mature structure. Mature receptors are then transported to their functional destination in the plasma membrane in vesicles that bud off from the Golgi.[89]

1.4.3 OXTR and AVPR1a/b Activation and Signalling

The OXT and AVP peptides interact with binding pockets on their cognate receptors involving the N-terminus, ECL 1 and TMs 1-2.[90] Once activated, OXTR, AVPR1a and AVPR1b all preferentially interact with, and activate, the $G\alpha_{q/11}$ subfamily of G proteins (Figure 2). Activated $G\alpha_{q/11}$ G proteins, in turn, stimulate the enzyme phospholipase C (PLC), which catalyses the conversion of membrane phospholipids (phosphatidylinositol 4,5-bisphosphate) to membrane-associated diacylglycerol (DAG) and water-soluble inositol trisphosphate (IP_3).[83] These second messengers can elicit a number of downstream cellular responses. For example, DAG stimulates activation of the enzyme protein kinase C (PKC), which can phosphorylate and modify the activity of various target proteins. The IP_3 diffuses intracellularly where it interacts with specific ligand-gated ion channel receptors located on the endoplasmic reticulum (ER), initiating the release of ER-retained calcium ions (Ca^{2+}).[91] Depending on the cellular context, the increase in intracellular Ca^{2+} can stimulate cellular effects/processes such as contractility or calcium-mediated exocytosis. Calcium ions can also interact with the protein calmodulin, which, in turn, interacts with and activates other target proteins, such as protein kinase B (Akt/PKB) and various transcription factors, to induce downstream cellular responses and altered gene expression.[91]

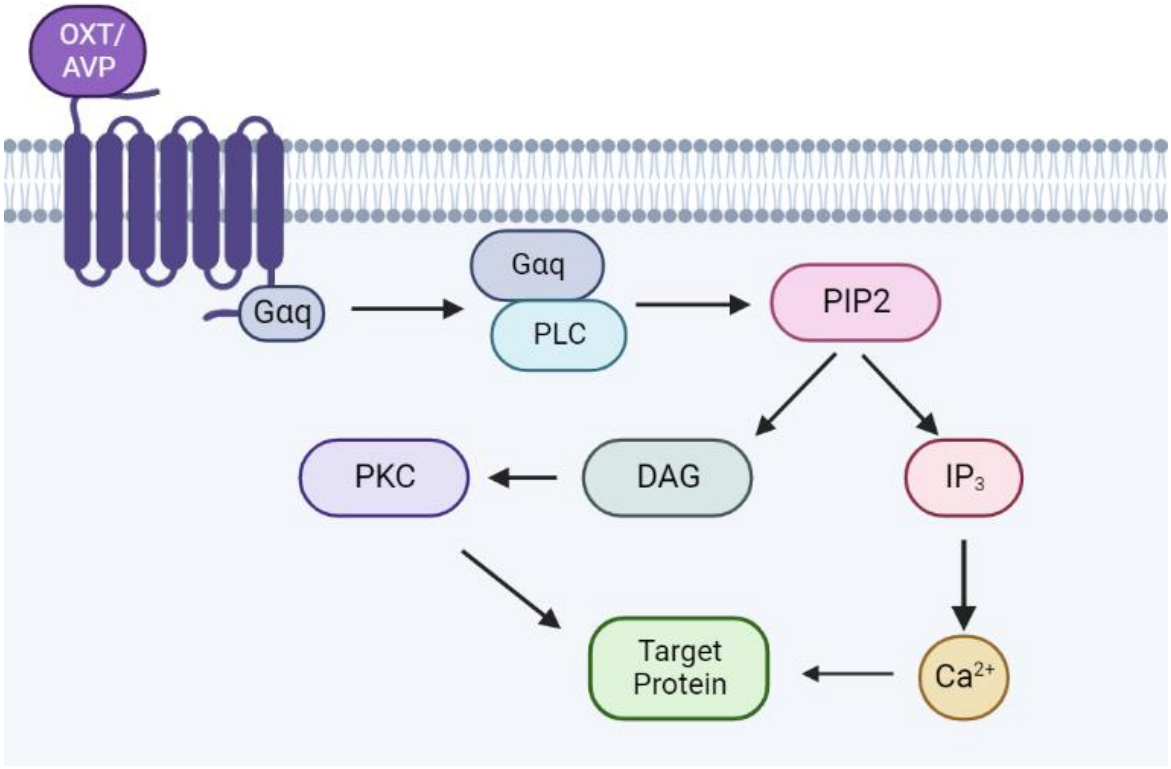


Figure 2: Signal transduction cascade stimulated by activation of oxytocin (OXTR) and arginine vasopressin 1a (AVP1a) and 1b (AVP1b) receptors

The binding of hormone ligand to OXTR, AVPR1a or AVPR1b stimulates the activation of phospholipase C (PLC) via interaction with $G\alpha_{q/11}$ G proteins. PLC catalyses the conversion of phosphatidyl inositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). Protein kinase C (PKC), activated by DAG, instigates phosphorylation of substrates which will characterise the response of the specific cell type. The IP_3 relays the influx of Ca^{2+} into the cytoplasm via opening of ligand-gated calcium (Ca^{2+}) channels on the endoplasmic reticulum. The increased Ca^{2+} stimulates cellular processes and modulates activity of various downstream target proteins. (Figure created with BioRender.com)

1.5 Pathogenic Genetic Variants of OXTR and AVPR1a/1b

Genetic variants of GPCRs such as the OXTR and AVPR1a/b can take several forms, from insertions, frameshifts, deletions, inversions, and single nucleotide

polymorphisms (SNPs; the single substitution of a nucleotide in a gene). These can either have no effect (silent) or be pathogenic, by causing loss-of-function or gain-of-function phenotypes. With respect to GPCRs, such variants can affect the expression and/or function of the encoded receptor protein and, thus, may result in the inhibition or over stimulation of a cellular response. As such, SNPs can act as biological markers for disease and can aid in implicating or excluding certain genes as contributing to certain disorders.

Studies investigating variants in the *OXT* gene in various populations have identified 14 non-coding SNPs in disorders ranging from ASD, harm avoidance, social cognition impairment to mood disorders.[92] Similarly, through studies analysing the gene encoding the OXTR several SNPs have been also identified. Many of these are in non-coding or regulatory regions of the gene, however, several are in the coding region (which will directly alter the amino acid sequence of the encoded protein after splicing).[93] Genetic association studies have linked genetic variants in the OXTR with several different disease phenotypes. Many of these are, not surprisingly, related to OXTRs effects on parturition including preterm birth (one of the primary causes of perinatal mortality)[20] and stalled labour.[25] Changes in the OXTR expression may affect the sensitivity of the receptor towards OXT, which may contribute to a variety of responses observed in clinical practice. Over expressed OXTR may lead to pre-term birth, where dysfunctional OXTR may lead to delayed labour. Both of these scenarios may leave the mother and the unborn child at risk of adverse complications during and after birth. The unpredictability of an individual sensitivity to exogenous OXT and subsequent prolonged OXT exposure increases the risks of uterine rupture, haemorrhage, hyponatremia, tachysystole, and foetal hypoxia, acidaemia, and abnormal heart rate tracings.[25] Furthermore, the variability of responsiveness to OXT may contribute to the increasing national caesarean delivery rate for the indication of labour dystocia. Numerous psychological disorders including excessive aggression/antisocial behaviour,[94] ASD, social cognition disorders, depression and schizophrenia have also been associated with OXTR variants (See Chapter 4 for more details).[19,44,94–97]

Population studies have identified more than 50 variants in the *AVP* gene which have

been linked to the pathogenic diabetes insipidus state.[98] This is a result of impaired signalling via the AVPR2 in the kidney. While no studies have yet implicated AVP mutations in other disease states, including psychological disorders, genetic variants within the gene encoding AVPR1a (*AVPR1A*, located on human chromosome 12q14-15) have been linked to cognitive/behavioural traits. Like the OXTR, several of these are associated with the non-coding regions of the gene. For example, studies have linked variants exhibiting different lengths of varying sequence repeats found in the promoter region of the gene (GT_{25}, RS1 ($[GATA]_{14}$) and RS3 ($[CT]_4TT[CT]_8[GT]_{24}$)) to behavioural traits, suggesting that they are relevant for brain functions related to emotional arousal and social behaviour. Individuals with shorter RS3 showed less altruistic behaviour while longer RS3 alleles were associated with greater levels of prepulse inhibition to a startle response (a neurological phenomenon where a weak stimulus (prepulse) can suppress the startle response to a subsequent stronger startle stimulus (pulse).[40] Population studies have also indicated an association between AVPR1a/b coding region SNPs and psychological disorders including ASD, aggression, depression, panic disorder and mood disorder (see Chapter 6 for details).[99]

Although the AVP receptors are expressed both centrally and peripherally, (see Section 1.3.1 and 1.3.1.2), it should be noted that variants found in AVPR1a and AVPR1b have only been associated with psychological traits and disorders. This emphasises the need to look at potential functioning of these gene variants in the neurobiological processes of normal physiology and pathophysiological states.

With regards to OXTR and AVPR variants linked to psychological disorders, the majority of the variants have not been analysed to determine whether they are indeed non-functional/pathogenic, nor have the mechanisms or the causes of their non-functionality been determined. The alteration in the gene sequence of a GPCR may impact a certain function due to the variant's location. For example, variants located in the coding sequence may affect the amino acid sequence (responsible for the folding, assembly, and trafficking), while other variants may be located in the regulatory/non-coding region of the genes and therefore affect biosynthesis (mRNA stability, post-translational modifications, etc.)

Loss-of-function genetic variants of GPCRs, such as the OXTR and AVPR1a/b, can affect receptor expression and/or function in a variety of different ways (Illustrated in Figure 3). A five-level classification system has been developed to categorise different types of suspected loss-of-function GPCR variants (see Section 1.4.1). Class I describes variants that result in defective receptor biosynthesis, Class II variants are those that lead to defective trafficking of the synthesised receptor protein to the cell membrane, Class III variants result in defective ligand binding, Class IV variants result in disrupted receptor activation/ problems in signalling, and Class V comprises of variants with no known defects (these variations can usually be disregarded as the cause of the disease in patient groups).[84]

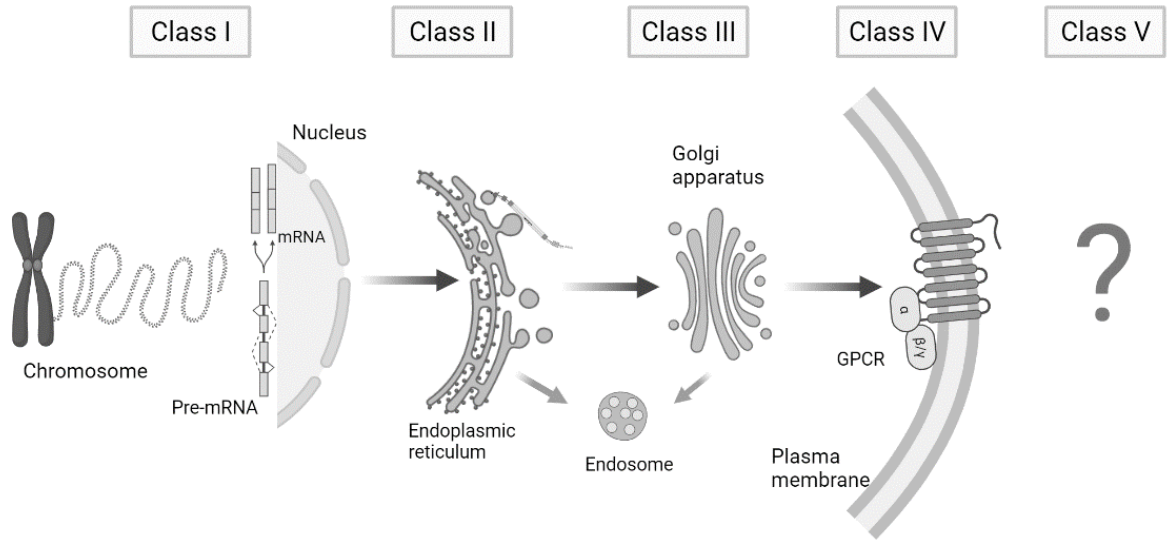


Figure 3: Classification of loss-of-function G protein-coupled receptor variants

Class I variants affect receptor biosynthesis, Class II variants affect receptor trafficking to the cell surface, Class III variants affect ligand binding, Class IV variants result in defective signalling and Class V variants have no known functional defects. (Figure created with BioRender.com)

Although little/no information is known regarding the pathogenicity and/or cause of non-functionality of the OXTR/AVPR1a/b mutations linked to psychological disorders, analysis of variants of several other GPCRs (including the AVPR2) has found that the most common pathogenic loss-of-function coding-region GPCR SNPs are Class II variants.[100–102] These receptors are synthesised, but the variants prevent the nascent protein from folding into its correct conformation with in the ER, resulting in 'misfolding' of the polypeptide (this can be due to changes such as amino acid hydrophobicity, size or polarity). Such misfolded proteins are detected by an ER-resident quality

control system.[103] This results in their retention in the ER (prohibiting further trafficking and insertion of the GPCR in the plasma membrane) while additional rounds of folding are attempted in a process termed the unfolded protein response (UPR). Should misfolding persist, the affected proteins are subjected to ER-associated-degradation (ERAD), whereby they are translocated to the cytosol where they are degraded by the proteasome. Should the UPR and ERAD processes not be able to deal with the volume of misfolded protein, misfolded GPCRs may aggregate and accumulate, which results in cell death/apoptosis.[104]

The balance of the dynamic processes of intracellular trafficking (i.e., synthesis, export from the ER/golgi, endocytosis/internalisation, recycling, and degradation) ultimately dictates the level of receptor expression at the plasma membrane, which, in turn, influences the magnitude of the cellular response to a given signal. When misfolding occurs, this prevents GPCR trafficking to the cell surface and thus reduces the response of the cells to hormone stimulation.

1.6 Research Problem

The emerging novel neuronal and cognitive effects of OXT and AVP have sparked interest with relation to the potential aetiology and treatment of psychological disorders and social impairments. Although a large body of research (ranging from rodents to human studies) has suggested a potential association between OXT/AVP and psychological disorders and has highlighted the possible genetic contributions of OXTR and AVPR1a/1b polymorphisms, there have been very few *in vitro* studies to determine the actual impact of these receptor polymorphisms on receptor function.

In some cases, *in silico* prediction programs have been used to attempt to verify whether the identified variants are indeed pathogenic. However, these tools have limited reliability/accuracy. Indeed, variants may be found only in diseased patient groups (and not in control groups) and yet do not cause dysfunction ('silent' variants), so their association with the disease is merely coincidental. Furthermore, even if a deleterious effect is predicted,[105] these *in silico* methods do not provide any information regarding the nature of the receptor dysfunction that is induced.

Understanding the nature of any defects in receptor function (e.g., impairments in the trafficking of the receptor (Class II variants), hormone binding (Class III variants) or receptor signalling (Class IV variants)) could aid in selecting or developing appropriate future treatment options to correct/overcome these deficiencies.

Therefore, this study aimed to examine the expression, hormone binding and signalling of several OXTR and AVPR1a/1b receptor SNPs that have been identified in patient groups suffering from a variety of psychological disorders. The results from these *in vitro* studies were compared to those obtained using a range of available *in silico* variant effect prediction tools to determine the effectiveness of these tools with regards to predicting functional outcomes of variants in these receptors. For comparison OXTR variants linked to birth-disorders (in which OXTR has a more indisputable role) were also examined.

As previous studies have demonstrated that the majority of GPCR variants (including those in the AVPR2[106]) result in receptor misfolding and loss of cell surface expression (Class II variants), it is hypothesised that the same will be observed for the pathogenic OXTR and AVPR1a/b variants. Thus, a novel class of therapeutic agents, termed pharmacological chaperones (PCs), may provide an attractive therapeutic approach for restoring function to these variant receptors, which could be beneficial for the treatment of their associated pathological disorders. PCs are cell-permeant receptor-selective small-molecule agents that are believed to interact with nascent proteins in the ER and act as molecular scaffolds, allowing stabilisation of variant-induced misfolding and bypassing of the cellular quality control processes, which enables restoration of their cell surface trafficking and, thus, function (see Section 1.4.1 for details). Therefore, the ability of existing small molecule cell-permeant ligands targeting these receptors to act as pharmacological chaperones was explored by examining the effects of such compounds on the cell surface expression of identified Class II variant receptors.

2. Aim and Objectives

2.1 Aim

The primary aim of this study was to characterise *in vitro* the function and expression of a number of naturally occurring variants of OXTR, AVPR1a and AVPR1b, identified in human patients with psychological disorders, in order to determine their pathogenicity and to interrogate the reliability of *in silico* variant effect prediction tools. A secondary aim was to explore the potential of existing small-molecule receptor-targeting compounds to act as pharmacological chaperones for these receptors.

2.2 Objectives

The objectives of this research study were:

i. To utilise *in silico* structure-function prediction tools to predict deleterious effects of variants of OXTR, AVPR1a and AVPR1b associated with psychological disorders or related impairments
ii. To generate mammalian expression vectors encoding the identified OXTR, AVPR1a and AVPR1b variants by site-directed mutagenesis for expression in an *in vitro* cell system
iii. To examine the ability of the OXTR, AVPR1a and AVPR1b variants to respond to cognate ligand stimulation using an inositol phosphate accumulation assay
iv. To examine the ability of the OXTR, AVPR1a and AVPR1b variants to interact with native hormone ligands using radioligand binding assays
v. To quantify cell surface expression levels of OXTR, AVPR1a and AVPR1b variants to identify those which cause intracellular retention (Class II mutants) using a cell surface receptor ELISA
vi. To determine whether small-molecule ligands can alter cell surface expression of identified Class II variants

3. Materials and Methods

3.1 Materials

3.1.1 Cell lines

HEK 293T cells (a human embryonic kidney cell line) were available in the Centre for Neuroendocrinology and were originally obtained from the American Cell and Tissue Culture Collection (ATCC; Manassas, Virginia, United States). HEK 293T cells have been engineered to promote expression of exogenous DNA. They contain the simian virus 40 (SV40) large tumour (T) antigen, which increases the replication of vectors containing the SV40 origin of replication.[62,63] These cells do not endogenously express OXTR and AVPRs,[107] and therefore provide a suitable blank background for examination of exogenous WT and variant receptor functionality.

3.1.2 Plasmids

A mammalian expression vector (pcDNA3.1+) encoding WT (WT) OXTR containing an N-terminal HA epitope tag (WT HA-OXTR) was available in the Centre for Neuroendocrinology and was originally obtained from the cDNA Resource Centre, Bloomsburg University, United States. Mammalian expression vectors (pcDNA3.1+) encoding WT AVPR1a or AVPR1b containing an N-terminal HA epitope tag (WT HA-AVPR1a or WT HA-AVPR1b) were obtained from Prof Mark Wheatley (Faculty Research Centre for Sport, Exercise and Life Sciences, Coventry University, England).

3.1.3 Oligonucleotide Primers

Site-directed mutagenesis primers were designed using a specialised online primer design program[108] (Appendix A, Table 17). Receptor-specific sequencing primers were also designed (Appendix A, Table 18) to ensure that, along with universal sequencing primers (T7 promotor and BGH reverse) directed at sites flanking the vectors multiple cloning site, double sequence coverage of the entire receptor cDNA

was obtained. All sequencing and site-directed mutagenesis primers were purchased from Integrated DNA Technologies (Coralville, Iowa, United States).

3.1.4 Antibodies

Mouse anti-HA primary antibody (0.1 µg/µL) was purchased from Life Technologies (Carlsbad, California, United States, Catalogue #32-6700) and goat anti-mouse secondary antibody conjugated to horseradish peroxidase (0.3 µg/µL) was purchased from Bio-RAD (Hercules, California, United States, Catalogue #1706516).

3.1.5 Ligands

Peptides: Native OXTR and AVPR peptide ligands, OXT (Oxytocin acetate salt hydrate) and AVP ([Arg^{8}]-Vasopressin acetate salt) were obtained from Sigma-Aldrich (St. Louis, Missouri, United States). Both peptides were prepared in deionised water to a stock concentration of 1 mM and were stored in aliquots at -20^{o}C prior to use.

Small molecules: Selective non-peptide OXTR agonist, WAY 267 464, and antagonist, L-371,257, were purchased from Tocris (Bristol, UK) and were prepared in 100% dimethyl sulfoxide (DMSO) to stock concentrations of 10 mM and 5 mM, respectively. Stock solutions were stored in aliquots at -20^{o}C prior to use

Radiolabelled peptide: Radiolabelled [^{3}H]-AVP (Vasopressin (8-L-Arginine), [Phenylalanyl-3,4,5-^{3}H(N)], 25 µCi, 61.2 Ci/mmol) was purchased from Perkin Elmer (Waltham, Massachusetts, United States). Stock was diluted to 0.1 mCi/ml in a solution of ethanol: 0.05 N acetic acid (in a 7:3 ratio) and stored at -20^{o}C prior to use.

3.1.6 General laboratory chemicals

All general laboratory chemicals were obtained from Sigma-Aldrich Ltd (St. Louis, Missouri, United States) unless otherwise stated.

3.2 Methods

3.2.1 Literature search and biological database analysis

Variants of OXTR, AVPR1a and AVPR1b were selected for this study through extensive literature research and analysis of biological databases, National Centre for Biotechnology Information SNP database,[109] and DisGenNet.[110] The searches included restrictions to ensure that the variants were naturally occurring coding-region SNPs identified in human patients and have been associated with psychological disease states.

3.2.2 *In silico* analyses

3.2.2.1 Amino Acid Properties Analysis

The properties of the amino acids altered in the variant receptors were compared to their WT counterparts to infer whether the change in amino acid was conservative or not and thus whether change could be likely to have a major impact on the structure and function of the receptor in question. This focused on the size, polarity, charge, and hydrophobicity, which all contribute towards the function and properties of an amino acid.

3.2.2.2 Conservation of Residues Analysis

For those variants located within the TM regions of the receptor, residue conservation analysis was undertaken using the conservation analysis tool, GMoS (GPCRs Motif Searcher, obtained and from created by the Laboratory of Computational Medicine, Universitat Autonoma de Barcelona, Spain[111]), which searches for conserved sequence motifs in the most recent sequence alignment of GPCRs. For this analysis, the Ballesteros–Weinstein numbering scheme,[87] was utilised. This numbering system is based on the presence of highly conserved residues in each of the seven TM helices. It consists of two numbers where the first denotes the helix, 1–7, and the

second the residue position relative to the most conserved residue, which is assigned number 50. The Ballesteros-Weinstein number for all the TM-located variants were determined using the GPCR database.[112] These values were then entered into the GMoS search tool, which provided information regarding residue conservation at this position across all Rhodopsin-family GPCRs. The results are classified according to the percentage of occurrences within different subfamilies.

Conservation of residues in the affected locations were further analysed using a Clustal Omega alignment tool,[113] where the human receptor genes of OXTR, AVPR1a or AVPR1b were aligned with those of each other and the corresponding receptors from other mammalian species (sheep, cattle, dog, rhesus monkey, Norway rat, and house mouse). The site of each mutation's amino acid position was located in the human receptor gene, to deduce whether the same residue was present in the other mammalian species. The conservation of residues in each location across the different human receptors for oxytocin and vasopressin (OXTR, AVPR1a, AVPR1b and AVPR2) were also compared to comment on the conservation of the sites of interest amongst these receptors as they are evolutionarily conserved. The Clustal Omega Gonnet PAM 250 point accepted mutation matrix mathematical tool that accounts for varying rates of acceptance of amino acid substitutions over evolutionary periods, was also utilised to predict conservation of substitutions.[114]

3.2.2.3 Variant Effect Prediction Programs

Multiple *in silico* variation effect prediction programs were then used to analyse the various OXTR/AVPR variants identified.[115] Seven different online tools were used in order to predict whether a deleterious effect will be caused by the variant. These tools were: sorting intolerant from tolerant (SIFT), PolyPhen2, likelihood ratio test (LRT), Mutation Taster, Mutation Assessor and Functional Analysis Through Hidden Markov Models (FATHMM) and were accessed using the Ensemble Variant Effect Predictor (VEP) platform.[116]

In general, prediction tools obtain information on amino acid conservation directly from

alignment with homologous and distantly related sequences. SIFT computes a combined score derived from the distribution of amino acid residues observed at a given position in the sequence alignment and the estimated unobserved frequencies of amino acid distribution calculated from a Dirichlet mixture. PolyPhen-2 uses a naive Bayes classifier to utilise information derived from sequence alignments and protein structural properties (e.g., accessible surface area of amino acid residue, crystallographic beta-factor, etc.). Mutation Assessor captures the evolutionary conservation of a residue in a protein family and its subfamilies using combinatorial entropy measurement.[117]

The comparison of *in silico* analysis results and *in vitro* analyses was utilised to comment on the accuracy of these prediction programs.

3.2.3 Production and propagation of mammalian expression vectors encoding variant receptors

3.2.3.1 Site Directed Mutagenesis

Site-directed mutagenesis was used to introduce the selected variants into mammalian expression vectors encoding OXTR/AVPRs. Two oligonucleotide primers containing the desired variants (Appendix A, Table 17) were used to amplify the variant receptors, using plasmids containing the WT receptor open-reading frame as a template. PFU Ultra HF DNA polymerase (Agilent, Santa Clara, California, United States), which is a high-fidelity enzyme with a low error rate, was used to amplify the variant plasmids by polymerase chain reaction (PCR). For the negative control reactions, the PFU Ultra HF enzyme was replaced with nuclease free water. Reactions were prepared as described in Table 1, The samples were then centrifuged, placed into a thermocycler and a PCR was performed using the cycling parameters described in Table 2.

Table 1: Reaction Components of Site-Directed Mutagenesis PCR

Reagent	With PFU	Without PFU
Nuclease free water	To 50uL	To 50uL
10x Reaction Buffer	5µl (1x)	5µl (1x)
Template dsDNA (50 ng/µl stock)	1 µl (50 ng)	1µl (50 ng)
dNTPs (10 mM stock)	1µl (200 µM)	1µl (200 µM)
DMSO	3µl (6%)	3µl (6%)
PFU enzyme (2.5 U/ µL stock)	1µl (2.5 U)	-
Primers (100ng/µl stock)	1.25µl (125 ng of each)	1.25µl (125 ng of each)

Table 2: Cycling parameters for Site-Directed Mutagenesis PCR

Temperature	Time	Cycles
95°C	1 minute	1
95°C	50 seconds	
50-70°C *	50 seconds	20
68°C	8 minutes	
68°C	7 minutes	1
4°C	∞	

[annealing temperature was adjusted based on calculated melting temperatures of each mutagenesis primer pair shown in Appendix A, Table 18]*

The resultant products were then incubated in the presence or absence of DpnI restriction endonuclease (0.5 IU/µl) (Thermo Fisher Scientific, Waltham, Massachusetts, United States) to digest the template DNA. Agarose gel electrophoresis was used to visualise the products and confirm successful amplification.

3.2.3.2 Agarose Gel Electrophoresis

Agarose was dissolved in TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 8) to a final concentration of 1% (w/v) supplemented with a 1/30 000 dilution of GelRed (Biotium; Hayward, California, United States). Once set, 5 µL of the PCR products were transferred to each well and were electrophoresed at 100 V for 30 minutes, alongside a 1 kb molecular weight ladder (New England Biolabs). DNA bands were visualised using a ChemiDoc XRS+ Gel Imaging System (Bio-Rad, Hercules, California, United States) to confirm the presence of PCR product of the desired size indicating successful generation of the plasmids encoding the variant receptors.

3.2.3.3 Transformation

The generated plasmids encoding the variant receptors were then transformed into chemically competent *E. coli* for plasmid propagation. 45 µl of XL 10 gold *E. coli* (Agilent Technologies, Santa Clara, California, United States) and 5 µl of DpnI-treated PCR reactions were added to chilled microcentrifuge tubes and incubated on ice for 30 minutes. The bacteria were then heat-shocked for 30 seconds in a water bath at 42°C, followed by 2 minutes on ice. 450 µl of Luria Bertani broth (LB; 1% (w/v) Tryptone, 0.5% (w/v) yeast extract, 170 mM NaCl, pH 7.5) was added and bacteria allowed to recuperate in a shaking incubator at 37°C and 250 rpm for 45 minutes. Tubes were then centrifuged for 3 minutes at 5 000 x *g* to collect the bacteria. 400 µl of the supernatant was carefully removed and the bacterial pellet resuspended in the remaining 100 µl of solution, which was then spread onto LB agar plates (LB supplemented with 1.5 % (w/v) agar)) containing 100 µg/ml ampicillin selection antibiotic. Agar plates were wrapped in parafilm and incubated overnight at 37°C before propagation and purification of the plasmids.

3.2.3.4 DNA Propagation and Purification

Colonies were selected from each agar plate for amplification and DNA purification by

mini prep. Colonies were selected using a sterile pipette tip and transferred to 5 ml of LB supplemented with 100 µg/ml ampicillin in a 50 ml centrifuge tube and were placed in a shaking incubator (250 rpm, 37°C) overnight. Glycerol stocks were prepared from each culture, by mixing 80% glycerol and an aliquot of the overnight bacterial culture at a 1:1 ratio and were stored at -80°C. Plasmid DNA was extracted and purified from the remaining culture using a QIAprep Spin Miniprep Kit (Macherey-Nagel, Düren, Germany), as per the manufacturer's instructions. The purified plasmid DNA was re-suspended in 30 µl of nuclease-free water and the concentration determined using a nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

3.2.3.5 Sanger Sequencing

To confirm successful introduction of the variants, the generated plasmid DNA was sequenced. Samples were prepared for Sanger sequencing using a BigDye reaction. Sequencing primers were designed to ensure double coverage of the full OXTR/ AVPR1a/b coding sequence (Appendix 1A, Table 18). For each reaction, plasmid DNA (50 ng) was added to a microcentrifuge tube with the relevant sequencing primer (100 ng). Each primer was used in a separate reaction, resulting in four reactions per plasmid. 1x sequencing buffer (Thermo Fisher Scientific, Waltham, Massachusetts, United States), and 1 µl BigDye v3.1 Ready Reaction Mix (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was added to the sample, along with PCR grade nuclease-free H_2O to make up the volume to 10 µl. Reactions were incubated in a thermocycler where a dye terminator cycle reaction was run using the reaction conditions described in Table 3.

Table 3: Cycling parameters of Sequencing reactions

Temperature	Time	Cycles
96°C	1 minute	1
96°C	10 seconds	
53°C	5 seconds	25
60°C	6 minutes	
4°C	∞	

Sequencing products were then precipitated by the addition of 1.5 µl 3 M sodium acetate (pH 4.6) and 31.25 µl 95 % molecular grade ethanol, and the volume made up to 50 µl with RNAse/DNAse -free water. Following vortexing, samples were incubated for 15 minutes at room temperature. DNA was pelleted by centrifugation at 15 000 x *g* for 20 minutes followed by careful aspiration of the supernatant. The DNA pellet was washed with 70% ethanol and centrifuged at 15 000 x *g* for 5 minutes. After aspiration of supernatant, the pellets were left to dry for 30 minutes in an oven at 37°C. Samples were then sequenced at the University of Pretoria's DNA Sequencing Facility.

Resultant sequence analysis files were obtained and software analysis tools Sequence Scanner Software v2.0 (SeqScanner, Applied Biosystems, Waltham, Massachusetts, United States)[118] and BioEdit (Manchester, England),[119] were used to analyse sample sequences. SeqScanner was used to view and trim all four reads to ensure that only the parts with high confidence base callings were included. The trimmed readings were then transferred to BioEdit where contigs were created and aligned to be compared to their respective NCBI wild type reference sequence (OXTR: NM_000916.4, AVPR1a: NM_000706.5, and AVPR1b: NM_000707.5). Each sequence was analysed to verify that the DNA plasmids contained the desired variant, with no other changes to the receptor sequence (Appendix 1B).

3.2.4 Cell culture

All receptor expression, ligand binding and signalling assays were performed using HEK 293T cells transiently transfected with the receptors of interest.

3.2.4.1 Cell Maintenance and Seeding

Cells were maintained in Complete Media (Dulbecco's modified Eagle's medium (DMEM; Thermo Fischer Scientific, Waltham, Massachusetts, United States) supplemented with 10% (v/v) Foetal Bovine Serum (FBS; Thermo Fischer Scientific, Waltham, Massachusetts, United States) in an incubator at 37℃ with 5% CO_2.

Cells were washed using phosphate buffered saline; PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, PH 7.4) and passaged with trypsin-EDTA (Thermo Fischer Scientific, Waltham, Massachusetts, United States) and Complete Media in a 1:10 ratio (v/v), three times a week to maintain a cell density of less than 90% confluence. Before seeding into multi-well culture plates for experimental analyses, the cells were counted using a Countess Automated Cell Counter (Thermo Fischer Scientific, Waltham, Massachusetts, United States). Plates were coated with Matrigel matrix (Corning, New York, United States) at a 1:30 dilution prior to cell seeding to aid cell attachment.

3.2.4.2 Transient Transfection

Transfection of the cells with plasmid DNA was performed to enable them to exogenously express the receptors of interest. In this process DNA-transfection reagent complexes are formed and added to the cells where they are engulfed via endocytosis, allowing the exogenous DNA to enter the nucleus, and be transcribed. Following seeding into culture plates, cells were grown to 60-70% confluence before transient transfection with the appropriate plasmid DNA using X-TREME GENE-HP (XTG) transfection reagent (Sigma-Aldrich, St. Louis, Missouri, United States) as per manufacturer's instructions. In brief, transfection complexes were prepared by mixing DNA with XTG HP at a 1:2 ratio (µg DNA:µl XTG) in serum free-DMEM. Following incubation for 15 minutes at room temperature, the complexes were added to the cells and the cells were incubated for 24 hours (to allow adequate receptor expression) prior to use in functional assays.

3.2.5 Cell number and viability analyses

3.2.5.1 Cell Visualisation by Light Microscopy

In order to monitor the growth rate and morphology of the cells, light microscopy was used to visualise cells. Light microscopy is a key tool in modern cell biology available for the observation of cells. Although limited in the fineness of detail that it can reveal,

gross cell morphology and density are clearly visible which allows the study of qualitative cell growth and viability. Cells were seeded in 24-well tissue culture plates, at a density of $1.2x10^5$ cells/well. After 24 h of incubation, the cells were then transfected with plasmids (0.25 µg DNA/well) encoding HA-tagged WT or variant receptors or empty vector (as described in Section 3.2.4.2) and were visualised using a Zeiss Axiovert A1 light microscope and ZEN Blue software (Zeiss; Oberkochen, Germany) at 4x magnification. After a further 48 hours of incubation, cells were visualised again.

3.2.5.2 Crystal Violet Cell Viability Assay

A simple method to quantify cell number is through the staining of attached cells with crystal violet dye. As there appeared to be variation in cell number following transfection with the different variant receptors, crystal violet staining was performed to quantify number of cells adhered to the plates.

Cells were seeded in 24-well tissue culture plates, at a density of $1.2x10^5$ cells/well. After 24 h of incubation, the cells were then transfected with plasmids (0.25 µg DNA/well) encoding HA-tagged WT or variant receptors or empty vector (as described in Section 3.2.4.2). After a further 24 hours of incubation, media was aspirated and replaced with Media 199 (Thermo Fischer Scientific, Waltham, Massachusetts, United States) supplemented with 1% (v/v) FBS, where after cells were incubated for 16 hours at 37°C. The Media-199 was removed and cells were fixed with 1% glutaraldehyde (in distilled water) and incubated at room temperature for 15 minutes. The glutaraldehyde was carefully discarded, and fixed cells were stained with 0.1% (w/v) crystal violet (in phosphate buffered saline; PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, PH 7.4)) and incubated for 30 minutes at room temperature. The plates were then thoroughly rinsed in deionised water to remove all excess crystal violet and left to dry overnight. The following day, the crystal violet stain was solubilised with 600 µl of 0.2% Triton X-100 and incubation for 30 minutes at room temperature. The samples (100 µl) were then transferred to clear flat-bottomed 96-well plates for measurement of absorbance at 595 nm using an iMark microplate reader (BioRad,

Hercules, California, United States).

3.2.5.2.1 Data analysis

All data analyses were performed using GraphPad Prism Software (Version 8 for Windows; GraphPad Software, San Diego, California, United States). Data are presented as bar charts with mean ± SEM of three independent assays (biological replicates) in which each data point was performed in triplicate. To control for inter-assay variability, the sum of all data generated in each replicate was calculated, and each data point within that replicate normalised to this value. Normalised data were then calculated as a percentage of the average value for empty vector transfected cells across all replicates. The statistical significance of differences in staining obtained between empty vector and WT/variant-transfected cells was determined using a one-way ANOVA followed by Dunnett's test, with $p<0.05$ considered significant.

3.2.5.3 Trypan Blue Cell Viability Assay

Membrane integrity is the feature most often used to detect whether cells cultured *in vitro* are alive or dead. Cells that have lost membrane integrity and allow the movement of otherwise non-permeable molecules are classified as non-viable or dead. For this study, viable vs non-viable cell determination was accomplished with the use of a Trypan blue, a well-known “vital dye”. Trypan blue is typically not permeable to viable cells but enters dead cells through damaged membranes, hence the selective staining of dead cells and quantitative measurement of cell viability.

Cells were seeded into 48-well cell culture plates at $0.5x10^5$ cells/ml and after 24 hours of incubation, were transfected with mammalian expression vectors (0.3 µg DNA/well) encoding WT or variant receptors or empty vector (as described in Section 3.2.4.2). 24 hours post transfection the media was aspirated, and 50 µl trypsin added to detach cells. Trypsin was neutralised after 2 minutes by addition of 450 µl Complete Media. Cells were then scraped and collected into microcentrifuge tubes. A sample of the cell

suspension was then stained by addition of trypan blue (Thermo Fisher Scientific, Waltham, Massachusetts, United States) in a 1:1 ratio. Stained cells were then counted, and viable cell percentage calculated using a Countess Automated Cell Counter (Thermo Fischer Scientific, Waltham, Massachusetts, United States).

3.2.5.3.1 Data analysis

All data analyses were performed using GraphPad Prism Software. Data are presented as bar charts. Live cell count is presented as a percentage of the total cell count as calculated by the Countess Automate Cell Counter. The statistical significance of differences in living cells obtained between empty vector and WT/variant-transfected cells was determined using a one-way ANOVA followed by Dunnett's test, with $p<0.05$ considered significant.

3.2.6 Inositol Phosphate Accumulation Assay

The activity of $G\alpha_{q/11}$ – coupled GPCRs, such as the OXTR and AVPR1a/1b, can be measured by the cell-based radiometric inositol phosphate (IP) accumulation assay. Activation of $G\alpha_{q/11}$ results in the generation of the second messenger IP3 from PIP2 (See Section 1.4.1 and Section 1.4.3). The IP3 is metabolised to inositol bisphosphate (IP2) and inositol monophosphate (IP1), whereby further breakdown can be prohibited by the inclusion of lithium chloride in the assay media, which results in an accumulation of IPs. In the IP accumulation assay, cells are loaded with a radiolabelled precursor of PIP2 (^{3}H-myo-inositol), thereby all generated IPs are also radiolabelled. These can be isolated by ion exchange chromatography and their radioactivity measured, with the amount of radiolabelled IP generated (i.e., the level of radioactivity measured) being proportional to the signalling activity of the receptor.

Cells were seeded in 24-well tissue culture plates, at a density of $1.2x10^5$ cells/well. After 24 h of incubation, the cells were then transfected with plasmids (0.25 µg DNA/well) encoding HA-tagged WT or variant receptors or empty vector (as described in Section 3.2.4.2). After a further 24 hours of incubation, media was aspirated and

replaced with Media 199 supplemented with 1% (v/v) FBS and 0.25 µCi/well [^{3}H] myo-inositol (Perkin Elmer, Waltham, Massachusetts, United States), where after cells were incubated for 16 hours at 37°C. The media was then aspirated, and cells incubated for 30 minutes with Buffer I (DMEM supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM LiCl and 0.1 % (w/v) bovine serum albumin (BSA; VWR; Radnor, Pennsylvania, United States)). Following aspiration, cells were then incubated with 300 µl of compound dilutions or appropriate vehicle prepared in Buffer I and were incubated for 1 hour at 37°C. For single concentration analyses, cells were treated with 10 nM OXT, 10 nM AVP or vehicle and for dose response assays, cells were treated with a range of concentrations of OXT (100 nM to 10 pM), AVP (100 nM to 10 pM) or vehicle. Cells were then lysed by incubation for 1 hour in 10 mM formic acid at 4°C, before collection of radiolabelled inositol phosphates by ion exchange chromatography.

Radiolabelled inositol phosphates were separated by ion-exchange chromatography using Dowex resin beads (100-200 mesh, Sigma-Aldrich, St. Louis, Missouri, United States). 800 µl of the formic acid lysates were transferred from the cell plates to tubes containing 2.5 ml of formate-resin (prepared as a 50% slurry in deionised water). Samples were gently vortexed twice followed by a 5-minute incubation period, and then the supernatants were aspirated without disturbing the beads. The beads were washed successively with two washes of 2 ml of distilled water and then two washes of 2 ml of Wash Buffer (60 mM ammonium formate, 5 mM sodium tetraborate). The radioactive IPs were then eluted from the beads by the addition of 1 ml of Elution Buffer (1M ammonium formate, 0.1 M formic acid). Without disturbing the beads, 800 µl of the supernatant was transferred into scintillation vials (6 ml Pony vials, Perkin Elmer, Waltham, Massachusetts, United States), to which 2.5 ml Optiphase HiSafe scintillation fluid (Perkin Elmer, Waltham, Massachusetts, United States) was added. Vials were vortexed well before radioactivity was measured with a Tri-Carb 2810TR beta counter (Perkin Elmer, Waltham Massachusetts, United States) (1 minute counts).

3.2.6.1 Data analysis

All data analyses were performed using GraphPad Prism Software. To control for inter-assay variability, the sum of all data generated in each replicate was calculated, and each data point within that replicate normalised to this value. To control for cell number variability between cells expressing the different receptors, each data point was normalised to the average crystal violet absorbance measured for each receptor (see Section 3.2.5.2). Non-specific signal (measured in the presence of cells transfected with empty vector) was subtracted from all readings, and data were calculated as percentage of the average maximal WT response across all replicates.

For single concentration responses, data are presented as bar charts with mean ± SEM of three independent assays (biological replicates) in which each data point was performed in triplicate. The statistical significance of differences in responses obtained between WT and variant-transfected cells or between vehicle and OXT/AVP-stimulated responses for each receptor was determined using a one-way ANOVA followed by Dunnett's post-test, respectively, with $p<0.05$ considered significant.

For dose response analyses, data were fitted to sigmoidal dose response curves with a Hill coefficient of unity and pEC_{50} (negative log concentration of ligand required to elicit 50% maximal response), E_{max} (maximal responses elicited) and basal response (response elicited in absence of stimulating ligand) values were calculated. Data are presented as mean ± SEM from three independent assays (biological replicates) in which each data point was performed in triplicate. The statistical differences between the basal responses, E_{max} and pEC_{50} for the WT receptor was compared to the corresponding variant receptors using a one-way ANOVA followed by Dunnett's test, with $p<0.05$ considered significant.

3.2.7 Ligand Binding Assay

Radiolabelled hormone binding assays were used to determine the effects of receptor variants on hormone binding. Radioligand binding assays provide sensitive and

quantitative information about GPCR expression and affinity, making them essential for GPCR research. A radioactively labelled compound is used to bind to the target site (receptor). A filtration step is then followed to separate free radioligand from the receptor-ligand complex before the measurement of incorporated radioactivity is undertaken.

3.2.7.1 Membrane preparation

Membrane preparations were generated to maximise the number of cells used in the radioligand binding assays to ensure a sufficient signal-to-noise ratio. Cells were seeded in T75 tissue culture flasks at $1.4x10^5$ cells/flask and were transiently transfected with plasmids (0.25 µg DNA/flask) encoding the WT and variant receptors or empty vector (see Section 3.2.4.2). 48-hours post-transfection, the media was removed, and cells were washed with and scraped into PBS. Cells were collected by centrifugation at 200 x *g* for 10 minutes. The supernatants were aspirated and the cell pellets frozen at -20^0C. The following day, cells were thawed and resuspended in Membrane Preparation Buffer (20 mM HEPES, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), pH 7.4) and were homogenised using a Potter Elvehjem glass homogeniser. Homogenates were then further ruptured by sonication using a Model 3000 Ultrasonic Homogeniser (BioLogics Inc, Cary, North Carolina, United States). The homogenates were centrifuged at 4000 x *g* for 5 minutes at 4°C to remove cell nuclei and any remaining whole cells. The supernatants were then collected and centrifuged at 11 400 x *g* overnight at 4°C (16 – 18 hr) to collect the cell membranes. The following morning, the supernatants were aspirated, and the resulting membrane pellets were resuspended in Membrane Preparation Buffer at 4°C. The homogenisation and sonication steps were then repeated to ensure a homogenous mixture. The protein concentration was determined using a Pierce BCA Assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) as per manufacturer's instructions. The membrane preparations were then aliquoted and stored at -80°C until required.

3.2.7.2 [^{3}H]-AVP binding assay

Membrane preparations (AVPR1a: 33.5 ng/µl and AVPR1b: 50 ng/µl) were incubated in deep 96 well plates with [^{3}H]-AVP in Buffer B[119] (20 mM HEPES, 24 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 400 mM NaCl, 0.4% (w/v) BSA, pH 7.4) in a final volume of 350 µl. Concentrations of [^{3}H]-AVP of 2.5 nM (AVPR1a) or 1.5 nM (AVPR1b) were utilised. These concentrations were selected to ensure that they were 2-3x the reported affinity (Kd) for AVP at the WT receptors (AVPR1a: 1.1 nM and AVPR1b: 0.7 nM [120]). The reactions were initiated by addition of membrane proteins and, after mixing on a plate-shaker, were incubated for 3 hours at 25°C. Reactions were terminated by rapid filtration through Whatman glass microfibre GF/B filters using a PHD cell harvester (Brandel Inc, Gaithersburg, Maryland, United States), followed by six 1 ml washes with Harvest Buffer (20 mM HEPES, 3.7 mM $MgCl_2$, pH 7.4) at 4°C. Filter discs were then cut out, put into 6 ml Pony Vials, and soaked in 2 ml Ultima Gold™ MV scintillation fluid (PerkinElmer, Waltham, Massachusetts, United States) overnight, before the radioactivity was determined by liquid scintillation counting using a Tri-Carb 2810TR beta counter (1-minute counts).

3.2.7.3 Data Analysis

All data analyses were performed using GraphPad Prism Software. To control for inter-assay variability, the sum of all data generated in each biological replicate was calculated, and each data point within that biological replicate normalised to this value. Non-specific ligand binding measured in the presence of cells transfected with empty vector was then subtracted from all readings, and data was calculated as percentage of average WT signal across all replicates. No normalisation for cell number was required as equal amounts of membrane proteins were included for the WT and each variant receptor. Data were presented as bar charts representing all data as a mean ± SEM of three independent assays (biological repeats), in which each data point was performed in triplicate. Statistical significance of differences between binding measured for WT and variant receptors was determined using a one-way ANOVA followed by Dunnett's test, with $p<0.05$ considered significant.

3.2.8 Receptor Enzyme Linked Immunosorbent Assay

A receptor enzyme-linked immunosorbent assay (ELISA) was performed to determine whether the variants affect receptor expression at the cell surface (using total cellular expression levels as a control to ensure successful transfection/expression of the variant receptors and to examine effects on receptor biosynthesis). An ELISA is an immunological assay commonly used to measure specific antigens in biological samples. A primary antibody (mouse anti-HA) was used to target the N-terminal epitope tags (HA-tags) on the receptors of interest, which are attached to the extracellular portion of the receptor. After several washes to remove excess primary antibody, goat anti-mouse secondary antibodies conjugated to horseradish peroxidase were then used to label the bound primary antibody. Addition of substrate enables quantification of the amount of bound antibody (and thus amount of the target antigen – HA-tagged receptor in this case). Using intact cells enables quantification of receptor at the cell surface only (as antibodies are not cell permeant), while use of permeabilised cells enables quantification of total cellular receptor levels.

Cells were seeded into 48-well cell culture plates at $0.5x10^5$ cells/ml and, after 24 hours of incubation, were transfected with mammalian expression vectors (0.3 µg DNA/well) encoding WT or variant receptors or empty vector (as described in Section 3.2.4.2). 48-hour post-transfection cells were washed with PBS supplemented with 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ (PBS+). Cells were then fixed with 2% (w/v) paraformaldehyde (PFA) at room temperature for 15 minutes. Cells were washed with PBS before incubation for 5 minutes at room temperature with either PBS (for measurement of cell surface expression) or PBS supplemented with 0.2% Triton X-100 (v/v) (for measurement of total receptor expression). Cells were then thoroughly washed with PBS before the addition of Blocking Solution (DMEM supplemented with 10% FBS (v/v), 5% (w/v) bovine serum albumin (BSA) (w/v) and 5% (w/v) skim milk powder (Sigma-Aldrich, St. Louis, Missouri, United States) and incubation for 1 hour at 37°C. Following three washes with PBS, cells were incubated with primary antibody (mouse anti-HA, diluted 1:5000 in DMEM supplemented with 10% FBS) overnight at 4°C. Cells were then washed three times with PBS and incubated with secondary

antibody (horseradish peroxidase–conjugated goat anti-mouse 1:5000 in DMEM supplemented with 10% FBS) for 1 hour at 37°C. Cells were then washed an additional three times with PBS before incubation for 20 minutes in the dark with 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine in phosphate citrate buffer (5 mM citric acid, 102 mM $Na_2HPO_4.2H_2O$, pH 5.0), supplemented with 0.006% (v/v) H_2O_2 immediately before use. Sulfuric acid (1M final concentration) was then added to stop the reactions. Samples (150 µl) were then transferred to clear 96-well plates and absorbance was measured 450 nm using an iMark microplate reader (BioRad, Hercules, California, United States).

The remaining sulfuric acid was then immediately aspirated from the plates and the cells were stained with 0.1% (w/v) crystal violet (in PBS) and incubated for 30 minutes at room temperature. The cells were then thoroughly rinsed in deionised water to remove all excess crystal violet and were left to dry overnight. The following day, the bound crystal violet dye was dissolved by addition of 600 µl of 0.2% (v/v) Triton X-100 and incubation for 30 minutes. Samples (100 µl) were then transferred to clear flat-bottomed 96-well plates and absorbance was measured at 595 nm using an iMark microplate reader (BioRad, Hercules, California, United States).

To test the capability of pharmacological chaperones to rescue the trafficking of variant receptors, the assay was conducted as described above, but with the following modification: 24 hours post-transfection, media was aspirated, and cells were incubated for 24 hours with either the pharmacological chaperone test compound (10 µM L 371 257 or 1 µM WAY 267 464) or vehicle, in Complete Media before the washing and fixation steps.

3.2.8.1 Data analysis

All data analyses were performed using GraphPad Prism Software. To control for cell number variability between cells expressing the different variants, each data point was normalised to the crystal violet absorbance measurement from the same well. To control for inter-assay variability, the sum of all data generated in each biological

replicate was then calculated, and each data point within that biological replicate normalised to this value. Non-specific signal measured in the presence of cells transfected with empty vector was then subtracted from all readings, and data was calculated as percentage of average WT signal across all replicates. A bar chart was then produced representing all data as a mean ± SEM of three independent assays (biological repeats), in which each data point was performed in triplicate. Statistical significance of differences between WT and variant-transfected total/cell surface expression was determined using a one-way ANOVA followed by Dunnett's test, with $p<0.05$ considered significant. For the pharmacological chaperone rescue ELISA, a statistical significance of differences between vehicle and pharmacological chaperone treated cells for cell surface expression was determined using a one-way ANOVA followed by Dunnett's test, with $p<0.05$ considered significant.

4. Oxytocin Receptor Variants

4.1 Foreword

It is becoming increasingly apparent that the OXTR/OXT system is integral to the psychosocial functioning of human behaviour. Indeed, the OXT/OXTR signalling has been associated with a variety of behavioural, emotional, and cognitive functions and the dysregulation of these pathways has been implicated in several psychological disorders (see Section 1.3.1.2.1). In 2022, the WHO reported that 1 in every 8 people in the world live with a physiological (mental) disorder, involving significant disturbances in thinking, emotional regulation, or behaviour.[121] Increased understanding of the molecular basis of these disorders may aid in the development of more targeted therapies.

Population genetics concentrating on gene-disease associations, is the study of variation within populations of individuals. The collection and analysis of human genetic data in population-based research, can help interpret variations in health and disease among people and across populations. This aids in understanding the cause and effects of diseases as well as identification of groups of people at increased risk for particular disorders. Indeed, genetic association studies have linked genetic variants in the OXTR with several different disease phenotypes. Many of these are, not surprisingly, related to OXTRs effects on parturition (see Section 1.3.1.1.1) including preterm birth (one of the primary causes of perinatal mortality)[20] and stalled labour.[25] Thus, defects in the OXTR will have a direct effect on the initiation of labour. For example, if an SNP causes the OXTR to overexpress or inappropriately signal, this may cause the premature onset of labour, alternatively, an SNP which results in a defective/ under expressed OXTR may result in stalled labour which can increase the risk of uterine rupture, haemorrhage, hypernatremia, tachysystole and foetal hypoxia.[24,122] Both circumstances may result in serious complications of both the mother and the unborn child. Consequently, synthetic OXT is currently one the most frequently used medication in obstetrics, administered to induce or augment labour.

However, the effectiveness of a given OXT dose is variable amongst women.[25] The unpredictably of an individual's response to exogenous OXT can reflect competence of their OXTR signalling. Indeed, a gene-association study identified potentially inactivating OXTR gene variants in a comparative study examining labouring women requiring either low or high OXT dosage requirements. Novel variants which were unique to the high dose requiring group included M133V, R150L, R150S, H173R and I266V.[25]

One particular study examined the role of rare genetic variation in susceptibility to pre-term birth. They conducted direct sequencing analysis on the OXTR in 1394 cases and 1112 controls form the US, Argentina, Denmark, and Finland, where they identified 6 common genetic variants predisposing to pre-term birth: V45L, P108A, V172A, W203R, L206V and V281M.[123] Despite their important biological effects, it is yet unclear how the mutations may contribute to the disease as it is counterintuitive that attenuated OXT signalling leads to PTB. However, it is possible that the variants may act indirectly to stimulate uterine contractions. Given that the regulation of OXTR function is dependent on steroids such as estradiol, progesterone, and its metabolites,[124] it is probable that the mutations may affect receptor-hormone interaction in a way that interferes with the timely onset of labour.[123]

Much of the evidence of the potential roles of OXT/OXTR in psychological functioning has also come from population genetics studies. Indeed, with respect to psychological disorders, of the studies investigating variants in the *OXT* gene in various populations, 14 non-coding SNPs have been detected in disorders ranging from ASD and attention deficit disorder,[125] to schizophrenia,[126] however the effects of these SNPs on the peptide expression/functionality are, as yet, unknown.[92] From studies analysing the gene encoding OXTR (*OXTR,* positioned on human chromosome 3p26) several SNPs have been also identified. Many of these are in non-coding or regulatory regions of the gene, however, several are in the coding region (which will directly alter the amino acid sequence of the encoded protein after splicing).[93] This change in amino acid sequence may ultimately affect the expression, transportation and/or function of the protein. Numerous psychological disorders including excessive aggression/antisocial

behaviour,[94] ASD, social cognition disorders, depression and schizophrenia have been associated with OXTR variants.[19,44,94–97] For example, a case control study in a Japanese population using 132 ASD subjects and 248 control subjects identified variants A63G, A63V, A218T, A238T, G252A, T273R, R376C and R376G as being potentially linked to ASD.[127]. Another study looked at variants with possible relation to harm avoidance in Caucasian men and women (sample of 99 people), in conditions marked by an increased sensitivity to perceived threat. This study identified variants A217T, A218T, A238T, E242K and G252A in subjects considered to be highly harm avoidant.[56] Another genetic study used family-based association testing in 200 families and found variant A218T to be significantly associated with core characteristics of ASD such as social withdrawal and repetitive behaviour.[128] The A218T variant has also been associated with emotional empathy in a non-clinical Chinese sample, where CC homozygotes were found to have less cognitive and trait empathy than the CT heterozygotes in their sample.[129]

4.2 Results

4.2.1 Identification and *in silico* analysis of OXTR variants

Psychological disorder-linked variants of the OXTR were identified via extensive literature and database research (as described in Section 3.2.1). Following shortlisting using the inclusion criteria, 11 missense coding-region SNP variants were selected (Table 4 and Figure 4 A). In some cases, more than one variant was identified for a given amino acid position. For comparison, a selection of a similar number of OXTR variants implicated in other (non-psychological) disorders were also chosen (Table 5 and Figure 4 B). These variants are associated with birth disorders related to the OXTRs role in labour, a physiological function for which the role of the OXTR has been clearly established, unlike physiological disorders. It is interesting to note that some variants (A218T, A238T and G252) have been linked to both psychological and birth disorders (indicated in bold text in Table 4). It is also interesting that, for the variants associated with psychological dysfunction, there is a distinct clustering of the majority of variants within the TM 5/ICL3/TM 6 region, while the birth disorder variants are more

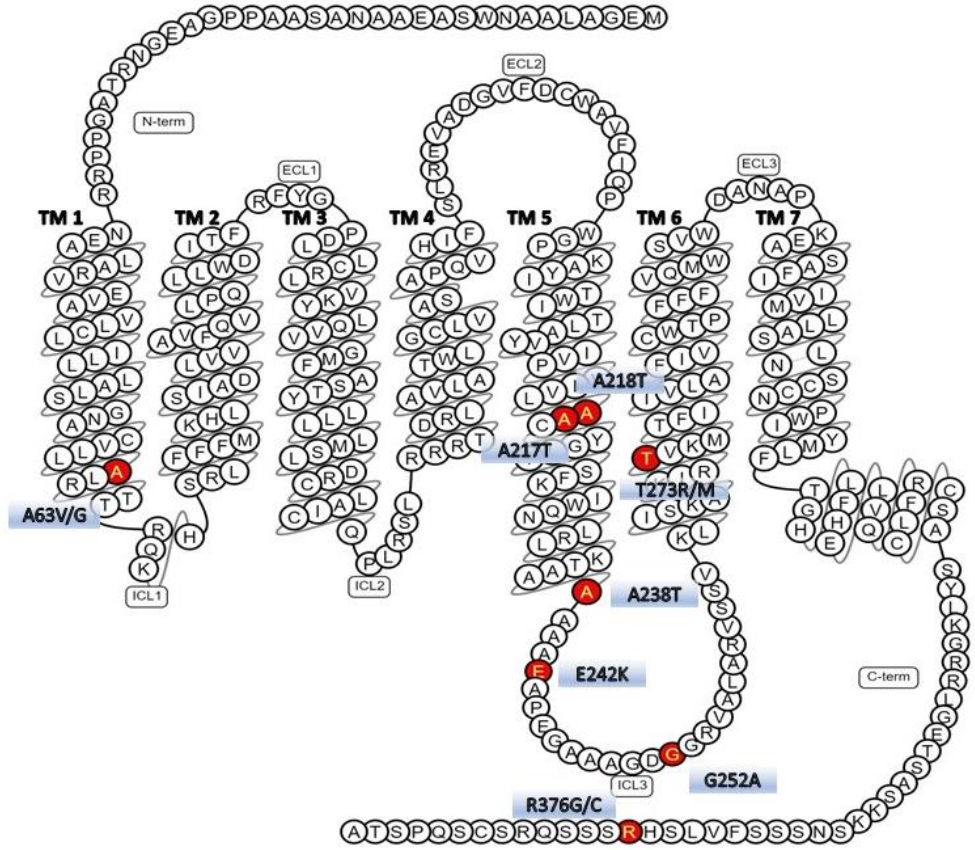
dispersed throughout the receptor (Figure 4 A and B).

Table 4: Selected OXTR variants linked to Psychological Disorders

Variant	rs number	Location in OXTR	Disease Phenotype and Reference
A63G	rs237901	TM 1	ASD[127]
A63V	rs171114	TM 1	ASD[127]
A217T		TM 5	Harm Avoidance[56]
	rs4686302		ASD[128–130] and Empathy[129]
A218T		TM 5	Harm Avoidance[56]
A238T	rs61740241	ICL3	ASD[127] and Harm Avoidance[56]
E242K	rs757022912	ICL3	Harm Avoidance[56]
G252A	rs151141371	ICL3	Harm Avoidance[56]
T273R	rs237901	TM 6	ASD[127]
T273M		TM 6	ASD[127]
R376C	rs35062132	C-term	ASD[127,131]
R376G	rs35062132	C-term	ASD[127,131]

Mutation refers to the amino acid position and change. The rs number is a locus accession for a mutation type assigned by dbSNP for the NCBI database. It must be noted that some variants do not have an assigned rs number. The location indicates where the mutation is located with regards to the GPCR structure. TM, transmembrane; ICL, intracellular loop. The variants in bold indicate variants which have been linked to both psychological and birth disorders.

A



B

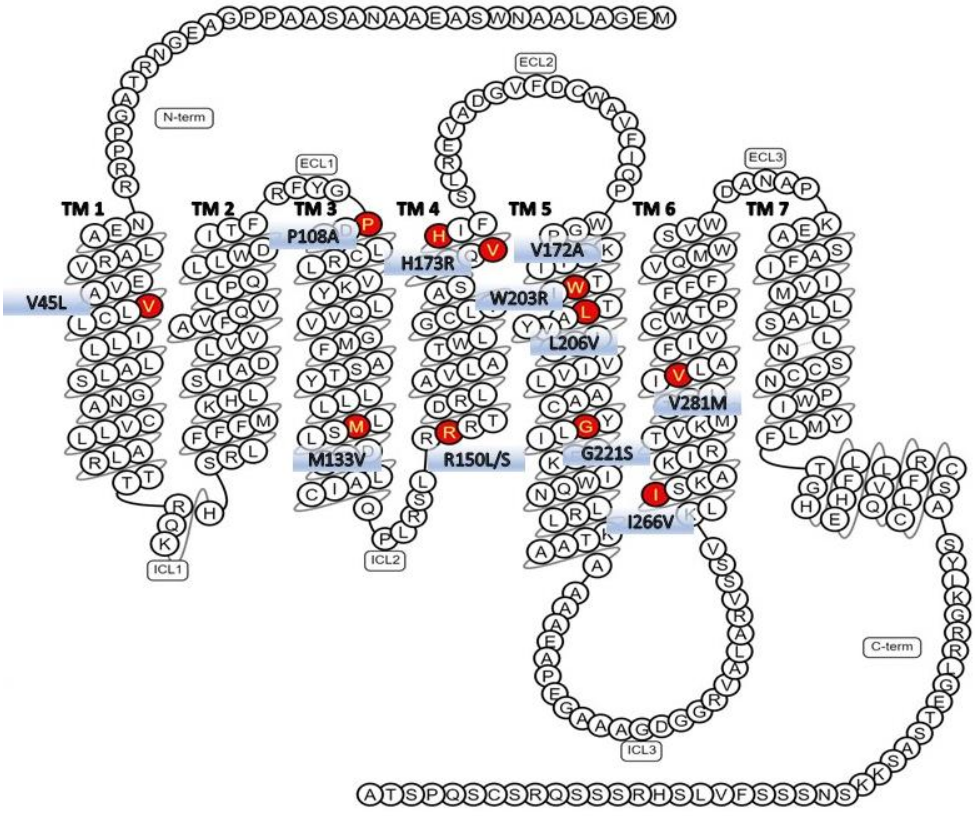


Figure 4: Two-dimensional schematic of the oxytocin receptor (OXTR) showing location of the selected variants linked to psychological disorders and birth disorders

The OXTR is a G protein-coupled receptor that consists of a single polypeptide chain of amino acids (labelled using standard single letter codes) consisting of a transmembrane domain (TMD) that comprises seven-hydrophobic transmembrane alpha helical domains (TM1-7, labelled in bold)), connected by three intracellular loops (ICL1-3) and three extracellular loops (ECL1-3). This structure is flanked by an extracellular N-terminus (N-term) and an intracellular C- terminus (C-term). Locations of the residues affected by ***A:*** *psychological disorder-linked variants (A63, A217, A218, E242, G252, T273 and R376) and* ***B:*** *birth disorder-linked variants (V45, P108, M133, R150, V172, H173, W203, L206, G221, I266 and V281) selected for this study, are labelled and indicated in red. Created using free online software provided by the GPCR database.* [111]

Table 5: Selected OXTR variants linked to Birth Disorders

Variant	rs number	Location in OXTR	Disease Phenotype and Reference
V45L	rs201689053	TM 1	Pre-term birth
P108A	rs202138705	TM 3/ ICL 3	Pre-term birth[123]
M133V	rs201782300	TM 3	High-dose OXT in labour[25]
R150L		TM 4/ ICL 2	High-dose OXT in labour[25]
R150S	rs547238576	TM 4/ ICL 2	High-dose OXT in labour[25]
V172A	rs115324487	TM 4	High-dose OXT in labour[25] and pre-term birth[123]
H173R		TM 4	High-dose OXT in labour[25]
W203R		TM 5	Pre-term birth[123]
L206V	rs150746704	TM 5	High-dose OXT in labour[25] and pre-term birth[123]
G221S	rs143908202	TM 5	High-dose OXT in labour[25]
I266V	rs770798571	TM 6/ ICL 3	High-dose OXT in labour[25]
V281M	rs144814761	TM 6	Pre-term birth[123]

Mutation refers to the amino acid position and change. The rs number is a locus accession for a mutation type assigned by dbSNP for the NCBI database, it must be noted that some variants do not have an assigned rs number. The location indicates where the mutation is located with regards to the GPCR structure. TM, transmembrane; ICL, intracellular loop.

As a simple initial means to infer the likelihood of variants affecting receptor functionality, the properties of the WT and variant amino acids were analysed and compared (Appendix 1C). This analysis showed that 8/23 OXTR variants (A63G, A63V, P108A, V172A, H173R, W203R, G252A and I266V) resulted in quite conservative changes, with only minor changes in amino acid properties (e.g. a slight change in the size of the amino acid reside for A63V). There is a change in residue polarity (non-polar to polar), hydrophobicity (hydrophobic to hydrophilic) and size (small to medium) for variants V45L, M133V, L206V, A217T, A218T, G221S, A238T and V281M, but the WT and variant residues would still be considered quite similar. For the remaining variants, the changes were less conservative. The two variants analysed at residue R150L/S show a large decrease in size as well as a change in charge and polarity. For variant E242K, there are alterations in residue charge, with an acidic residue being substituted with a basic residue. For variant T273R a medium sized uncharged residue is replaced with a large basic residue. For variant T273M a medium sized hydrophilic residue is replaced with a large hydrophobic residue. For variants R376C and R376G, a large hydrophilic basic residue is replaced with small/medium sized hydrophobic non-polar residues. Therefore, 16/23 variants experience a significant change in residue size, charge, polarity and/or hydrophobicity, which may have a negative impact on the conformational and functional competence of the OXTR.

Conservation of residues across species, or among protein families can be a good indicator of the importance of a specific residue in receptor structure/functionality. Analysis of the conservation of variant residues in the OXTR among several mammalian species and between the different members of the phylogenetically related human neurohypophyseal hormone receptor subfamily (which comprises the OXTR, AVP1a, AVPR1b and AVPR2) was therefore performed. It was observed (Appendix 1C), Figure 35 and Figure 38; summarised in Table 6 and Table 7) that all of the birth disorder-linked residues were highly conserved across all the mammalian species (V45, P108, M133, R150, V172, H173, W203, L206, G221, I266 and V281) and that four of the psychological disorder linked residues (A63, A217, A238 and T273) display conservation across all mammalian species. While residue A218 shows

quite strong conservation, with the same or similar residue being present at this position in all species examined. Amongst the neurohypophyseal hormone receptor subfamily there was also strong conservation at positions A63, A217, A218 and T273, with all members having the same or similar amino acids at these positions. However, residue A238 was not conserved within the family, indicating that perhaps this residue might be responsible for receptor-specific interactions/functionality. Interestingly, for residues E242 and G252, there was high species conservation, however, the human receptor was different from all/the majority of the other species. Also of note is that the human OXTR residues A217, A218 and G252 express the variant amino acid in at least one of the other OXTR mammalian species or other neurohypophyseal hormone receptor family member (at the position corresponding to human OXTR position 217, A is replaced with T in the AVPR1b sequence, at the position corresponding to human position 218, A is replaced with T, in the rhesus monkey sequence and the AVPR1a sequence, and at the position corresponding to human position 252, G is replaced with A in all other species examined). This could indicate that the change in amino acid may not have any harmful effect on receptor function. The most inter-species residue variability was observed at position 376, while large intra-family variability was observed at positions 238, 242, 252 and 376.

Table 6: Summary of residue conservation across OXTRs from different species

Amino Acid Position (Human)	Mammalian Species						
	Human	Sheep	Cattle	Dog	Rhesus Monkey	Norway Rat	House Mouse
45	V	V	V	V	V	V	V
63	A	A	A	A	A	A	A
108	P	P	P	P	P	P	P
133	M	M	M	M	M	M	M
150	R	R	R	R	R	R	R
172	V	V	V	V	V	V	V
173	H	H	H	H	H	H	H
203	W	W	W	W	W	W	W
206	L	L	L	L	L	L	L
217	A	A	A	A	A	A	A
218	A	A	T	A	T	A	A
221	G	G	G	G	G	G	G
238	A	A	A	A	A	A	A
242	E	A	A	E	A	A	A
252	G	A	A	A	A	A	A
266	I	I	I	I	I	I	I
273	T	T	T	T	T	T	T
281	V	V	V	V	V	V	V
376	R	H	Y	H	Y	R	R

The amino acid sequence of human OXTR was aligned with other mammalian species (sheep, cattle, dog, Rhesus monkey, Norway rat, and house mouse) to determine conservation of the residues of interest. The colours indicate the degree of conservation (red: very different, yellow: different, green: similar; white: conserved, based on Gonnet PAM 250 matrix).

Table 7: Summary of OXTR residue conservation among the neurohypophyseal hormone receptor family

Amino Acid Position (OXTR)	Human Gene			
	OXTR	AVPR1a	AVPR1b	AVPR2
45	V	V	V	L
63	A	A	T	A
108	P	P	P	P
133	M	M	M	M
150	R	Q	Q	H
172	V	Y	V	L
203	W	W	W	W
206	I	V	V	V
217	A	G	T	A
218	A	T	A	A
221	G	G	S	V
238	A	R	W	R
242	E	-	-	D
252	G	K	T	P
266	I	I	I	V
281	V	V	V	V
273	T	T	T	T
376	-	-	R	R

The amino acid sequence of human OXTR, AVPR1a, AVPR1b and AVPR2 were aligned to determine conservation of the residues of interest. The colours indicate the degree of conservation (red: very different, yellow: different, green: similar; white: conserved, based on Gonnet PAM 250 matrix). - indicates that no corresponding residue is present in that sequence.

To further examine residue conservation at the different positions, the Ballesteros-Weinstein GPCR numbering system was utilised to categorise the TM-located residues so that a GMoS conservation tool could be employed to examine the amino acid conservation at the corresponding position in all Rhodopsin-family GPCRs. This analysis ((Appendix 1C); summarised in Table 8) revealed that V45 (1.38), L206 (5.44) and V281 (6.41) are the only three of the examined residues with high conservation within this GPCR family. Although residues A63 (1.56), M133 (3.46), W203 (5.41),

A217 (5.55) and T273 (6.33) are conserved within the neurohypophyseal hormone receptor subfamily, they are not highly conserved within the GPCR family as a whole. Indeed, the majority of the variant OXTR residues are not recognised as highly conserved with regards to the GPCR family, with a variety of different residues being expressed at these positions, although in most instances the residues have similar properties. In all cases, the residues expressed in the WT OXTR are only expressed in a subset of GPCRs. It is interesting to note that for residue A63 (1.56) the variant amino acids (G and V) are found at that position in a similar (G) or higher (V) proportion of GPCRs than the WT residue. The WT and variant amino acid are also found in similar proportions of GPCRs for residues M133 (3.46), H173 (4.62), W203 (5.41), A218 (5.56) and G221 (5.59). For residues A217 (5.55), and T273 (6.33), although the variant residues were found to be at that position in some GPCR members, the proportion of GPCRs with these residues present was lower than that of the WT residues.

Table 8: GMOS and In silico analysis summary for OXTR variants

Variant	Location	Ballesteros-Weinstein number	Conserved residue? (WT amino acid present in ≥ 25% GPCRs)	Variant residues present in < 10% GPCRs?	Number of different residue variants located at that position in all GPCRs	Number of VEP programs predicting deleterious outcome
V45L	TM 1	1,38	Yes	No	14	6/7
A63G	TM 1	1.56	No	Yes	18	5/7
A63V	TM 1	1.56	No	No	18	5/7
P108A	TM 3/ ICL 3	3.21		*ND*		6/7
M133V	TM 3	3.46	No	No	7	6/7
R150L	TM 4/ ICL 2	4.39		*ND*		6/7
R150S	TM 4/ ICL 2	4.39		*ND*		5/7
V172A	TM 4	4.61	No	No	14	7/7
H173R	TM 4	4.62	No	Yes	19	7/7
W203R	TM 5	5.41	No	Yes	13	7/7
L206V	TM 5	5.44	Yes	No	18	3/7
A217T	TM 5	5.55	No	Yes	19	1/7
A218T	TM 5	5.56	No	Yes	18	0/7
G221S	TM 5	5.59	No	No	18	6/7
A238T	ICL3	-		*ND*		0/7
E242K	ICL3	-		*ND*		1/7
G252A	ICL3	-		*ND*		0/7
I266V	TM 6/ ICL 3	6.26		*ND*		6/7
T273R	TM 6	6.33	No	Yes	18	6/7
T273M	TM 6	6.33	No	Yes	18	6/7
V281M	TM 6	6.41	Yes	Yes	13	5/7
R376C	C-term	-		*ND*		1/7
R376G	C-term	-		*ND*		1/7

The selected variants in the OXTR and their specific location and Ballasteros-Weinstein number. A residue conservation analysis tool (GMOS) could only predict the conservation of the residue if located in the core of the transmembrane portion of the receptor. Consequently, ND: Not Defined - indicates residues located in the intra- or extracellular loops (ICL or ECL), or the beginning or end of the transmembrane domain (TM) and therefore have no Ballesteros-Weinstein number assigned precluding this analysis. The mutations were analysed using a series of mutation effect prediction programs (SIFT, Polyphen, FATHMM, LRT, Mut assessor, Mut Tester and PROVEAN) with the score representing how many programs predicting the variant to be deleterious. The red text indicates those variants for which majority of prediction tools predicted a deleterious effect.

The residue conservation analyses were followed by use of a suite of variant effect prediction programs ((Appendix 1C); summarised in Table 8). These online tools predict whether the variants would result in a deleterious effect on protein function using a variety of different algorithms (see Section 3.2.2.3). Interestingly, all the variants located in TM 1, 3, 4 and 6 were predicted to be deleterious in 5 or more of the prediction programs. Of the seven variant prediction programs used, the majority predicted that only 4/11 psychological disorder-linked variants (A63V, A63G, T273R and T273M) would result in a deleterious effect. The variants linked to the birth disorders which were predicted to be deleterious in five or more of the programs included V45L, P108A, M133V, R150L, H173R, W203R, I266V and V281M. It should be noted that the FATHMM program predicted all the variants to be tolerated and Provean predicted the majority of the variants to be moderately deleterious or neutral.

4.2.2 *In vitro* analysis of OXTR variants

To confirm the (non)pathogenicity of the variant receptors *in vitro* functional analyses were conducted in HEK 293T cells transiently transfected with WT and variant OXTRs.

4.2.2.1 Variant effects on cell growth

Upon commencement of the *in vitro* analyses, it was observed that there was a difference in cell growth of the of HEK 293T cells 48 hours post-transfection with empty vector when compared to WT OXTR, with cells transfected with the WT OXTR appearing to be fewer in number. It was therefore postulated that the OXTR may be somehow affecting cell growth and that cells transfected with the different variant OXTRs may exhibit different rates of growth depending on the activity of the variant receptor. If this was the case, it would be important for two reasons. Firstly, examination of cell growth following transfection may provide information about variant functionality and, secondly, if differences in cell number were observed then it would be important to account for this in downstream *in vitro* activity/expression analysis, such that data generated could be normalised to viable cell number. Therefore, images of cells were captured after 48 hours of transfection to confirm any cell number

differences visually (Appendix 1D: Cell Images 48 hours post-transfection). In order to quantify cell number, crystal violet staining was also performed where the amount of crystal violet staining reflects the number of viable cells (Figure 5A). It was found that compared to the empty vector (control) the WT OXTR transfected cells had only 61% of the control cell number. For the psychological disorder-linked variants 9 out of the 11 resulted in a significant decrease in cell number ($p<0.05$, one-way ANOVA followed by Dunnett's post-test) compared to the control, with all resulting in cell numbers not different to that seen with the WT OXTR (A63V: 67%, A63G: 64%, A217T: 70%, A218T: 59%, E242K: 57%, G252A: 63%, T273R: 66%, T273M: 61% and R376C: 65% of the control cell number). Conversely, when transfected with variants A238T or R376G there was no significant difference in cell number compared to the empty vector transfected cells. When examining the effects of the birth disorder-linked variants (Figure 5B), 9 of the 12 variants also resulted in a significant decrease in cell number when compared to control to levels not different to that seen with WT OXTR transfected cells (V45L: 85%, P108A: 66%, M133V: 67%, R150L: 67%, R150S: 65%, V172A: 61%, H173R: 66%, W203R: 64%, L206V: 55%, G221S: 83%, I266V: 64% and V281M: 89% of the control cell number). When transfected with variants V45L, G221S and V281M, there was no significant difference in cell number compared to the control cells.

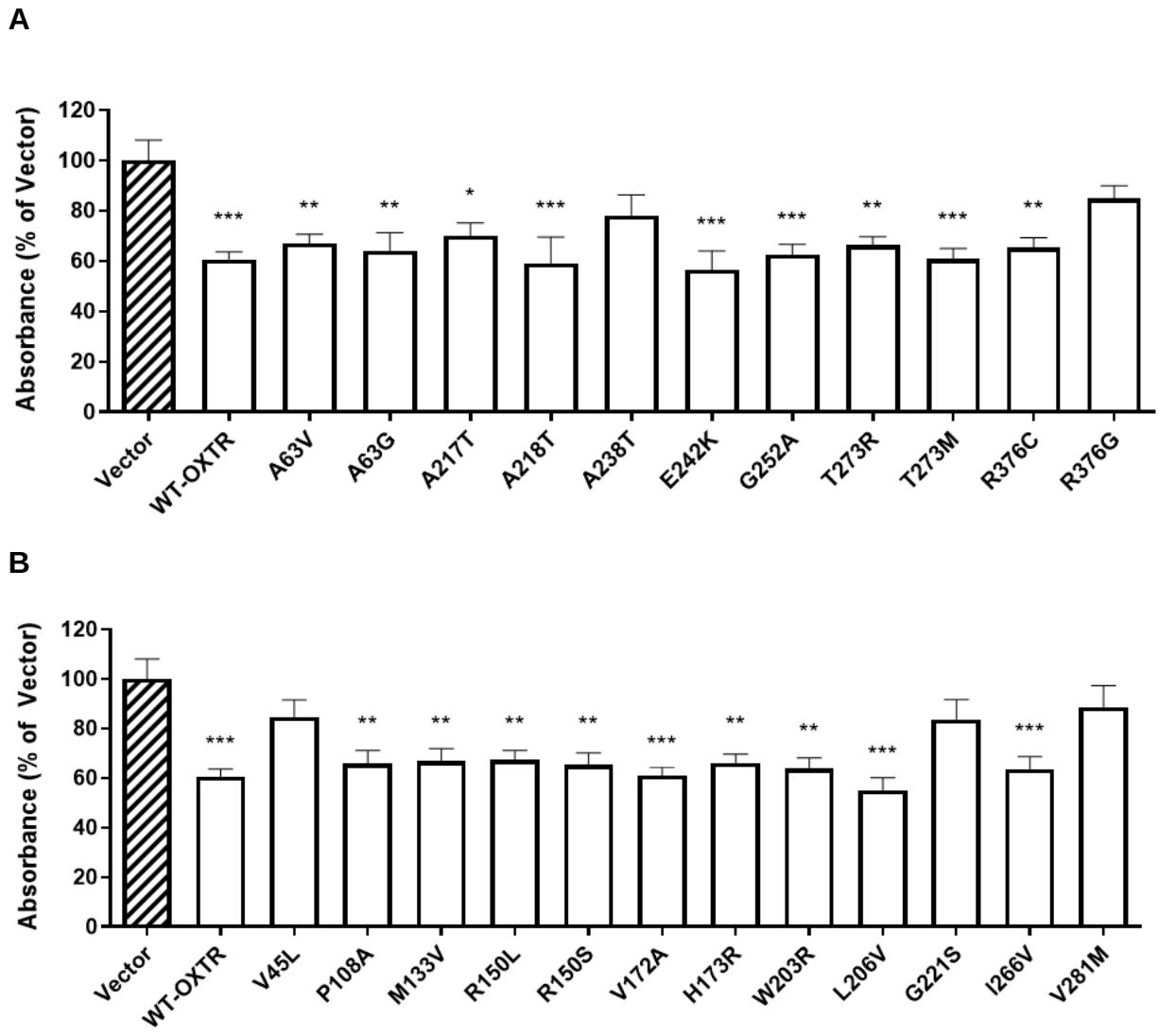


Figure 5 : Crystal violet staining of HEK 293T cells transfected with empty vector, WT OXTR or variant OXTRs

*Cell number was quantified by crystal violet staining following transfection of **A:** psychological disorder-linked OXTR variants or **B:** birth disorder-linked OXTR variants. Cells were fixed and stained 48 hours post transfection with WT or variant OXTRs. HEK 293T cells transiently transfected with empty vector (vector) were used as a control. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as % of the average crystal violet staining observed in cells transfected with empty vector only. ***p<0.001, **p<0.01 and *p<0.05, 1-way ANOVA followed by Dunnett's multiple comparison post hoc test.*

A thorough examination of the mechanism behind the reduced growth of cells transfected with the OXTR is outside the scope of the present study. However, in order to determine whether the decreased growth rate was as a result of OXTR expression having a cytotoxic effect, a cell viability assay was performed using trypan blue staining to quantify the ratio of living cells to dead cells 24 hours after transfection following careful removal of the culture media (Appendix 1E). It was found that transfection with the WT OXTR did not result in a difference in living cell percentage when compared to empty-vector transfected cells. Likewise, cells transfected with the majority of the variant receptors exhibited similar cell viabilty. There is a hint that transfection with variants A63G and A238T may possibly cause a slight reduction in cell viability. However, as this analysis was only performed once, statistical confirmation was not possible.

4.2.2.2 Variant effects on receptor signalling

To determine the functionality of the variant OXTRs, their response to hormone stimulation was measured. However, before signalling competence of the variant receptors was examined, the ideal concentration of OXT ligand to be used when testing the signalling capabilities of the OXTR variants was first determined. The OXTR signals via coupling to $G\alpha_{q/11}$ G proteins, which, in turn, activate the enzyme PLC that catalyses the conversion of membrane lipids to produce IPs and DAG. Thus, to measure the dose-dependent signalling of the OXTR, cells transfected with WT OXTR were grown in media containing [^{3}H]-myoinositol such that any generated IPs would be radioactively labelled. Cells were then stimulated with OXT before capture and measurement of the generated radiolabelled IPs. Dose-response analyses using cells expressing WT OXTR (Figure 6) demonstrated that OXT stimulated IP production in these cells with a potency (EC_{50}) of 3.4 nM (pEC50 ± SEM = 8.5 ± 0.24).

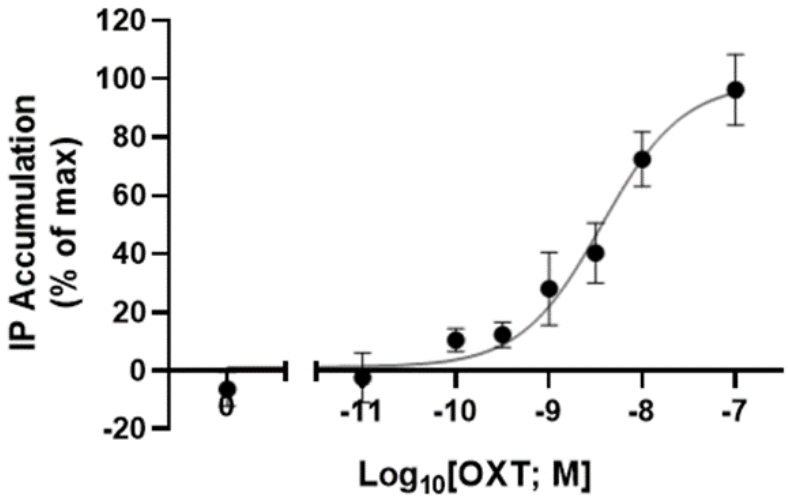


Figure 6: Dose-response analysis of WT OXTR signalling in response to OXT stimulation

Signalling response in HEK 293T cells transiently transfected with WT OXTR was measured by radiolabelled inositol phosphate (IP) accumulation assay following stimulation with vehicle (0) or a range of OXT concentrations. Data presented are from three independent experiments (N=3). Data have been fitted to a sigmoidal dose response curve from which the potency (EC_{50}) of oxytocin was calculated to be 3.8 nM.

The signalling activity of all twenty-three (11 psychological disorder and 12 birth disorder-linked) selected OXTR variants were then compared to the WT OXTR using a single concentration screen. As a result of the OXT dose response analysis (Figure 6), it was decided that the ideal concentration to use for examining variant receptor signalling would be 10 nM, as defects in potency or efficacy of activation of variant receptors are best detected when using concentrations of ligand of 3-10x EC_{50} [132], which achieve just sub-maximal responses at the respective WT receptor. HEK 293T cells were transfected with either WT OXTR, variant OXTRs or empty vector (negative control). Transfected cells were then treated with vehicle (to measure basal receptor activity and examine any alterations in possible basal/constitutive activity of the OXTR as a result of the variants) or 10 nM OXT (to measure alterations in OXT-induced OXTR signalling). As variations in cell number were observed between cells transfected with the different variant receptors, the relative cell numbers (percentages) measured by crystal violet staining (Figure 5) were used to normalise measured

signaling responses to cell number variability in these signalling (IP accumulation) assays.

There was no significant difference in the basal activity measured in cells expressing any of the variants when compared to cells expressing the WT OXTR ($p>0.05$, one-way-ANOVA followed by Dunnett's post-test; data not shown). When treated with OXT, 4/11 psychological disorder-linked variants (A63V, A238T, T273R and T273M) displayed significantly decreased IP accumulation when compared to cells expressing the WT receptor ($p<0,05$; one-way ANOVA followed by Dunnett's post-test) (Figure 7A). Variant T273R showed no IP accumulation (signalling) at all, while variants A63V, A238T and T273M resulted in a significant reduction of signalling capacity (42%, 48% and 17% of WT, respectively). It should be noted that variants A63G, A217T, R376C and R376G also showed a decrease in signalling compared to WT OXTR (74%, 65%, 64% and 62% of WT, respectively), however these differences were not significant, probably as a result of large variability. With regards to the variants linked to birth disorders, a significant decrease in signalling response to OXT was seen in 8/12 variants (V45L, P108A, M133V, H173R, W203R, G221S, I266V and V281M) when compared to WT OXTR ($p<0,05$; one-way ANOVA followed by Dunnett's post-test) (Figure 7A). Variant W203R showed no IP accumulation (signalling) at all, while variants V45L, P108A, M133V, H173R, G221S, I266V and V281M resulted in a significant reduction of signalling capacity (21%, 10%, 41%, 38%, 42%, 47% and 28% of WT, respectively). Again, it should be noted that variants R150L and R150S showed a decrease in signalling compared to WT OXTR (to 64% and 68% of WT respectively), however these differences were not significant. The remaining three psychological disorder-linked (A218T, E242K and G252A) and two birth disorder-linked variants (V172A and L206V) did not show a decrease in IP signalling compared to WT OXTR. Therefore, these variants were deemed to be unimpaired and were excluded from further functional analysis.

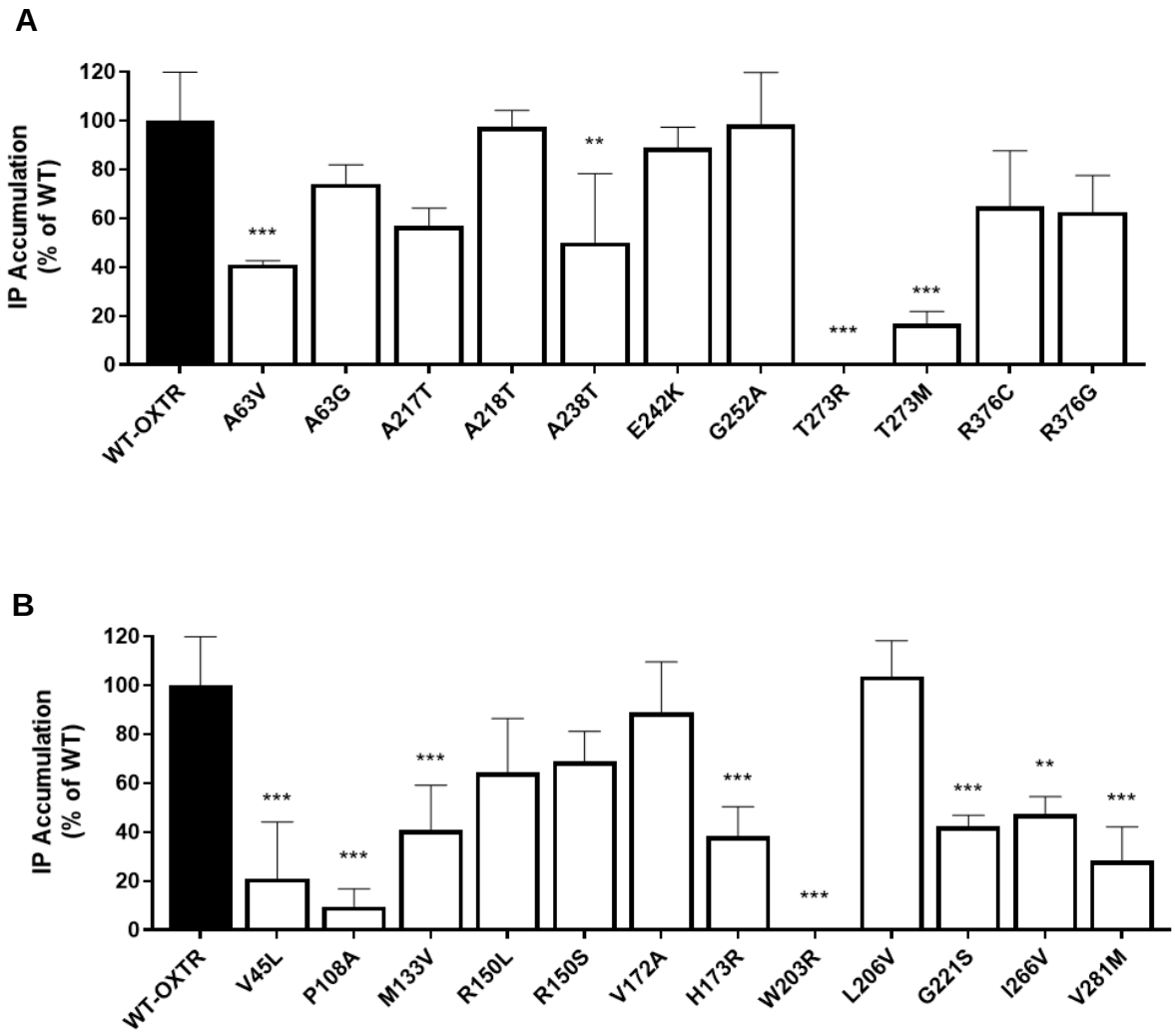


Figure 7: OXT-stimulated Inositol phosphate accumulation in cells expressing variant OXTRs

Signalling responses in HEK 293T cells transiently transfected with WT OXTR, ***A*** *psychological disorder-linked OXTR variants or* ***B*** *birth disorder-linked OXTR variants were measured by radiolabelled inositol phosphate (IP) accumulation assay following stimulation with 10 nM OXT. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT OXTR after subtraction of non-specific signal measured in cells transfected with empty vector and normalisation to cell number (determined via crystal violet staining, Figure 5).***p<0.001, **p<0.01 and *p<0.05, 1-way ANOVA followed by Dunnett's post-test for comparison with WT OXTR.*

Further, more detailed, examination of the signalling responses of the four OXTR variants (A63V, A238T, T273R and T273M) that displayed impairment in these single

concentration experiments were then conducted by dose response analyses following stimulation with a range of OXT concentrations (Figure 8). Variants R376C and R376G, which had suggestion of a marginal, but not significant, decrease in signalling response in the single concentration experiments were also included as a control. Data were fitted to sigmoidal dose response curves and potency (pEC_{50}) and maximal responses (E_{max}) were calculated. Due to the very high costs associated with the IP accumulation assay, which had been inflated as a result of the COVID-19 pandemic and the container crises experienced in 2021-2022, only the psychological disorder-linked variants were included in these dose-response analyses.

As seen in the single concentration analyses, variant T273R was completely non-responsive and did not signal across the array of OXT concentrations. Therefore, no E_{max} or pEC_{50} could be determined (Figure 8C and Table 9). When comparing the maximal responses (E_{max}) for variants A63V and T273M (Figure 8A and D and Table 9) there was a significant decrease when compared to WT OXTR (44% and 24% of WT, respectively). Variant A238T (Figure 8B and Table 9) also showed a decrease in maximal response when compared to WT OXTR, although, in this case, this was not significant. Interestingly, none of the variants resulted in reduced OXT potency but variant T273M rather showed a significant increase in potency compared to the WT OXTR (5.8 nM vs 3.4 nM). For variants R376C and R376G (Figure 8E and F and Table 9), there was no difference observed in signalling when compared to WT OXTR thus confirming that these variants are not functionally impaired.

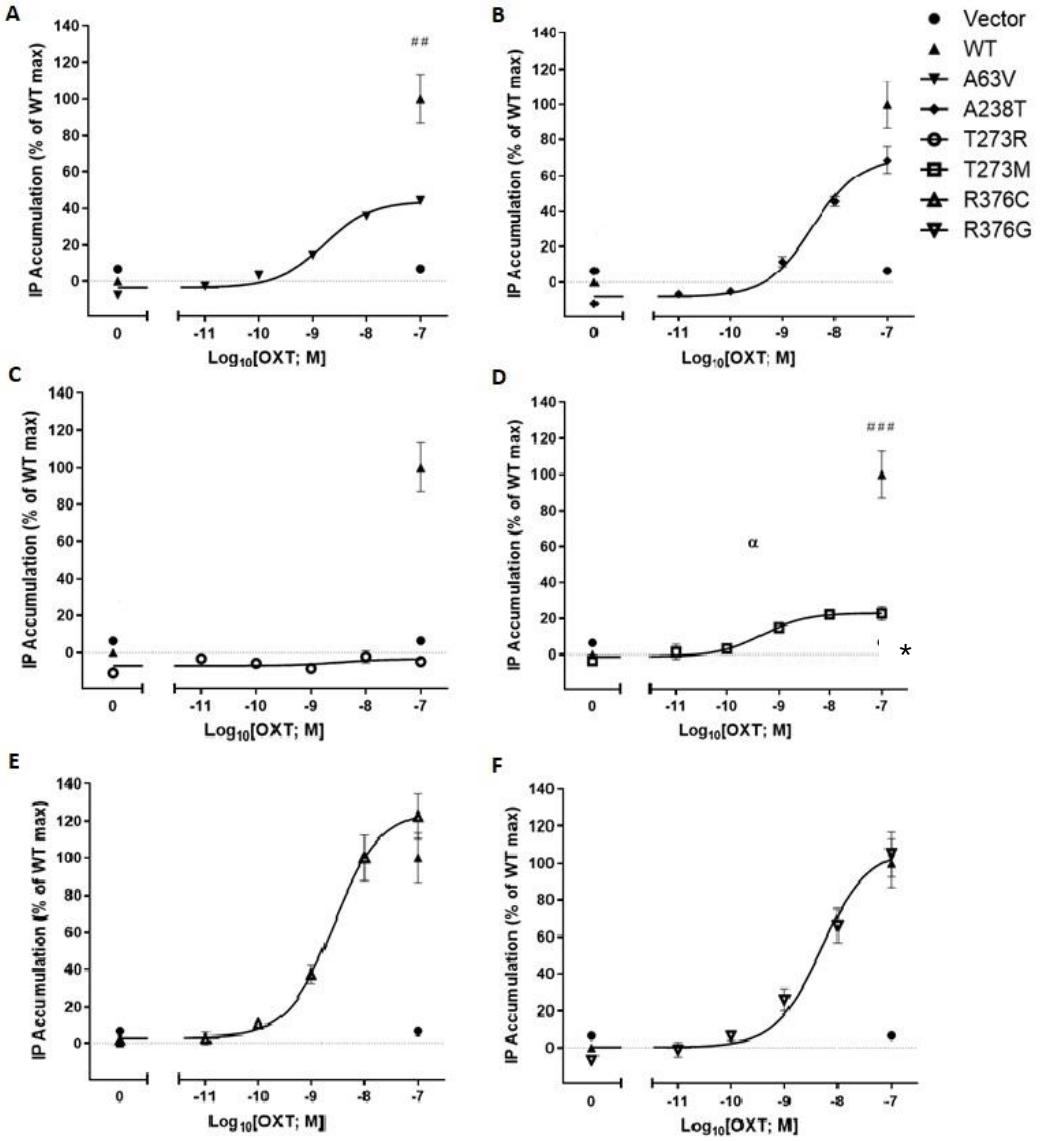


Figure 8: Dose-response analysis of variant OXTR signalling in response to OXT stimulation

*Signalling response in HEK 293T cells transiently transfected with empty vector, WT OXTR or **A:** A63V, **B:** A238T, **C:** T273R, **D:** T273M, **E:** R376C and **F:** R376G variant OXTRs was measured by radiolabelled inositol phosphate (IP) accumulation assay following stimulation with vehicle (0) or a range of OXT concentrations. Data presented are from three independent experiments (N=3). Data have been fitted to sigmoidal dose response curves from which potencies and E_{max} were calculated (Table 9). Data are presented as a % maximal activity measured in cells transfected with the WT OXTR (stimulated with 0.1 µM OXT) following subtraction of the average basal activity (0) measured in cells expressing the WT receptor and normalisation to cell number. Data are presented as mean ± SEM from three independent experiments.*

Table 9: Statistical analysis of E_{max} and Potency of Dose-response signalling in response to OXT stimulation

Receptor	E_{max} (% of WT)	pEC_{50} (EC_{50} compared to WT)
WT OXTR	100 ± 13	8.47 ± 0.24 (3.4 nM)
A: A63V	44 ± 1 $^{\#\#}$	8.77 ± 0.12 (1.7 nM)
B: A238T	71 ± 8	8.42 ± 0.09 (3.8 nM)
C: T273R	ND	ND
D: T273M	24 ± 2 $^{\#\#\#}$	9.24 ± 0.21 (5.8 nM) *
E: R376C	125 ± 15	8.63 ± 0.9 (2.5 nM)
F: R376G	110 ± 12	8.27 ± 0.18 (5 nM)

ND- Not Defined (no measurable response elicited). $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{*}p<0.05$, one-way ANOVA followed by Tukey's post-test for comparison with WT.

4.2.2.3 Variant effects on hormone binding

The signalling analysis of the selected OXTR variants showed that four psychological disorder-linked variants and eight birth-disorder-linked variants had reduced OXT-induced signalling responses compared to the WT OXTR (Figures 8 and 9). This reduced response could be a result of several factors including reduced receptor expression/cell surface localisation, reduced ligand binding or reduced receptor signal transduction/coupling to downstream signalling partners. Radioligands can be used to measure ligand binding to receptors. These should ideally have high affinity, low non-

specific binding, high specific activity (to ensure high signal-to -noise ratio), and specificity for the receptor of interest. Due to the cost and licence limitations with regards to purchase of certain radiolabelled ligands, it was only possible to purchase radiolabelled [^{3}H]-AVP to examine binding competence of the OXTR variants. Due to the high similarity between OXT and AVP, AVP is able to interact with the OXTR and both AVP and OXTR bind to the OXTR in similar manner/at a similar site,[133] thus, it should, in theory, be possible to utilise this radioligand to examine effects of variants on ligand binding of the OXTR. Unfortunately, despite extensive optimisation, a low signal-to-noise ratio was observed in cells expressing the WT OXTR making examination of the variant receptors unfeasible (Appendix 1G: Radioligand Binding Assay Optimisation).

4.2.2.4 Variant effects on receptor expression

In most cases, GPCRs must be expressed at the cell surface in order to be accessible to their ligands. As described in Section 1.4, GPCR variants have been found to often cause receptor conformation complications (misfolding of the receptor protein) and subsequent loss of cell surface expression of the receptor protein due to intracellular retention (in the ER) of nascent receptor proteins by cellular quality control processes. This is a common cause of loss-of-function induced by GPCR variants. Thus, examination of the OXTR variant effects on receptor cell surface expression was undertaken.

When studying receptor variants, structural changes that result from introduction of the mutations may affect antibody recognition. To circumvent this, an ELISA-based assay exploiting HA-tagged (YPYDVPDYA) variant OXTRs was used to measure and compare total cellular and cell surface expression of each of the selected variant receptors. This epitope tag antigen will remain unaffected by introduction of the mutations to the variant receptors allowing for an unbiased measure of receptor expression. A highly specific anti-HA antibody can then be used to label the receptors, allowing quantification of expression by incubation with HRP-conjugated secondary antibody and colorimetric analysis following application of the HRP substrate. When

the receptor is correctly placed in the plasma membrane, the N-terminal HA tag protrudes from the extracellular surface, so in intact cells, the only receptors accessible for the specific binding of HA antibodies are those expressed at the cell surface. Permeabilisation of the cells to allow for penetration of the antibodies into the cells then enables total (internal and cell surface) receptor expression to be measured.

Optimisation was first performed in order to determine the most appropriate post-transfection incubation period as well as the primary and secondary antibody concentrations for the ELISA to ensure the best signal-to-noise ratio was achieved (Appendix 1F)

The cell surface expression of the variant receptors was then determined. After the elimination of variants which did not show a significant decrease in IP signalling, 12 selected OXTR variants (4 psychological disorder-linked variants (A63V, A238T, T273R and T273M) and 8 birth disorder-linked variants (V45L, P108A, M133V, H173R, W203R, G221S, I266V and V281M)) were compared to the WT OXTR. Cell surface expression of all of the psychological disorder linked OXTR variants (A63V, A238T, T273R, T273M) (Figure 10 A) displayed a statistically significant decrease in cell surface expression when compared to WT OXTR (to 73%, 74%, 27% and 28%, of WT, respectively). For the birth disorder linked OXTR variants (Figure 9 B) 7/8 variants (V45L, M133V, H173R, W203R, G221S, I266V and V281M) showed a significant decrease in cell surface expression (to 34%, 74%, 59%, 23%, 58%, 41% and 31% of WT, respectively). Interestingly, variant P108A showed a significant increase in cell surface expression (to 137% of WT).

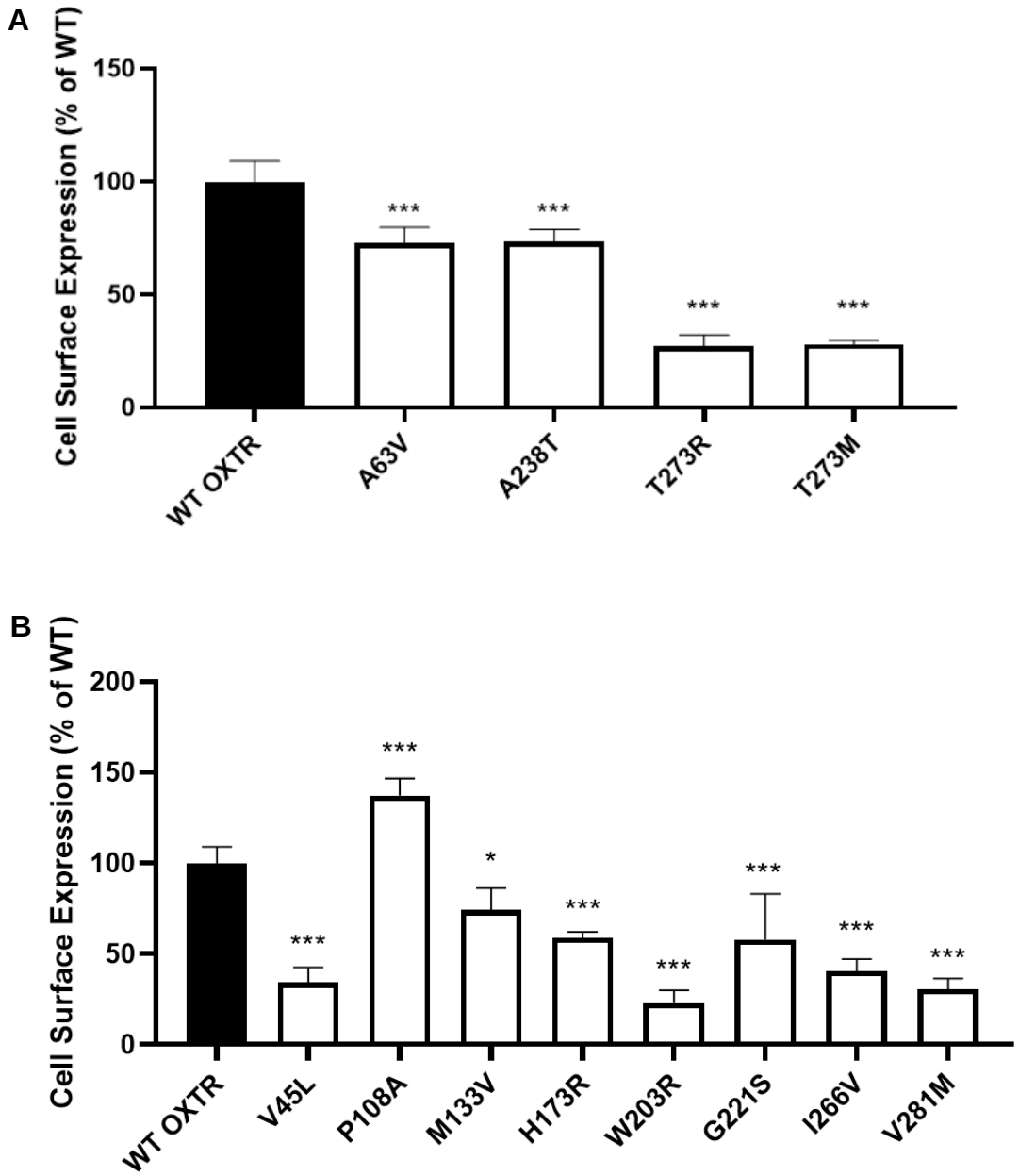


Figure 9: Cell surface expression of WT and variant OXTRs

HEK 293T cells were transiently transfected with either empty vector, WT OXTR, ***A*** *psychological disorder-linked variant OXTRs, or* ***B*** *birth disorder-linked variant OXTRs. Cell surface expression of was measured by a receptor ELISA with intact cells. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT OXTR after subtraction of non-specific signal measured in cells transfected with empty vector and following normalisation for cell number. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA followed by Dunnett's post-test for comparison with WT OXTR.*

To determine whether the altered cell surface expression observed was due to altered receptor trafficking to the cell surface or due to altered receptor biosynthesis and/or degradation (or a combination), total cellular receptor expression was examined using permeabilised cells. When comparing the total receptor expression of the psychological disorder-linked variants to the WT OXTR (Figure 10 A), it was found that 3/4 of the variants (A238T, T273R and T273M) showed a slight, but statistically significant, decrease (to 75%, 72% and 69% of WT, respectively) ($p<0.05$; one-way ANOVA followed by Dunnett's post-test). With regards to the birth disorder-linked OXTR variants, 5/8 variants (V45L, H173R, W203R, G221S and V281M) showed a significant decrease in total expression, with variants V45L and V281M showing a large decrease (to 46% and 52% of WT, respectively) and variants H173R, W203R, G221S a more modest decrease (to 69%, 65% and 82% of WT, respectively). Interestingly, as observed for the cell surface expression, variant P108A also showed a significant increase in total expression of almost 1.5-fold (to 145% of WT).

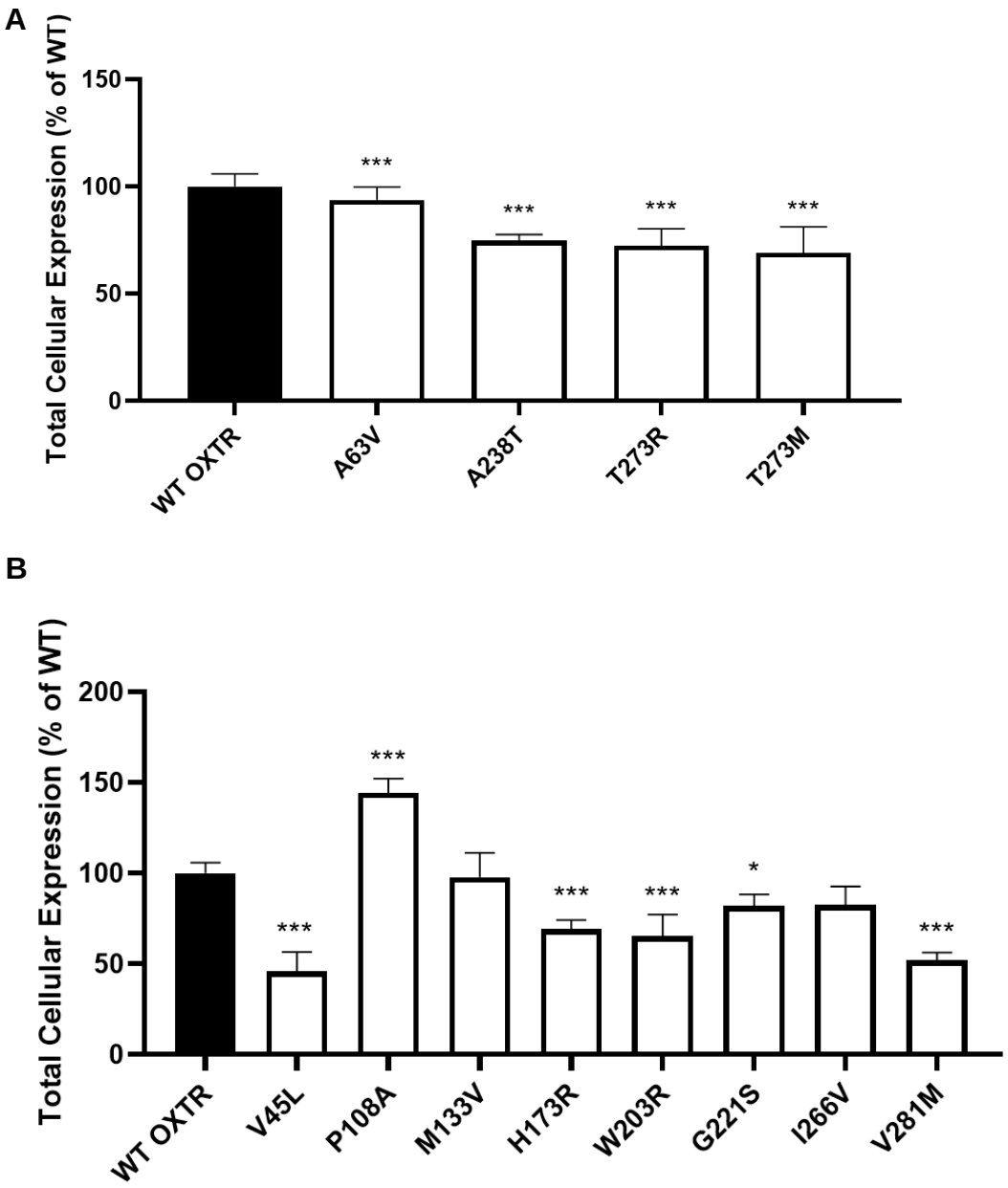


Figure 10: Total cellular expression of WT and variant OXTRs

HEK 293T cells were transiently transfected with either empty vector, WT OXTR, ***A*** *psychological disorder-linked variant OXTRs, or* ***B*** *birth disorder-linked variant OXTRs. Total cellular expression was measured by a receptor ELISA with permeabilised cells. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT OXTR after subtraction of non-specific signal measured in cells transfected with empty vector and following normalisation for cell number. *p<0.05, and ***p<0.001, one-way ANOVA followed by Dunnett's post-test for comparison with WT OXTR.'*

4.3 Discussion

The initial phase of this study aimed to characterise OXTR coding-region variants suspected to result in psychological disorders such as ASD and harm avoidance. An extensive literature search as well as database exploration, resulted in the identification of eleven missense OXTR coding-region variants that have been putatively linked to psychological disorders. Previous research within the Centre for Neuroendocrinology conducted a similar review to identify OXTR variants linked to birth disorders and identified a far larger number of variants (a selection of which were examined in the recent study alongside the psychological disorder-linked variants). This is perhaps not surprising given the well characterised role of OXTR in labour, while its potential roles in psychological disorders are still being elucidated.

It is interesting to note that many of the variants identified in both psychological and birth disorders are situated in the transmembrane domains of the receptor, suggesting that this region is more sensitive to disruption as opposed to the intracellular/extracellular domains of the receptor. Conservation analysis indicates that highest homology between the human neurohypophysis receptor subtypes is found in the ECLs and TM helices, while the N-terminus and the C-terminus have lower similarities, and the ICLs are the least conserved.[16] Again, this is perhaps not surprising as the TM domains make up a large proportion of the receptor structure and are comprised of important secondary and tertiary structures (e.g., TM helices) that can easily be disrupted and are the location of many of the sites involved in hormone binding, G protein-interaction and receptor signal transduction.

Of all the variants examined, only a minority (36%; 4/11) of the psychological disorder-linked variants were found to have a significantly reduced response to OXT stimulation, indicating loss-of-function. Conversely, the majority (67%; 8/12) of birth-disorder linked variants showed impaired responses. For those variants linked to both disorder categories (A281T, A238T and G525A) it is interesting to note that only variant A238T displayed an impaired signalling response. Thus, their identification in different disease populations linked to/potentially dysfunctional OXT/OXTR signalling

appears to be coincidental and these variants may actually just be silent variants/polymorphisms present within the general population as a whole. It is important to note that no previous functional analysis has been reported for several of these variants and these are therefore novel findings.

4.3.1 The majority of loss-of-function OXTR variants exhibit reduced cell surface expression

When cell surface expression of the loss-of-function variants was examined, all of the psychological disorder linked variants had significantly reduced expression at the cell surface (Figure 9A), with 50% (2/4; T273R and T273M) having significantly impaired cell surface expression of <50% of WT levels and the other two (A63V and A238T, only moderately decreased expression (of approximately 75% of WT levels). Similarly, 7/8 (88%) of the birth disorder variants had significantly reduced expression at the cell surface (Figure 9B), with 4/7 (V45L, W203R, I266V and V281M) displaying severe effects and one (M133V) a moderate decrease. The majority of variants with severely decreased cell surface expression had a more moderate (L206V, T273R, T273M and V281M) or no (I266V) decrease in total cellular expression. Variants A63V, M133V and G221S, which had moderately reduced cell surface expression, also had little (G221S) or no (A63V and M133V) concurrent reduction in total cellular receptor expression (Figure 10A and B). The remaining variants showed a severe (V45L) or moderate (H173R and A238T) reduction in cell surface expression and a parallel decrease in total receptor expression (Figure 10A and B).

When a cell detects an incorrectly folded protein, the protein is either sent back through the folding cycle or marked for the ER associated degradation (ERAD).[134] In many physiological and pathological circumstances, intracellular trapping of the conformationally abnormal receptor in the ER is seen to cause stress. In order to alleviate the ER stress, cells activate the unfolded protein response (UPR), a dynamic signalling network that, depending on the severity of the damage, either orchestrates the restoration of homeostasis or initiates degradation.[134] Thus, that some variants have reduced total expression could possibly reflect the degree to which they are

misfolded such that a greater portion is targeted for degradation. Alternatively, those that are affecting total receptor expression could also be affecting the biosynthesis of the receptor. Confirmation of the cause of decrease in expression of these variants is beyond the scope of this study. Regardless of the cause, with the exception of variants V45L, H173R and A238T, they have more of an effect on cell surface expression than on total expression. This suggests that trafficking to the cell surface plays at least a part in their non-functionality.

4.3.2 Non-Functionality of Variants V45L, G221S, I266V, T273M and V281M is due to Reduced Expression/Cell Surface Localisation

After considering the ratio of cell surface-to-total expression as well as the OXT-induced receptor signalling measured, it may be inferred that variant V45L (birth disorder-linked), affects receptor biosynthesis and/or degradation (Class I/II variant) that accounts for the reduction in cell surface expression and thus signalling observed. Likewise, variants G221S (birth disorder-linked), I266V (birth disorder-linked), T273M (psychological disorder-linked) and V281M (birth disorder-linked) appear to primarily result in intracellular retention/abnormal receptor trafficking (Class II variants), which, again, accounts for their parallel reduction in signalling capacity.

Residue V45 is a conserved residue (Table 6 and Table 7) located in TM 1. It is positioned near N57, which is a known polar pocket site and variants here may therefore interfere with the folding of the receptor.[135] This substitution is not a particularly conservative one, with introduction of a large, polar hydrophilic amino acid in place of a smaller non-polar hydrophobic one. Indeed, six out of the seven VEP programs predicted this variant to be deleterious (See Appendix, Table 21). A recently published study performed *in vitro* assays which demonstrated that the introduction of V45L, did not affect OXT-induced Ca^{2+} signalling.[136] This is contrary to the present study, which showed a decrease in $G\alpha_q$-mediated IP accumulation. However, it is important to note that different signalling outputs were utilised, with Ca^{2+} signalling being further downstream, and thus perhaps more sensitive to receptor reserve,[137] which may account for lesser effects being observed by this measure. A previous

study showed only a very small decrease in cell surface localisation of this variant receptor using quantitative flow cytometry, [136] which is surprising given the large effects seen here. The previous study also examined β-arrestin interactions, desensitization, internalization and found these to be impaired for this variant. They also used an artificial intelligence program (DiffNets) to identify structural changes that were common to V45L but not present in OXTRs with normal internalization and desensitization. The structural differences observed explain how this variant disrupts β-arrestin interactions.[136]

Variant G221S is located in TM 5, while TM 6 is host to variants T273M and V281M, and variant I266V is located in the ICL3/TM6 interface. [123] All four of these variants are highly conserved (Table 6 and Table 7) and the majority of the VEP's predicted a deleterious effect (6/7 for G221S, 6/7 for T273M, 6/7 for I266V and 5/7 for V281M) (See Appendix, Table 21). It appears that substitution of these residues has a large effect on folding of the receptors, suggesting that they may be important conformational residues. Residue G221 is on the intracellular end of TM5 and variants here (such as substitution of the small non-polar hydrophobic glycine residue with a slightly larger, polar hydrophilic serine) may consequently interfere with folding of this helix and the closely located ICL 3, possibly through disruption of important hydrophobic interactions. Interestingly, a previous study found no large effect of this variant on OXT-induced signalling,[136] which is contrary to the findings of the present study. Again, this may be a result of the more-sensitive Ca^{2+} signalling output being utilised in the previous study that may make identification of small differences in signalling induced by decreased cell surface receptor expression more difficult to detect.[137]

Like, residue G221 the substitution of residues I266, T273 and V281 likely disrupt the correct conformation and/or intramolecular interactions of TM 6. For variant I266V the amino acid change is quite conservative, while for variants T273M and V281M there are changes in size, polarity and hydrophobicity that may account for such disruptions. In agreement with the present study, a recent study (published after commencement of the present study) reported that variant V281M decreased OXTR activation (OXT-

induced calcium signalling) and cell surface expression.[136] Furthermore, this previous study suggested that this variant may also affect receptor activation as modelling studies suggested steric inhibition of G protein by this variant,[136] although experimental verification would be required to confirm this. [135]

Variant I266V is located in the ICL3/TM6 interface. The switching from an inactive to an active conformational change is associated with the relative orientational change of TM 3 and 6, which unmasks G protein binding sites. It is hypothesised, that protonation of motifs results in the cytoplasmic exposure of buried sequences in ICL 2 and 3.[138] Therefore, it is perhaps surprising that this variant does not appear to have a large effect on receptor signalling in comparison to its cell surface expression.

4.3.3 Variants W203R and T273R have impaired cell surface trafficking in addition to impaired hormone binding/receptor signalling

Variants W203R (birth disorder-linked) and T273R (psychological disorder-linked) have a large effect on membrane trafficking of the receptor, but the signalling response elicited is lower than that expected based on the level of cell surface expression and, thus, these variants also appear to have effects on OXT binding/receptor signalling (combination Class II and II/V variants. Like those variants found to primarily disrupt cell surface trafficking (Section 4.3.2), introduction of these variants therefore appears to likely disrupt receptor conformation by disruption of the helices in which they are located (TM5 and 6, respectively). Both of these variants introduce a charged (basic) arginine residue, which possibly disrupts important intramolecular interactions). Indeed, the radical change from a large, non-polar, hydrophobic amino acid with a neutral charge, to a smaller, polar, hydrophilic amino acid with a positive charge for variant W203R, may disrupt the sensitive structure and folding of the α-helical transmembrane domain, which may stunt the maturation of the OXTR. These residues are conserved across mammalian species and human neurohypophyseal receptors and all of the VEP tools predicted a deleterious effect of these substitutions.

These variants also appear to affect OXT binding and/or receptor activation.

Radioligand binding assays were unfortunately unsuccessful, and this meant that the cause of non-functionality of these variants (i.e. determining whether they are Class III and/or IV variants) could not be elaborated further, however this would be an interesting avenue for future characterisation of these variant receptors. Although not confirmed experimentally, the location of the variants in the receptor structure can give some insight into the possible effects.

Given the location of residue T273 at the intracellular face of the receptor, it would be tempting to predict that this variant likely affects signal transduction (Class IV variant) rather than hormone binding. Indeed, T273 follows a string of alanine's in the ICL3/TM6 interface, which have been linked to receptor activation.[138,139] It is noteworthy that, at position T273, substitution with arginine had a detrimental effect on receptor signalling, while substitution with methionine actually resulted in increased potency. This may be as a result of a more hydrophobic amino acid which may favour the activation mechanism of the receptor and result in increased signalling. However, further *in vitro* studies would be necessary to fully elucidate the effect of these variants. There has also been no previous functional analysis published on T273R to confirm the findings of the present study.

Unlike residue T273, residue W203 is located close to the extracellular face of the receptor. Indeed, it is sandwiched between two hydrophilic residues known to be important for hormone binding interactions (T205 and T202).[16] This particular change in amino acid may also have an effect on the specificity of the receptor with regards to ligand binding. It is therefore feasible that introduction of the charged basic residue here may disrupt the oxytocin-receptor interactions (i.e. a Class III variant). It should also be noted that the residues on the extracellular regions of ECL 1, TM 5 and 6 account for the specificity of OXTR for OXT versus AVP.[140] A previous study verified that W203R caused nearly complete abolishment of specific hormone binding.[141]

4.3.4 OXTR Variants A63V, P108A, M133V, H173R and A238T Severely Impair Receptor Signalling/Binding

For the remaining loss-of-function variants, although some do seem to have a small effect on expression/cell surface localisation, their moderate reductions (or even increases) in total/cell surface expression cannot fully account for their severe reduction on signalling response. Variants H173R (a birth disorder-linked variant) and A238T (a variant linked to both birth and psychological disorders) appear to have minor effects on receptor biosynthesis and/or degradation but also more severe effects on either OXT binding/receptor signalling that contributes to the drastic decrease in signal despite the moderate amount of receptor available at the cell surface (therefore can be categorised as being primarily Class III/IV variants). Likewise, variants M133V (a birth disorder-linked variant) and A63G (a psychological-disorder-linked variant) appear to have an effect on intracellular retention/abnormal receptor trafficking, but their primary cause of non-functionality appears rather to be due to effects on either OXT binding/receptor signalling (Class III/IV variants). Variant P108A (a birth disorder-linked variant), appears to have increased expression and cell surface localisation, as discussed above. However, the signalling response elicited by this variant is severely impaired, thus also suggesting that this is a Class III/IV variant. Again, future radioligand binding assays would be required to confirm effects on OXT binding, but predictions can be made based on locations of the variants within the receptor structure.

Variant A63V is located in TM 1, which together with the N-terminus, ECL 1 and TM 2, makes up the binding pocket for the interaction with OXT. [90] However this variant is located on the intracellular side of the receptor and is therefore unlikely to affect the binding of the hormone. However, A63V folds in close proximity to N57 which is a known polar pocket site and may therefore interfere with the folding of the receptor, which could account for the slight reduction in cell surface expression observed.[135] After agonist binding, it is understood that the arginine side of the “polar pocket” is shifted intracellularly, thereby unmasking a G protein binding site. This variant was also predicted to be deleterious by 5/7 prediction programs (See Appendix, Table 21).

The residue is also known to be highly conserved across mammalian species and human neurohypophyseal receptors (Table 6 and Table 7), which supports the functional importance of this specific residue. Therefore, although this substitution is relatively conservative in terms of amino acid properties, it does appear that the introduction of an additional carbon chained residue into this highly conserved position may disrupt the activation and signalling of the OXTR receptor. It is interesting that the other variant with substitution at this position (A63G) did not have any effect on receptor function. Thus, substitution of another small hydrophobic residue is tolerated, suggesting that the effects seen with variant A63V may be sterically linked and result from introduction of the larger valine at this position.

The highly conserved variant M133V (Table 6 and Table 7)[25] was predicted by 6/7 VEP's to be deleterious (See Appendix, Table 21). Based on model GPCR studies, M133V, on the end of TM 3 in proximal distance to polar pocket residues D136 and R137, may influence the hydrophilic state and cytoplasmic exposure of residues.[142] Residues D136 and R137 form part of a highly conserved GPCR motif E/DRY (DRC in the OXTR). The switching from an inactive to an active conformation is associated with the relative orientational change of TM 3 and 6, which unmasks G protein binding sites[138] These residues are important for intermolecular interactions that are involved in this conformational switch.[141,142] Introduction of a residue in close proximity that interferes with their interactions may conceivably influence receptor activation. This would explain the drastic decrease in receptor signalling in the OXTR possessing this variant, however, additional assays would be needed to confirm this. Although this substitution is conservative in terms of amino acid properties, this highly important region of the receptor may be particularly sensitive to alterations in terms of effects on receptor activity.

The variant H173R is located in TM 4, at a site shown to be highly conserved amongst mammalian OXTR species (Table 6) and is predicted to be deleterious by all VEP's (See Appendix, Table 21). Residue H173 is located at the beginning of a domain known to interact with the cyclic part of OXT along the ECL 2.[143] It is also in close proximity to several conserved glutamines (Q), responsible for ligand binding.[144,145]

Thus it is predicted that, although this residue substitution is relatively conservative, it may be disrupting hormone-receptor interactions (Class III variant) which would account for the deficient signalling noted (Figure 7) however, ligand binding assays would be required to confirm this theory.

Variant A238T, is located at the ICL 3/TM 5 interface, and this position is also highly conserved amongst mammalian OXTR species (Table 6). Interestingly, it was predicted to be deleterious by none of the VEP's (See Appendix, Table 21) despite this substitution not being very conservative in terms of amino acid properties. In GPCR models, selective replacement of the intracellular loops has shown that on the intracellular receptor face,[144] ICL 2 and 3 are vital for the G protein interactions.[145] The probable disruption of the G protein signalling after the introduction of variant A238T (Class IV variant), would account for the lack of receptor signal seen in the present study (Figure 7). With the use of a heterologous expression system, a previous study supports and augments the present findings and shows that A238T reduces the capabilities of OXT binding (presumably because of reduced cell surface expression) and signalling.[123] Interestingly, a previous study examining this variant did not see any effect on OXT-induced signalling,[136] which is contrary to the findings of the present study. However, again, this may be a result of the more-sensitive Ca^{2+} signalling output being utilised in this previous study.

Variant P108A is the only variant to have showed a significant increase in the total and cell surface expression when compared to WT levels. Interestingly, a previous study has reported a similar increase in cell surface expression for this OXTR variant.[136] This residue is found in a highly conserved position of ECL 1, a region important for interacting with the linear C-terminal tripeptidic part of the OXT ligand.[143] Therefore it is tempting to predict that this substitution may affect hormone binding (Class III variant). Again, a previous study utilising Ca^{2+} accumulation as a signalling output reported no effect,[136,137] contrary to the findings of the present study. This previous study did however report effects of this variant on β-arrestin recruitment, OXTR desensitization, and OXTR internalization.[135] A possible explanation is that the variant may stabilise the expression of OXTR. The increase in cell surface expression

suggests some intrinsic instability due to intramolecular interactions that are not seen in the WT OXTR. However, the stabilisation of this variant results in very diminished receptor signal, which implies that this stable confirmation will interfere with either the binding, signalling transduction or G protein interaction of the OXTR.

4.3.5 The Functional Impacts (If Any) of Variants A63G, R150L, R150S, V172A, L206V, A217T, A218T, E242K, G252A, R376C and R376G Remain Unclear

The remaining psychological disorder-linked variants (A63G, A217T, E242K, R376C and R376G), birth-disorder linked variants (R150L, R150S, V172A and R206V) and variants linked to both disorder groups (A218T and G252A) were found to exhibit no significant decrease in receptor signalling when compared to the WT receptor (although variants R150S, R150L, A217T, R376C and R376G showed a partial decrease in receptor signalling, this was not significant). This suggests,[=- that they are not in fact pathogenic/cause loss-of-function. For variants L206V, A218T, V172A, G252A and R376G these findings are in agreement with a recently published study that also reported no effect on OXT-induced signalling elicited by these variants.[145] However, another study showed the elevation in calcium levels and IP_3 formation decreased in the cells expressing R376C and R376G tagged with enhanced green fluorescent protein (EGFP), in comparison with the cells expressing the WT OXTR tagged with EGFP,[127] which would suggest that there may be some effect on receptor function. In the present study, there was a small but not significant decrease in signalling response. It is also notable that examination of cell surface expression of these two variants (see Chapter 5) suggested a slight decrease so further studies on these variants to firmly confirm/refute their (non)functionality are required.

Variants R150L/S also had a slight but not significant decrease in signalling response that warrants further investigation in order to firmly conclude that these variants are functional. Particularly as residue R150 is also within a known polar pocket site for G protein coupling,[142] so has the potential to affect receptor signalling. The “polar pocket” residues are involved the conformational change of the receptor once the agonist has bound to the docking site, allowing for the unmasking of the G protein site

to allow receptor signalling.[16]

For the remaining variants, there has been no prior *in vitro* analysis. As no previous *in vitro* analysis has been published on several of these variants, functional inferences have been drawn from in silico analyses. Interestingly, for the majority of the variants the majority of the VEP tools predicted that the substitutions would be tolerated. The exceptions being variants A63G, R150L, R150S and V172A, for which the majority of programs predicted a deleterious effect (Table 8). For some variants, the substitutions are relatively conservative (A63G, V172A and G252A) such that it is perhaps not surprising that they are well tolerated. However, for others, there are larger differences in the properties of the WT and variant amino acids. For example, for variants R150L/S and R206V and R376C/G there are changes in size as well as loss of a positive charge, and for variant E242K, there is a change in charge from negative to positive. For variants A217T and A218T, the substitutions result in changes in polarity, hydrophobicity, and size. Thus, given that these substitutions are well tolerated these regions of the receptor may not be so important for function and/or conformation.

Perhaps surprisingly, several of these residues show strong conservation. For variants A63G, R150L/S, V172A and A217T the positions are highly conserved amongst the mammalian species while only partially conserved in the human neurohypophyseal receptors (Table 6 and Table 7). For variant L206V there is conservation within the human neurohypophyseal receptor family and for variants R376G/C this residue is conserved across both mammalian OXTR species and human neurohypophyseal receptors. However, there is little/no conservation for positions A218, E242 and G252. Also of note is that the human OXTR residues A217, A218 and G252 express the variant amino acid in at least one of the other OXTR mammalian species or other neurohypophyseal hormone receptor family member and, when looking at the whole rhodopsin-like GPCR family, for residues A63 and A218 the variant amino acid is found at that relative position in a similar proportion as the WT residue.

Although no effects on OXT-induced signalling were observed for these variant receptors when measuring IP accumulation, it is important to note that this does not mean that there will be no functional effects on other signalling outputs of aspects of receptor function. Indeed, although the human oxytocin receptor couples primarily to $G\alpha_q$ G proteins, coupling to $G\alpha_i$ G proteins has also been reported. OXTRs also interact with β-arrestins, which can mediate G protein-independent signalling events.[135] Thus, it would be important in future studies to examine the response of the various variants with respect to these other pathways.

Indeed, that this may be the case is supported by a study which showed that OXT-induced receptor internalization and recycling were faster in HEK-293 cells expressing R376G than in cells expressing R376C or the WT OXTR.[123139140 142124] The R376 residue is located in the C-terminal of the receptor which is critical for desensitisation, internalisation, and recycling of the OXTR.[145] R376 is linked with two structural components involved in these processes: firstly, this residue forms part of a PKC consensus motif (SHR), which is thought to interact with PKC after OXT stimulation.[141] Secondly, R376 is in conjunction with one of the two serine triplets in the C-terminus, which couple to the $G\alpha_q$ G protein and are primary sites of agonist-induced phosphorylation and β-arrestin-2 binding.[138] It has been shown that when variants are introduced at this site the stability of the β-arrestin-2-OXTR complex is altered, and the ability of β-arrestin-2 to internalise with the receptor was eliminated. Therefore, it is suggested that the R376C/G substitution in the OXTR might suppress the phosphorylation of the triplet motif or reduce the stability of β-arrestin binding to the triplets, resulting in faster receptor recycling. Thus, arrestin-mediated G protein-independent signalling by these variants may also be affected.

Variants V172A (located in TM4) and L206V (located in TM5) are situated next to the Q171 polar pocket site,[142] or within a known polar pocket site for G protein coupling.[142] respectively. Similarly, variants, E242K and G252A, are located on the ICL 3, which is known to be important for G protein coupling. Thus, these variants may be expected to interfere with receptor function, but this does not appear to be the case, at least with respect to OXT-induced $G\alpha_q$-mediated IP accumulation.

4.3.6 Other Noteworthy Observations and Limitations

Initially, it was observed that there was a significant decrease in cell number 48 hours post transfection with WT OXTR when compared to empty vector (Appendix 1D: Figure 39). This is very unusual as no previous research has expressed this observation. Transient transfections induce a stressful state in the cell and may have a direct impact on proliferation/apoptosis of the cell. It could be argued that the cells with high expression of these receptors would therefore undergo apoptosis as a protective measure and those remaining were not successfully transfected/poorly transfected, however this was disproven with high expression of WT receptor observed in both cell surface and total ELISA assays (Figure 9 and Figure 10). It is also unlikely that these cellular effects are simply due to the stress of trying to generate large amounts of exogenous protein, as, in this case, the effects would be expected to be similar for all the WT and variant receptors regardless of their functionality, which was not the case. Several cell viability assays were included to confirm that transfection did not lead to cell death. A simple experiment to determine the percentage of cell death in cells 24 hours post transfection (Figure 40 and Figure 41) showed that there was no significant decrease in living cell percentage when comparing empty vector transfected cells to the WT and variant OXTR transfected cells. It is therefore unlikely that the decrease in cell number may be as a result of apoptotic/cytotoxic effects but rather an anti-proliferative effect experienced by the transient transfection of WT OXTR and OXTR variants. Further investigation of the mechanisms behind this observation are of interest for future studies. Also, to prevent this complication in future studies examining OXTR variants *in vitro*, stable clones could be used for functional assays, an option which was not feasible at the time of this study. It is however interesting to note that the effects on cell number do not appear to be correlated with functionality (as measured by OXT-induced IP accumulation) of the variant receptors. Although many of the variants that elicited signalling responses not different from the WT receptor also had similar effects as the WT receptor on cell number, the exception was R376G which did not affect cell growth. Likewise, some variants (such as A63V, T273R and T273M) that were found to have reduced signalling response had a similar effect as WT receptor on cell number. Thus,

it may be that these effects on cell growth are mediated through another pathway/signalling response and further support the need for additional, more detailed examination of different signalling profiles/outputs for the variant receptors.[149,150]

When analysing the location of the variants for OXTR, it should be noted that majority of the variants (15/18) with impaired function of the receptor were located in the transmembrane domain of OXTR. When looking at certain clusters of functional impairment, we can see that variants located in TM 1, 3 and 4 as well as ICL 3 results in the severe disruption of OXTR signalling or binding (A63V, P108A, M133V, H173R and A238T). Variants which impact the folding of the OXTR are located on TM 5 and 6 (W203R and T273R), while the partial damage to the biosynthesis/degradation of the OXTR are linked to variants located on the C terminal (R376C and R376G). Lastly, the full disruption of OXTR function may be linked to variants located predominantly on TM 6 (I266V, T273M and V281M) as well as TM 1 (V45L) and TM 5 (G221S). The variants which resulted in inconclusive results with regards to receptor signalling and require further analysis include: A63G, R150L, R150S and A217T. The variants which showed no effect on the receptor function and therefore considered tolerated are: V172A, L206V, A218T, E242K and G252A. This valuable information provides a clearer understanding of the OXTR and its intricate pathways of binding, activation, and signalling.

5. The effect of small molecules on OXTR variant cell surface expression

5.1 Foreword

As discussed in Section 1.4.1, the folding of GPCR polypeptides is an intricate process and cellular quality control systems are in place to detect and deal with incorrectly folded proteins as the accumulation of misfolded proteins can result in aggregation and cell death. Cell surface expression is critical for the physiological function of most GPCRs, and misfolding and thus subsequent intracellular retention/degradation of the receptor protein can have severe impacts on hormone responses. As GPCRs are such important signalling molecules and are responsible for facilitating many physiological processes, their impaired signalling can have severe consequences. Indeed, misfolded GPCR variants have been implicated in several disorders including, but not limited to, retinitis pigmentosa, blindness, hypocortisolism, hypothyroidism, hypercalcaemia, diabetes insipidus, obesity and hypergonadotropisim. [146–151]

GPCRs are a major therapeutic target and, as a result, many licenced drugs target this family of signalling proteins. The majority of these drugs are agonists (which stimulate receptor activity) or antagonists (which block receptor activity), but both of these classes of therapeutics require receptor to be present with which to interact. Recently a novel class of therapeutics, PCs, have been identified, which can promote receptor trafficking to the cell surface. PCs are small molecules that can permeate the cell membrane, bind to a specific target protein within the ER and are believed to serve as a molecular scaffolding to support proper folding of the proteins. In the case of misfolded variant GPCRs this facilitates their evasion of the ER quality control systems and translocation into the secretory pathway and, thus, expression at the cell surface. In many cases, once trafficked to the cell surface, these ‘rescued’ receptors retain, at least some degree of, functionality and, therefore, their hormone response can also be ‘rescued’.[134]

The discovery of PC therapeutics is a relatively embryonic field, but, despite this, two PCs have already been approved by the U.S. Food and Drug administration, VX-809 and its analogue VX-661 (Lumacaftor and Tezacaftor respectively).[152] These compounds act to increase cell surface expression of a misfolded variant of the cystic fibrosis transmembrane conductance receptor (CFTR) that is one of the major causes of cystic fibrosis, and provide clinical benefit to patients harbouring this variant in terms of lung function.[153, 154] Although the CFTR is not a GPCR, the clinical development of these PCs provides a proof-of-principle for the therapeutic benefit of this class of compounds.[159]

Although no GPCR PCs have reached the clinic yet, several potential PCs have been identified and have yielded promising results in *in vitro* and, in some cases, in *in vivo* and in clinical studies. For example, PCs have been described to target misfolded variant LHRs implicated in reproductive dysfunction,[155] rhodopsin implicated in retinitis pigmentosa blindness,[156] gonadotropin-releasing hormone receptor,[157] follicle-stimulating hormone receptor,[158] and neurokinin 3 receptor.[159]

As discussed above (Section 1.3.1.1) OXT and its analogues are commonly administered clinically during labour. Numerous studies have also utilised OXT (and/or AVP) administration to provide valuable insight into the importance of the central OXT-AVP function to CNS function in humans (described in Section 1.3.1.2). However, OXT (and AVP), like most neuropeptides, are metabolically instable, have a short half-life and poor blood brain barrier penetration.[160] There is also considerable cross-activity of these peptides across all of the neurohypophysin hormone receptor members. This results in experimental limitations for the use of these peptides clinically and in animal and human models. Thus, there has been considerable effort made in the development of analogues with improved pharmacological properties. One area of interest in this respect is the development of “small molecule” (non-peptide) OXTR/AVPR-targeting compounds that aim to improve receptor selectivity, oral bioavailability, and brain penetration. Such orally active small molecules will be naturally cell permeant and are often designed to be selective for OXTR and/or specific AVPRs, and they have the potential to act as PCs for variant OXTR/AVPRs. Indeed,

several such OXTR/AVPR-targeting small-molecules have been shown to exhibit PC activity.

X-linked nephrogenic diabetes insipidus (NDI) is a well-studied condition caused by variants located in the AVPR2 gene. The most prevalent AVPR2 variants result in misfolding of receptors, retention in the ER and degradation (Class II). These receptors are unable to reach the plasma membrane to promote water reabsorption through the principal cells of the collecting ducts. The cell-permeable AVPR2 antagonists S 121463 and VPA 985 can act as PCs and stabilise ER-retained AVPR variants, allowing the receptors to be released from the ER and travel to the plasma membrane.[161,162] Other antagonists such as SR 49059 (AVPR1a antagonist with moderate affinity for AVPR2) have also been shown to act as PCs for AVPR2 variants. These *in vitro* observations have also been shown to translate to clinical efficacy as SR 49059 has been shown to have beneficial effects (in terms of decreased urine volume and water intake and increased urine osmolality)[163] when tested in patients harbouring Class II AVPR2 variants and therefore provides potential for development of future treatment options for X-linked NDI.[163165] Although there are many studies which have investigated the effects of pharmacological chaperones in rescuing AVPR2 loss-of function variants, no studies have investigated the effect of these PCs in variants found in AVPR1a and AVPR1b.

A recent study analysed several OXTR small-molecule ligands with the aim of identifying potential PCs that achieve an increase in OXT variant signalling responses. This study focused on the rescue of an OXTR variant (V281M) commonly found in the Swedish population,[164] and which has previously been shown to impair OXTR trafficking to the cell surface and thus significantly decrease cellular response to OXT (a Class II variant). [136]

Nine different compounds were screened for effects on OXTR cell surface expression (namely: TCOT 39, OPC 41061, WAY 26746, TASP 0390325, YM 087, OPC 21268, SSR 149415, SR 49059 and L 371 257). The results indicated that two OXTR/AVPR antagonists, SR 49059 and L 371 257, act as PCs for both variant and WT OXTR.

These PCs restored OXTR trafficking and oxytocin response in HEK 293T cells transfected with V281M OXTR and also mobilised endogenous WT OXTR to the cell surface in a translation-dependent manner. These compounds were also able to increase response to OXT in hTERT-HM cells and primary human myometrial cells from five individuals.[136]

Several of the OXTR variants examined in the present study (Chapter 4) with reduced functionality/signalling showed a reduction in cell surface expression (A63V, A238T, T273R, T273M, V45L, M133V, H173R, W203R, G221S, I266V and V281M) (Figure 9). For the majority of these variants, there was little/no parallel reduction in total cellular expression (A63V, M133V and I266V) (Figure 10), suggesting that the decreased cell surface expression is due to reduced cell surface trafficking. For the remaining variants, (V45L, P108A, H173R, W203R, G221S, A238T, T273R, T273M and V281M) a reduction in both cell surface and total cellular expression was observed (Figure 10). However, the decrease in total cellular expression was substantially smaller than the decrease in cell surface expression for variants T273R, T273M, W203R, G221S and V281M suggesting that, although reduced receptor biosynthesis/degradation may be contributing to the decreased cell surface expression of these variants, reduced trafficking also likely plays a role. For variants V45L, H173R and V281M, there are similar reductions in both cell surface and total expression so the role of potential deficits in receptor trafficking are less clear.

As the previous study examining potential OXTR PCs only examined one variant receptor, the ability of L 371 257 (the compound that showed the greatest rescue of OXTR signalling [136]) to act as a PC and restore cell surface expression of two other psychological disorder-linked variant OXTRs identified to have impaired trafficking in this study was also examined.

The small molecule, L 371 257, (Figure 11) is a potent and selective piperidino-benzoxazinone OXTR antagonist (Ki = 4.6 nM at human OXTR) that displays > 800-fold selectivity over human AVPR1a. [165] However, there is no information available regarding its selectivity over human AVPR1b.[168] Studies have shown this compound to antagonise OXT-induced contractions in isolated rat uterine tissue and in

anesthetised rats following intravenous and intraduodenal administration.[95] More recently, L 371 257 has been shown to act on central synapses to reduce chronic-pain-induced anxiety in a stress-induced mouse model indicating its central bioavailability.[96]

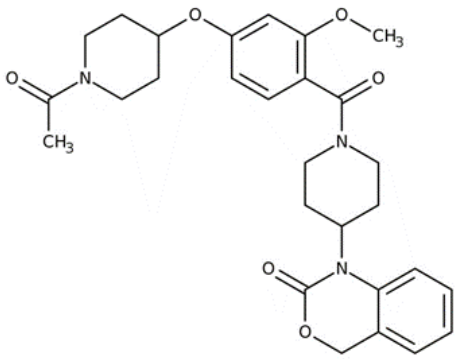


Figure 11: Structural image of the small molecule OXTR antagonist L 371 257

One potential downside of L 371 257 as a PC is its antagonist properties, which mean that it will compete with endogenous ligand for receptor activation and would therefore need to be removed following any receptor rescue to permit hormone activation. Therefore, the effects of another OXTR small-molecule compound, WAY 267 464, an OXTR agonist were also examined. WAY 267 464 (Figure 12) is one of the first generation of non-peptide agonists developed to target the OXTR. It is reported to be a potent, selective non-peptide OXTR agonist (K_i = 58.4 nM at the human OXTR). [166] It is also reported to exhibit anxiolytic-like effects and therapeutic potential for the treatment of psychological symptoms, again, conforming its efficacy at central sites.[166] The receptor pharmacology profile of WAY 267 464 in cell-based assays has revealed it to be a highly selective OXTR agonist that actually displays antagonist activity at AVPR1a and AVPR1b.[167–169,170] This compound has shown to increase pro-social behaviour and decrease anxiety in rodents and could therefore have the potential for treating psychological disorders.[169] In a four-plate test rodent model, WAY 267 464 has shown to dose-dependently reduce anxiety and prevent deficits in prepulse inhibition induced by amphetamine.[168] However, the compound did not affect immobility in the

ail suspension test (a rodent model of antidepressant action).[168, 170]

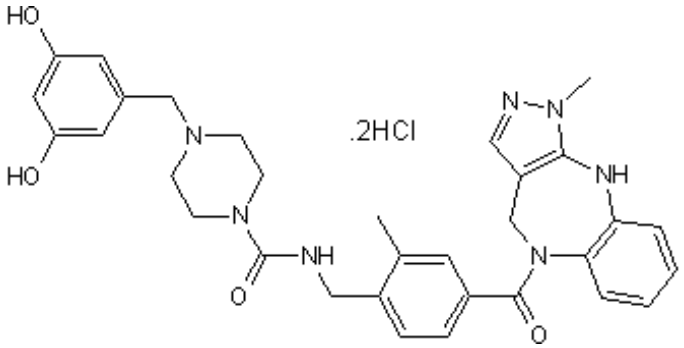


Figure 12: Structural image of small molecule OXTR agonist WAY 267 464

Although WAY 267 464 did not show PC activity with variant V281M in the previous study,[136] a PC's ability to rescue the trafficking of a receptor may depend on the location of the variant and it is therefore worthwhile to test different molecules for different variants and for this reason, the PC activity of this compound was also examined.

5.2 Results

As a preliminary proof-of-principle, two of the psychological disorder linked OXTR variants identified to have most severely impaired cell surface trafficking, T273R and T273M were used to examine the PC activity of L 371 257. HEK 293T cells were transfected with either the empty vector, wild type OXTR or variant OXTRs and were treated with 10 µM L 371 257 (the concentration identified as effective in the previous study[136]) or vehicle (0.1% DMSO) for 24 hours before measurement of receptor cell surface expression by receptor ELISA (Figure 13A). For comparison, two variants that showed no disruption of OXTR signalling were also included (R376C and R376G). A significant increase for T273R and T273M (from 16 to 64% and 13 to 51% of WT, respectively) in cell surface expression of both variant receptors ($p<0.05$, Student's t-test) was observed, while there was no effect observed on WT OXTR expression or expression of the 'functional' variants R376C and R376G). No effects were observed for the WT or any of the variant receptors upon treatment with 1 µM WAY 267 464 (Figure 13B).

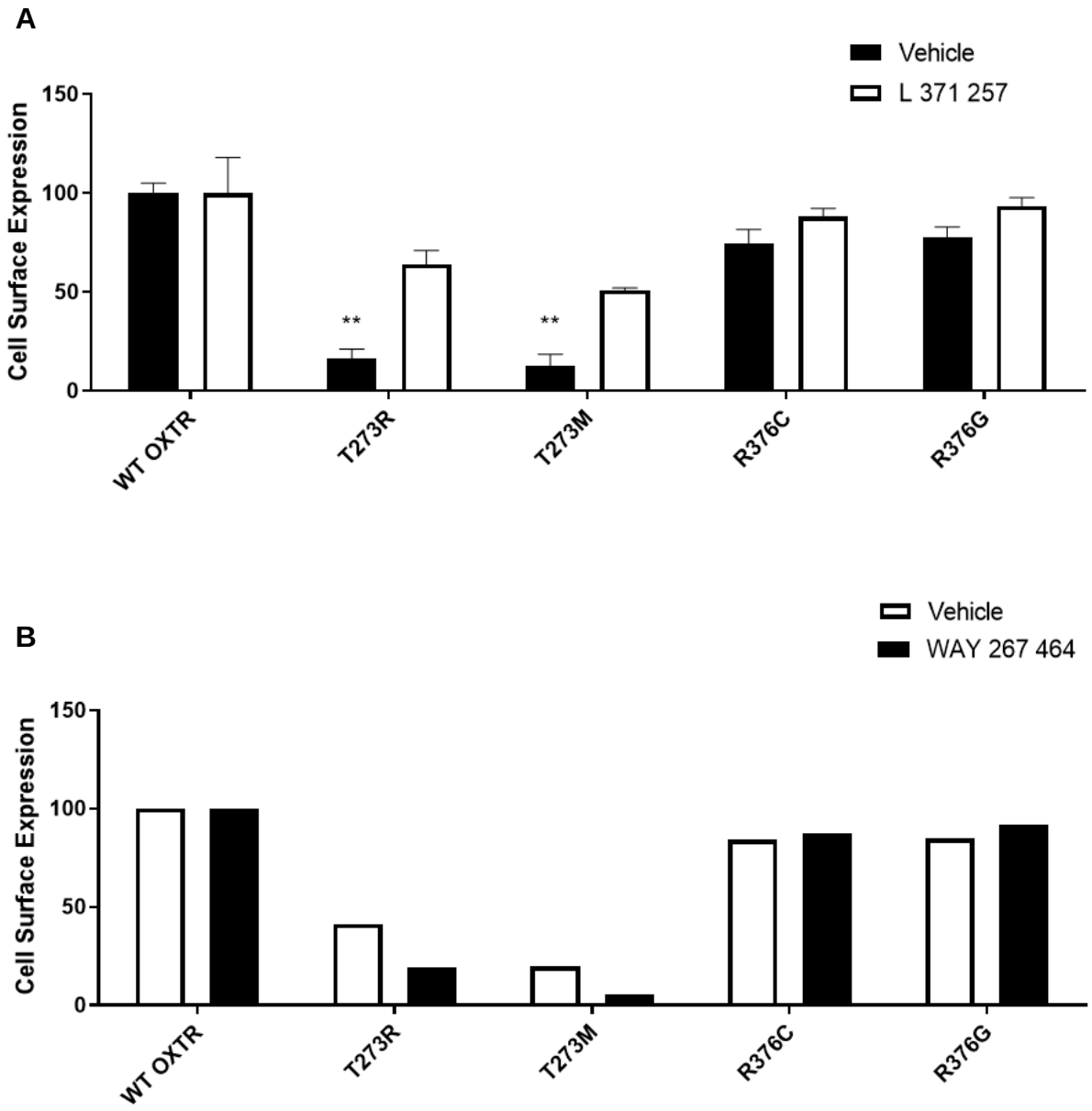


Figure 13: Effects of L 371 257 and WAY 267 464 on cell surface expression of OXTR variants with reduced cell surface expression

*Cell surface expression was measured, by receptor ELISA in intact cells, of wild type and variant OXTRs transiently expressed in HEK 239 T cells treated in the presence of **A** 10 µM L 371 257 or **B** 1 µM WAY 267 464 for 24 hours (black bars) or vehicle (white bars) for 24 hours. Data are presented as **A** mean ± SEM from three independent experiments (N=3) or **B** as mean from a single experiment (N=1) and have been calculated as the percentage of the average WT OXTR expression in the absence of treatment following subtraction of background signal (measured in cells transfected with empty vector). **p<0.01, Student's t-test for comparison between treated and untreated cells.*

As no effect on receptor cell surface expression was observed with WAY 267 464, to confirm that it was actually functional and able to interact with the OXTR, WAY 267 464 stimulation of OXTR signalling was determined. A WAY 267 464 signalling dose response was undertaken using the WT OXTR. Although it was not a robust response, a signalling response was measured, and the potency of WAY 267 464 was determined to be 7.9 nM (Figure 14). Thus, despite PC activity usually requiring a dose of at least 100-fold higher than the potency observed in signalling assays, the concentration employed in the receptor ELISA assay (1 µM) should be sufficient to observe a response if there was one.[167,168]

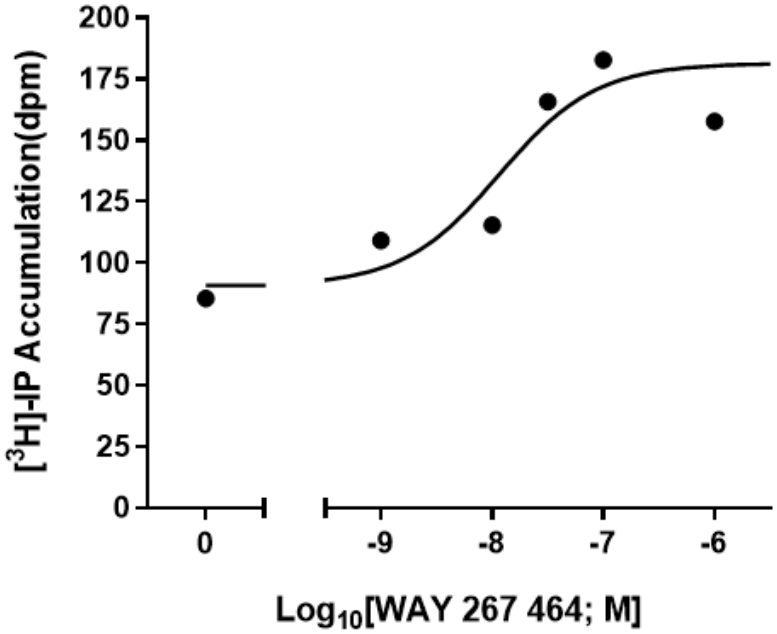


Figure 14: Dose-response analysis of WT OXTR signalling in response to WAY 267 464 stimulation

Signalling response in HEK 293T cells transiently transfected with WT variant OXTRs was measured by radiolabelled inositol phosphate (IP) accumulation assay following stimulation of vehicle (0) or a range of WAY 267 464 concentrations. Data presented are mean from a single experiment (N=1). Data have been fitted to a sigmoidal dose response curve from which the potency of WAY 267 464 was calculated to be 7.9 nM

5.3 Discussion

The present study utilised the nonpeptide OXTR agonist L 371 257 and antagonist WAY 267 464 to determine whether OXTR variants with impaired trafficking may be rescued to the cell membrane. This was an attempt to further characterise the apparent differences in these two compounds seen in previous pharmacological work and expand the number of variants for which PC activity of these compounds has been examined.[167] The novelty of this work is the attempt to rescue OXTR variants T273R, T273M, R376C and R376G which have previously been linked in genetic association studies to specific psychological disorders such as ASD as previous studies have only examined one variant (V281M) linked to birth disorders.[96,128]

The compound L 371 257 showed promising results in rescuing the trafficking of variants T273R and T273M, increasing their receptor expression by more than 30% when compared to the control (vehicle-treated cells). There was also a slight, but not significant, improvement in the cell surface expression of variants R376C and R376G (Figure 13).

Molecular modelling has revealed that residue A318 is a candidate discriminator required for high affinity binding and provides a direct hydrophobic contact with the methoxy group of compound L 371 257.[171]Another molecular docking and 3D structure model determined that F284 and F133 are the hydrophobic residues used in the receptor ligand complex when L 371 257 binds to OXTR.[172] It is therefore possible that T273 is located deep enough in TM 6 that it does not interfere with the docking of L 371 257 and thus is able to successfully assist in the localization of the receptor to the surface of the cell membrane. However, molecular modelling/docking or structural studies would be required to confirm this. It is important to note that the non-functionality of both rescued variants (as well as V281M) appeared to largely be due to their decreased cell surface expression (although variant T273R did also display some evidence of hormone binding/receptor signalling deficiency as well) (see Chapter 4). Thus, rescuing the cell surface of these variants (certainly T273M) could also potentially rescue their functionality (hormone responsiveness) as was seen with

variant V281M.[167] However, further functional studies examining variant functionality following PC rescue would be required to confirm this.

The downfall of L 371 257 is that it is unable to penetrate the blood brain barrier and would therefore be ineffective in the treatment of psychological disorder linked OXTR rescue.[173] Nevertheless, these results provide meaningful contribution to the effectiveness of this PC and potential development of a small molecules which may aid in the treatment of psychological disorders. [167]

Unlike L 371 257, treatment with WAY 267 464 had no effect on cell surface expression of any of the variants (Figure 13). The first point of concern would be the potency/activity of this compound. However, it was shown to be able to activate the WT OXTR in functional assays indicating its activity.[175] A recent study tested WAY 267 464 and its ability to rescue receptor expression of V281M and also saw no effect for his variant.[167] Therefore, it appears that this compound is not acting as a PC, at least for the small selection of variants that have been examined. It should be noted previous pharmacological experiments indicate that WAY 267 464 is a full agonist (with weak affinity) of the OXTR and a possible antagonist at the AVPR1a, which may make this PC unfavourable for the treatment of psychological linked disorders in any case.[169]

It has been found previously that variant location in relation to compound binding interactions plays a large role in the effectiveness of PC compounds.[155,158] This is postulated to be because either compound binding is affected by the presence of the variants, or they are located too far from the site of the binding interactions/in different helices not involved in compound binding so that the compound interactions are not able to stabilise the disruptions caused by the variants. Thus, a possible explanation for the absence of rescue seen in variants T273R and T273M is that WAY 267 464 binds with seven key residues located in TM 2, 3, 5 and 6, to successfully interact with the OXTR. The most relevant residue for this study is Q295, due to its close proximity to the variants T273R/M. Using molecular docking simulations, the Q295 residue is shown to interact with the central carboxyl oxygen (the one closest to the tail group,

see Figure 12) of WAY 267 464 and with a water molecule packed within this region as well.[174] This interaction mainly consists of water bridges and hydrogen bonding. It may be hypothesized that the change in molecular charge in T273R and polarity in T273M may interfere with the docking of WAY 267 464 to the TM 6 of the receptor. Variants R376C and R376G are located on the C terminal of the receptor which is not involved in docking of either compound and should therefore cause no interference to the binding of the compound but may be too distal from the binding site that any disruptions they cause cannot be stabilised. [172]

Future experiments should investigate the PC effects of L 371 257 (and other small-molecule OXTR selective agents) on the whole range of variants identified as having loss-of-function due to reduced expression/cell surface localisation. Any successful PC compounds identified *in vitro* could then form the basis of future drug development programs aimed at improving the pharmacological characteristics of these agents and their PC activity with the aim of developing agents that may be able to be used to improve oxytocin sensitivity in patients predicted to have reduced oxytocin responses due to genetic factors.

6. Arginine Vasopressin Receptor Variants

6.1 Foreword

Social behaviour is a crucial facet in the psychological network which constitutes human behaviour. Population studies have identified more than 50 variants in the *AVP* gene which have been linked to the pathogenic diabetes insipidus state.[98] This negatively impacts the functioning of AVP-AVPR2 signalling, while no research has yet determined the effects (if any) on AVP-AVPR1a/b responses. As mentioned previously (Section 1.5) many studies have linked variants in promotor and intronic regions of AVPR1a/b to symptoms experienced in psychological disorders. Genetic variants within the gene encoding AVPR1a (*AVPR1A*, located on human chromosome 12q14-15) have also been linked to cognitive/behavioural traits. Again, several of these are associated with the non-coding regions of the gene. For example, studies have linked variants exhibiting different lengths of varying sequence repeats found in the promoter region of the gene (GT_{25}, RS1 ($[GATA]_{14}$) and RS3 ($[CT]_4TT[CT]_8[GT]_{24}$)) to behavioural traits, suggesting that they are relevant for brain functions related to emotional arousal and social behaviour. Individuals with shorter RS3 showed less altruistic behaviour while longer RS3 alleles were associated with greater levels of prepulse inhibition to a startle response (a neurological phenomenon where a weak stimulus (prepulse) can suppress the startle response to a subsequent stronger startle stimulus (pulse).[40] A population study has also indicated an association between several intronic AVPR1a SNPs and severe long-term heroin addiction in African Americans.[99]

Two variants located in the coding region have also been linked to pair bonding and social behaviour. A long-term study of a random sample of 10,317 men and women who graduated from Wisconsin high schools in 1957 (Wisconsin Longitudinal Study - WLS) provided an opportunity to study the life course, intergenerational transfers and relationships, family functioning, physical and mental health and well-being, and morbidity and mortality from late adolescence through 2011. From the list of SNPs identified in this study, variant G6S was linked to impulsivity, ASD, and social

behaviour.[175] In a behavioural study which looked at AVPR1a and SLC684 polymorphisms in a sample size of 523 participants,[176] the common tandem repeats RS1 and RS3 were detected in the promoter region. However, a variant in the coding region (F136L) was also detected which may influence the social behaviour experienced by humans.

There is also mounting evidence with regards to the involvement of AVP in social behaviour via AVPR1b in both rodents and humans. Studies that have employed genetic knockouts or pharmacological manipulation of the AVPR1b point to the importance of central AVPR1b in the modulation of social behaviour. However, there continues to be a knowledge gap in our understanding of where in the brain this is occurring, as well as how and if the central actions of AVP acting via the AVPR1b interact with the stress axis, which ultimately induce social cues. Genetic association studies in Hungarian children (N=141) have shown a significant relationship between variant K65N and aggressive behaviour towards teachers.[177] A Ukraine genetic association study found variants G191R and G191S in families with offspring that have made suicide attempts, linked to mood and anxiety outcomes (depressive symptoms) and in suicidal behaviour.[178] Another study which genotyped 186 German subjects diagnosed with anxiety or panic disorder discovered variants R364H and R364L which may alter the susceptibility to anxiety disorder in human behaviour. It is important to understand the functional effect of these variants to aid the development of potential future therapeutics for these psychological disorders.

Although the AVP receptors are expressed both centrally and peripherally, (see Section 1.3.2), it should be noted that variants found in AVPR1a and AVPR1b have been associated with only psychological traits and disorders. This emphasises the need to look at potential functioning of these gene variants in the neurobiological processes of normal physiology and pathophysiological states.

6.2 Results

6.2.1 Identification and *in silico* analysis of AVPR variants

Potential psychological disease-causing variants of the AVPR1a/b were identified via extensive literature and database research (as described in Section 3.2.1). Following the inclusion criteria, 2 missense variants (G6S and F136L) were selected for AVPR1a, and 5 missense variants were selected for AVPR1b (K65N, G191R, G191S, R364H and R364L) (shown in Table 10). The structural position of the variants in AVPR1a and AVPR1b can be seen in Figure 15 and Figure 16 respectively.

Table 10: Variants Located in AVPR1a/b linked to Psychological Disorders

Receptor	Variant	rs number	Location	Disease Phenotype and Reference
AVPR1a	G6S	rs2228154	N-term	ASD and Social Behaviour[179,180]
	F136L	rs1042615	TM 3	Social Behaviour[176]
AVPR1b	K65N	rs35369693	ICL 1	ASD[181] and Aggression[177]
	G191R	rs33990840	ECL2	Depression[178]
	G191S	rs33990840	ECL2	Depression[178]
	R364H	rs28632197	C-term	Panic Disorder[182]
	R364L		C-term	Panic Disorder[182]

Table 10 summarises the mutation code which represents the original amino acid, the amino acid site in the receptor, and the new amino acid identified as a result of a SNP. The rs number is a locus accession for a mutation type assigned by dbSNP for the NCBI database, it must be noted that some variants do not have an assigned rs number. The location indicates where the mutation is located with regards to the GPCR structure.

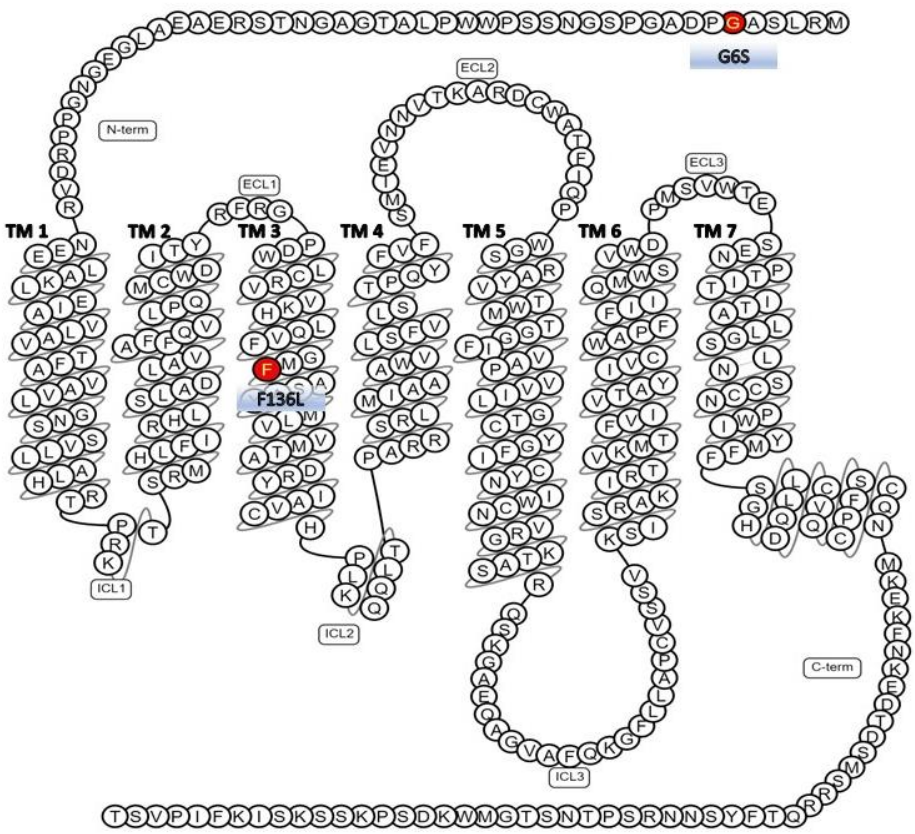


Figure 15: Two-dimensional schematic of the arginine vasopressin receptor 1a (AVPR1a)

The AVPR1a is a G protein-coupled receptor (GPCR) of the rhodopsin-like family. GPCRs are cell surface receptors characterised by a structure comprising of a single polypeptide chain of amino acids (labelled using standard single letter codes), with seven-hydrophobic transmembrane alpha helical domains (TM1-7), connected by three intracellular loops (ICL1-3) and three extracellular loops (ECL1-3). This structure is flanked by an extracellular N-terminus (N-term) and an intracellular C- terminus (C-term). Nucleotide positions (G6S) selected for this project are indicated in red. Created using free online software provided by the GPCR database.

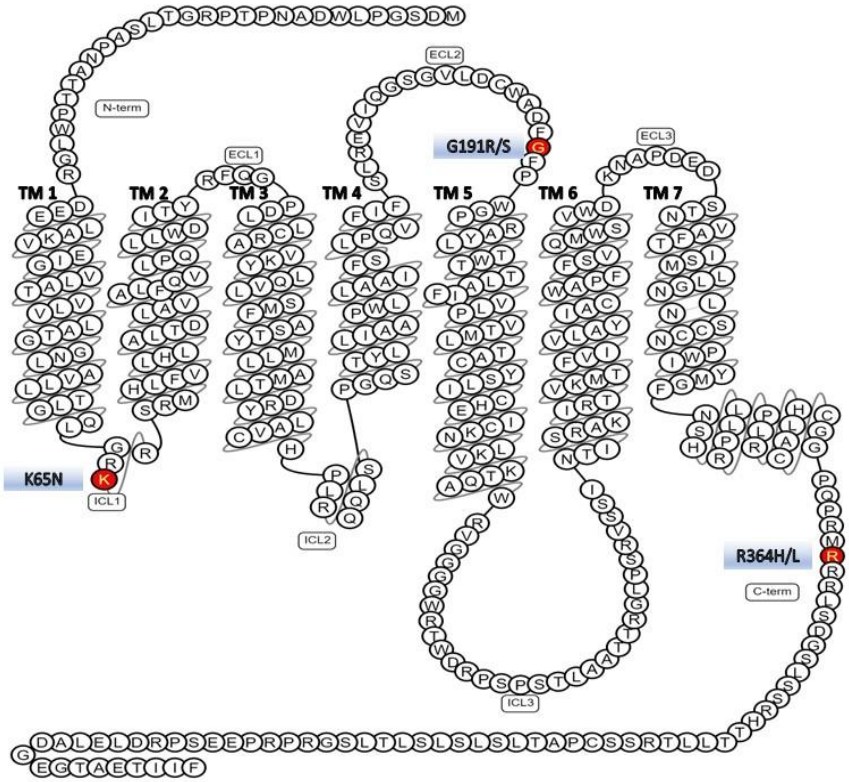


Figure 16: Two-dimensional schematic of the arginine vasopressin receptor 1b (AVPR1b)

The AVPR1b is a G protein-coupled receptor (GPCR) of the rhodopsin-like family. GPCRs are cell surface receptors characterised by a structure comprising of a single polypeptide chain of amino acids (labelled using standard single letter codes), with seven-hydrophobic transmembrane alpha helical domains (TM1-7), connected by three intracellular loops (ICL1-3) and three extracellular loops (ECL1-3). This structure is flanked by an extracellular N-terminus (N-term) and an intracellular C- terminus (C-term). Nucleotide positions (K65, G191 and R364) selected for this project are indicated in red. Created using free online software provided by the GPCR database.

As a simple initial means to infer the likelihood of variants affecting receptor functionality, as described in Section 3.2.2.1, the properties of the WT and variant amino acids were analysed and compared (Appendix 1C). For the AVPR1a variants, the change at residue 6 (G6S) shows a change from an aliphatic residue to a neutral residue with a large increase in molar mass, while the F136L shows a slight decrease in molar mass however this is probably not significant. Therefore, 1/2 variants in AVPR1a experience a significant change in size. For AVPR1b variants, 3/5 variants

(K65N, R364H and R364L) experience a change in the original hydrophilic state as well as a change in size which may be of significance for K65N and R364L, although, for R364H this is probably not significant. The 2 remaining variants (G191R and G191S) showed a change in polarity as well as an increase in size. Therefore, 4/5 variants in AVPR1b experience a significant change in either size, charge, polarity, or hydrophobicity which may have a negative impact on the conformational and functional legitimacy of their receptor.

The comparison of residues amongst recently diverged species and protein families also predicts the implication of a specific residue in receptor structure/functionality. The analysis of the conservation of residues following sequence alignment in AVPR1a/b among mammalian species was first performed (Appendix 1C), Figure 35 and Figure 38; summarised in Table 11) where it was observed that both AVPR1a residues and only 1/3 of the AVPR1b residues (K65) are considered highly conserved. Interestingly, human AVPR1b residues for G191 and R364 are shown to be very different in comparison the other mammalian species. The conservation amongst the human neurohypophoseal receptors showed F136 of AVPR1a and K65 of AVPR1b also to be highly conserved ((Appendix 1C), Figure 38, summarised in Table 13 and Table 14).

Table 11: Summary of residue conservation across AVPR1a from different species

Amino Acid Position (Human)	**Mammalian Species**						
	Human	**Sheep**	**Cattle**	**Dog**	**Rhesus Monkey**	**Norway Rat**	**House Mouse**
6	G	S	G	G	G	G	G
136	F	F	F	F	F	F	F

The amino acid sequence of human AVPR1a was aligned with other mammalian species (sheep, cattle, dog, Rhesus monkey, Norway rat, and house mouse) to determine conservation of the residues of interest. The colours indicate the degree of conservation (red: very different, yellow: different, green: similar; white: conserved, based on Gonnet PAM 250 matrix).

Table 12: Summary of residue conservation across AVPR1b from different species

Amino Acid Position (Human)	Mammalian Species						
	Human	Sheep	Cattle	Dog	Rhesus Monkey	Norway Rat	House Mouse
65	K	K	K	K	K	K	K
191	G	R	R	R	R	Y	Y
364	R	R	R	-	C	H	H

The amino acid sequence of human AVPR1b was aligned with other mammalian species (sheep, cattle, dog, Rhesus monkey, Norway rat, and house mouse) to determine conservation of the residues of interest. The colours indicate the degree of conservation (red: very different, yellow: different, green: similar; white: conserved, based on Gonnet PAM 250 matrix).

Table 13: Summary of AVPR1a residue conservation among the neurohypophyseal hormone receptor family

Amino Acid Position (AVPR1a)	Human Gene			
	AVPR1a	OXTR	AVPR1b	AVPR2
6	**G**	-	-	T
136	**F**	F	F	Y

The amino acid sequence of human OXTR, AVPR1a, AVPR1b and AVPR2 were aligned to determine conservation of the residues of interest. The colours indicate the degree of conservation (red: very different, yellow: different, green: similar; white: conserved, based on Gonnet PAM 250 matrix). - indicates that no corresponding residue is present in that sequence.

Table 14: Summary of AVPR1b residue conservation among the neurohypophyseal hormone receptor family

Amino Acid Position	Human Gene			
	AVPR1b	OXTR	AVPR1a	AVPR2
65	**K**	K	K	R
191	**G**	I	I	A
364	**R**	R	N	S

The amino acid sequence of human OXTR, AVPR1a, AVPR1b and AVPR2 were aligned to determine conservation of the residues of interest. The colours indicate the degree of conservation (red: very different, yellow: different, green: similar; white: conserved, based on Gonnet PAM 250 matrix). - indicates that no corresponding residue is present in that sequence.

To further examine residue conservation at the different positions, the Ballesteros-Weinstein GPCR numbering system was utilised to categorise the TM-located residues so that a GMoS conservation tool could be employed to examine the amino acid conservation at the corresponding position in all Rhodopsin-family GPCRs. This analysis ((Appendix 1C); summarised in Table 8) only includes residues located in the transmembrane domain, namely F136L (3.37) of AVPR1a, since the remaining variants are located on terminal tails or intra/extra cellular loops. This analysis indicated that it is not a particularly conserved site.

The residue conservation analyses were followed by use of a suite of variant effect prediction programs (Appendix C, summarised in Table 15). These online tools predict whether the variants would result in a deleterious effect on protein function using a variety of different algorithms (see Section 3.2.2.3). Interestingly, only the variants located on TM 3 and ICL 1 of AVPR1a and AVPR1b, respectively, were predicted to be deleterious (F136L and K65N). However, it should be noted that when running the AVPR1b variants through the prediction programs it was found that only SIFT, Polyphen, FATHMM and Provean could provide a prediction score. The reason for this is that the AVPR1b sequence does not meet the requirements for the nonsynonymous mutation to be scored in the excluded programs and could consequently not be recorded. Why this is the case is not clear and further investigation was outside the scope of the present study.

Table 15: GMOS and In silico analysis summary for AVPR variants

Receptor	Mutation	Location	Ballesteros-Weinstein number	Conserved residue? (WT amino acid present in ≥ 25% GPCRs)	Variant residues present in < 10% GPCRs?	Number of different residue variants located at that position in all GPCRs	Number of prediction programs with deleterious outcome
AVPR1a	G6S	N-term	-	No information from GMOS as the mutation is found in the N terminal			0/7
	F136L	TM 3	3.37	No	No	18	6/7
AVPR1b	K65N	ICL 1	-	No information from GMOS as the mutation is found in the ICL 2			3/4
	G191R	ECL2	-	No information from GMOS as the mutation is found in the ECL 2			0/4
	G191S	ECL2	-	No information from GMOS as the mutation is found in the ECL 2			0/4
	R364H	C-term	-	No information from GMOS as the mutation is found in the C terminal			0/4
	R364L	C-term	-	No information from GMOS as the mutation is found in the C terminal			0/4

A table showing the different AVPR1a/b variants and their specific location and Ballasteros-Weinstein number. A residue conservation analysis tool (GMOS) could only predict the conservation of the residue if located in the transmembrane of the receptor. Therefore, ND: Not Defined indicates residues located in the intra- or extracellular loops (ICL or ECL), or the beginning or end of the transmembrane domain (TM). The mutations were analysed using a series of mutation effect prediction programs (SIFT, Polyphen, FATHMM, LRT, Mut. Assessor, Mut. Tester and PROVEAN) with the score representing how many programs predict the variant to be deleterious

6.2.2 *In vitro* analysis of AVPR variants

6.2.2.1 Variant effects on cell growth

Similar to the OXTR (Chapter 4), it was observed that there was a difference in cell growth of the of HEK 293T cells 48-hours post-transfection with empty vector when

compared to WT AVPR1a/b, with cells transfected with the WT AVPR appearing to be fewer in number. It was therefore postulated that cells transfected with the different variant AVPRs may exhibit different rates of growth depending on the activity of the variant receptor. If this was the case, it would be important for two reasons. Firstly, this may infer information about variant functionality and, secondly, if differences in cell number were observed then it would be important to account for this in downstream *in vitro* activity/expression analysis, such that data generated could be normalised to viable cell number. In order to quantify cell number, crystal violet staining was also performed where the amount of crystal violet staining infers the number of viable cells (Figure 17 and Figure 18). It was found that compared to the empty vector (control), the WT AVPR1a transfected cells had only 49% of the control cell number, while cells transfected with both the AVPR1a variants also showed a similar significant decrease in cell number (G6S: 60% and F136L: 58%), where $p<0.05$, one-way ANOVA followed by Dunnett's post-test.

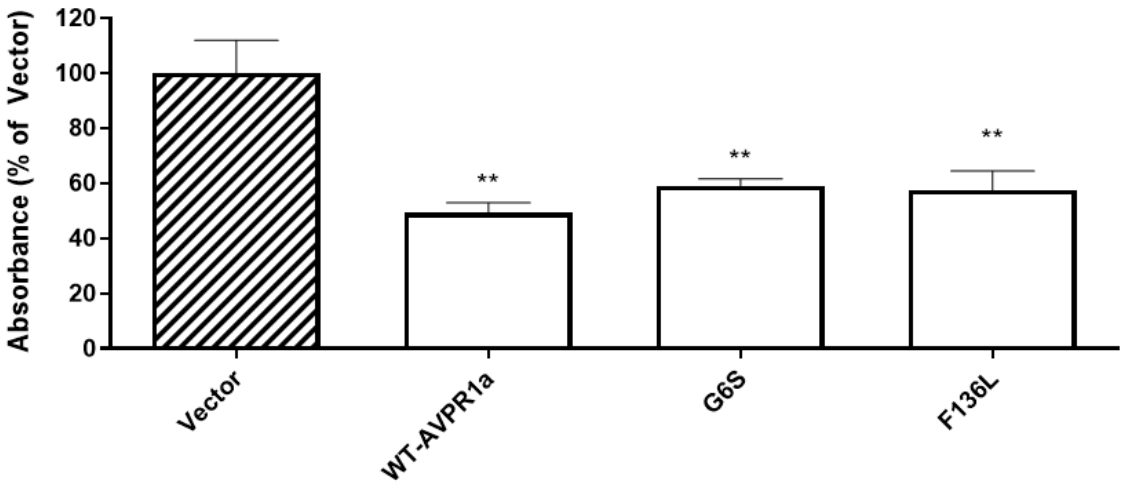


Figure 17: Crystal violet staining of HEK 293T cells transfected with empty vector, WT AVPR1a or AVPR1a variants

Cell number was quantified by crystal violet staining following transfection of WT AVPR1a or AVPR1a variants. Cells were fixed and stained 48 hours post transfection HEK 293T cells transiently transfected with empty vector were used as a control. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as % of the average crystal violet staining observed in

*cells transfected with vector only. **p<0.01, 1-way ANOVA followed by Dunnett's multiple comparison post hoc test.*

Similarly, compared to the empty vector transfected cells (Figure 18), it was found that WT AVPR1b transfected cells had only 53% of the control cell number 48 hours after transfection. It was found that 2/5 AVPR1b variants also resulted in a significant decrease in cell number when compared to empty vector transfected cells to levels not different to that seen with WT AVPR1b transfected cells (K65N: 58% and G191S: 60%). However, when transfected with variants G191R, R364H and R364L, there was no significant decrease in cell number compared to the empty vector transfected cells.

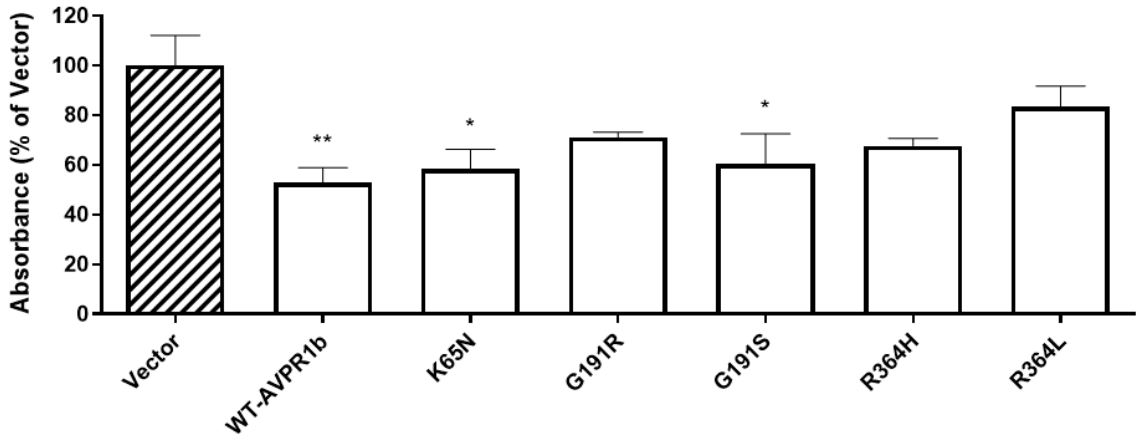


Figure 18: Crystal violet staining of HEK 293T cells transfected with empty vector, WT AVPR1b or AVPR1b variants

*Cell number was quantified by crystal violet staining following transfection of WT AVPR1b or AVPR1b variants. Cells were fixed and stained 48 hours post transfection. HEK 293T cells transiently transfected with empty vector were used as a control. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as % of the average crystal violet staining observed in cells transfected with vector. **p<0.01 and *p<0.05, 1-way ANOVA followed by Dunnett's multiple comparison post hoc test.*

To determine whether the decreased growth rate observed was as a result of cytotoxicity/cell death, a trypan blue cell viability assay was performed (described in 3.2.5.3) to quantify the ratio of living cells to dead cells 24 hours after transfection

(Appendix 1E). It was found that none of the AVPR variants showed a significant decrease in living cell percentage when compared to their respective WT receptor, where $p>0.05$, one-way ANOVA followed by Dunnett's post-test.

6.2.2.2 Variant effects on AVPR signalling

Before signalling competence of the variant receptors was examined, the ideal concentration of AVP ligand to be used when testing the signalling capabilities of the AVPR variants was first determined. The AVPR1a/b signals via coupling to $G\alpha_q$ G proteins, which, in turn, activate the enzyme PLC that catalyses the conversion of membrane lipids to produce IPs and DAG. Thus, to measure the dose-dependent signalling of the AVPR1a/b, cells transfected with WT AVPR1a/b were grown in media containing [^{3}H]-myoinositol such that any generated IPs would also be radioactively labelled. Cells were then stimulated with AVP before capture and measurement of the generated radiolabelled IPs. Data were then normalised to account for differences in cell number between the different transfected cells (see Section 6.2.2.1). Dose-response analyses using cells expressing the WT AVPR1a or AVPR1b demonstrated that AVP stimulated IP production in these cells with a potency (EC_{50}) of 0.5 nM (pEC50, 9.34 ± 0.03) for WT AVPR1a (Figure 19) and a potency (EC_{50}) of 3.7 nM (pEC50, 8.43 ± 0.14) for WT AVPR1b (Figure 20).

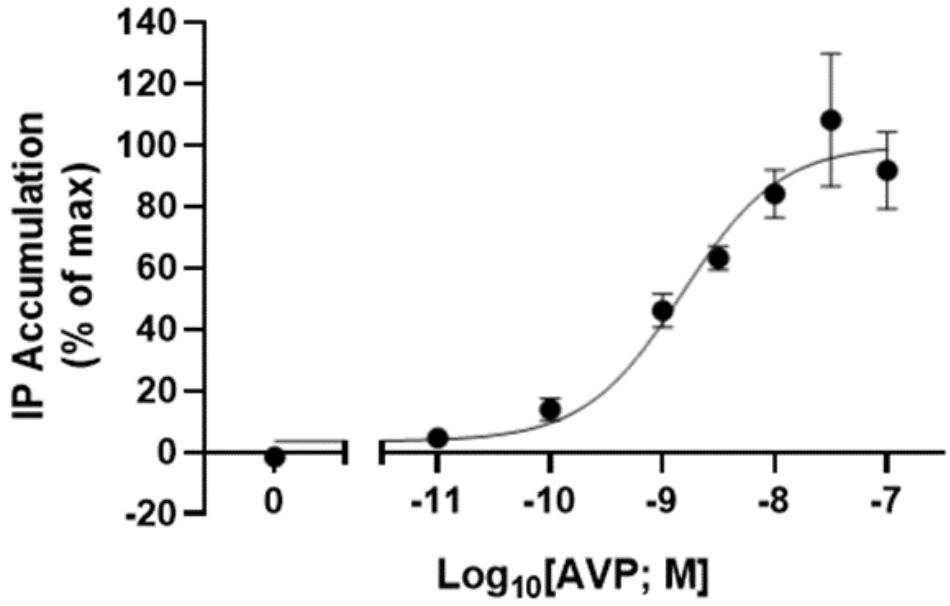


Figure 19: Dose-response analysis of WT AVPR1a signalling in response to AVP stimulation

Signalling response in HEK 293T cells transiently transfected with WT AVPR1a was measured by radiolabelled inositol phosphate (IP) accumulation assay following stimulation of vehicle (0) or a range of AVP concentrations. Data presented are from three independent experiments (N=3). Data have been fitted to a sigmoidal dose response curve from which the potency (EC_{50}) of oxytocin was calculated to be 0.5 nM.

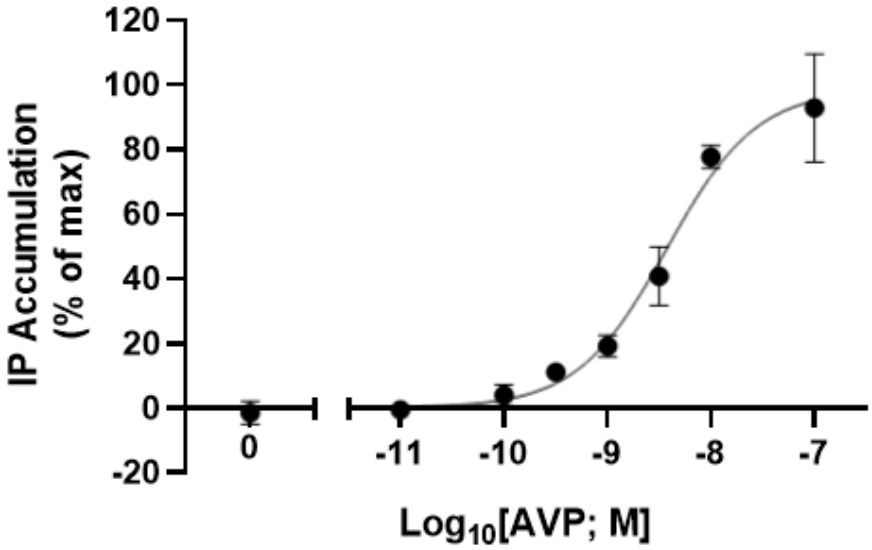


Figure 20: Dose-response analysis of WT AVPR1b signalling in response to AVP stimulation

Signalling response in HEK 293T cells transiently transfected with WT AVPR1b was measured by radiolabelled inositol phosphate (IP) accumulation assay following stimulation of vehicle (0) or a range of AVP concentrations. Data presented are from three independent experiments (N=3). Data have been fitted to a sigmoidal dose response curve from which the potency (EC_{50}) of oxytocin was calculated to be 3.7 nM.

As defects in potency or efficacy of activation of receptors is best detected when using concentrations of ligand of 3-10x EC_{50},[131] which achieve just sub-maximal responses at the respective WT receptor, it was decided that the ideal concentration to use for the investigation of vasopressin receptor signalling would be 10 nM for both the AVPR1a and AVPR1b variants.

There was no significant difference in the basal activity measured in cells expressing any of the variants when compared to cells expressing the WT AVPR1a or AVPR1b ($p>0.05$, one-way-ANOVA flowed by Dunnett's post-test; data not shown). When treated with AVP, 1/2 of the AVPR1a variants (Figure 21) and 4/5 AVPR1b variants (Figure 22) displayed a significant decrease in IP accumulation when compared to cells expressing the WT receptor ($p<0,05$; one-way ANOVA followed by Dunnett's post-test). For the AVPR1a variants, F136L elicited only 8% of the signal of the WT

receptor, while G6S did not show a decrease in IP signalling compared to WT AVPR1a. The AVPR1b variants K65N, G191R, R364H and R364L elicited signals of 21%, 41%, 10%, and 41% of the WT receptor response, respectively, while G191S showed no impairment in signalling response.

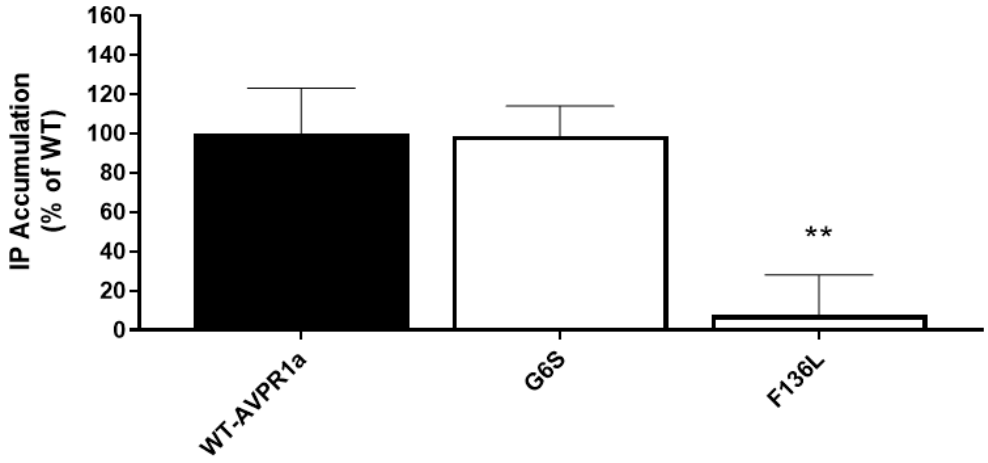


Figure 21: AVP-stimulated Inositol phosphate accumulation in cells expressing AVPR1a variants

*Signalling response in HEK 293T cells transiently transfected with WT or variant AVPR1a was measured by radiolabelled inositol phosphate (IP) accumulation assay following stimulation with 10 nM AVP. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT AVPR1a after subtraction of non-specific signal measured in cells transfected with empty vector treated with vehicle and normalisation to cell number (determined via crystal violet staining,Figure 17). **p<0.01, 1-way ANOVA followed by Dunnett's post-test for comparison with WT AVPR1a.*

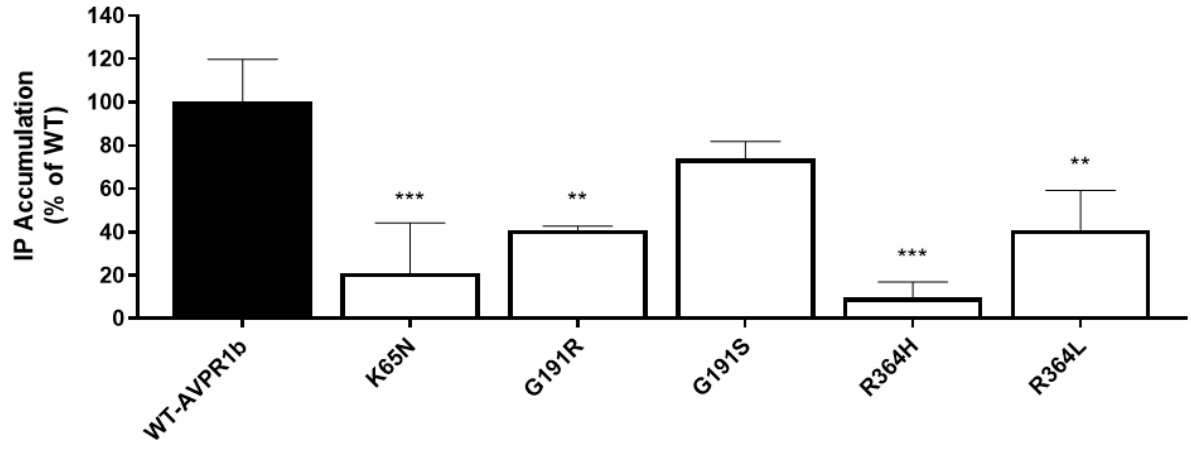


Figure 22: AVP-stimulated Inositol phosphate accumulation in cells expressing AVPR1b variants

*Signalling response in HEK 293T cells transiently transfected with WT or variant AVPR1b was measured by radiolabelled inositol phosphate (IP) accumulation assay following stimulation with 10 nM AVP. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT AVPR1b after subtraction of non-specific signal measured in cells transfected with empty vector treated with vehicle and normalisation to cell number (determined via crystal violet staining,Figure 18). **p<0.01 and ***p<0.001, 1-way ANOVA followed by Dunnett's post-test for comparison with WT AVPR1b.*

The signalling activity of the AVPR1a variant F136L with reduced signalling was then examined in more detail by generating dose response curves after stimulation with a range of AVP concentrations (Figure 23). Similar to the single concentration analyses, variant F136L was completely non-responsive and did not signal across the array of AVP concentrations. Therefore, no E_{max} or pEC_{50} could be determined (Figure 23).

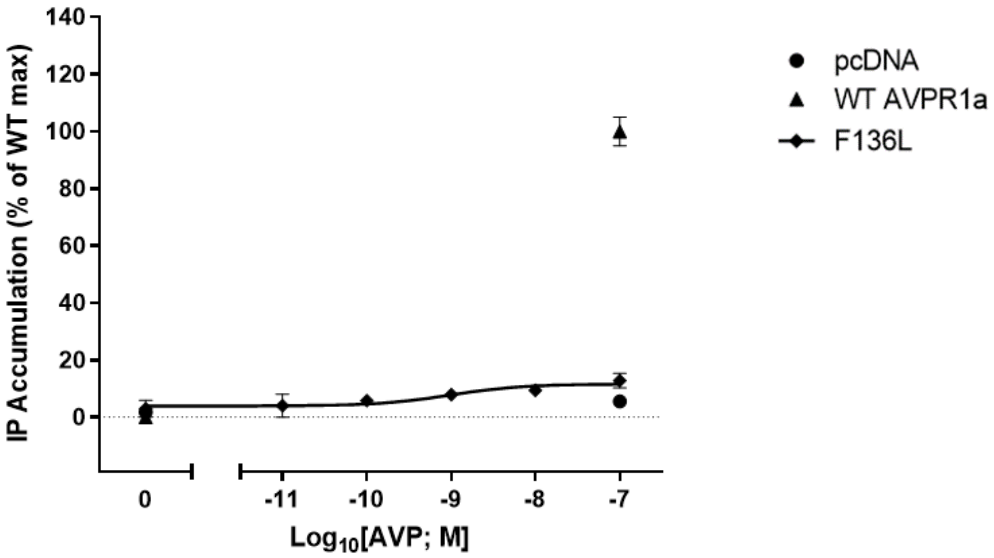


Figure 23: Dose-response analysis of variant AVPR1a signalling in response to AVP stimulation

Signalling response in HEK 293T cells transiently transfected with empty vector, WT AVPR1a or F136L variant AVPR1a was measured by radiolabelled inositol phosphate (IP) accumulation assay following stimulation of vehicle (0) or a range of AVP concentrations. Data presented are from three independent experiments (N=3). Data have been fitted to sigmoidal dose response curves from which potencies and E_{max} were calculated. Data are presented as a % maximal activity measured in cells transfected with the WT AVPR1a (stimulated with 0.1 µM OXT) following subtraction of the average basal activity (0) measured in cells expressing the WT receptor and normalisation to cell number. Data are presented as mean ± SEM from three independent experiments.

The signalling activity of the AVPR1b variants with reduced signalling (K65N, G191R, R364H and R364L) were then also examined in more detail by generating dose response curves after stimulation with a range of AVP concentrations. When compared to WT AVPR1b ($p<0,05$; one-way ANOVA followed by Dunnett's post-test) All of the AVPR1b variants resulted in a significant reduction of signalling capacity (69%, 65%, 58% and 55% of WT, respectively). When analysing the potency of AVP activation of the AVPR1b variants (Figure 24), it was found that K65N showed a small but significant decrease in potency (EC_{50}) of 3-fold, when compared to WT. Interestingly, none of the variants resulted in reduced AVP potency but variant K65N rather showed a significant increase in potency compared to the WT AVPR1b (1.5nM vs 4.3 nM).

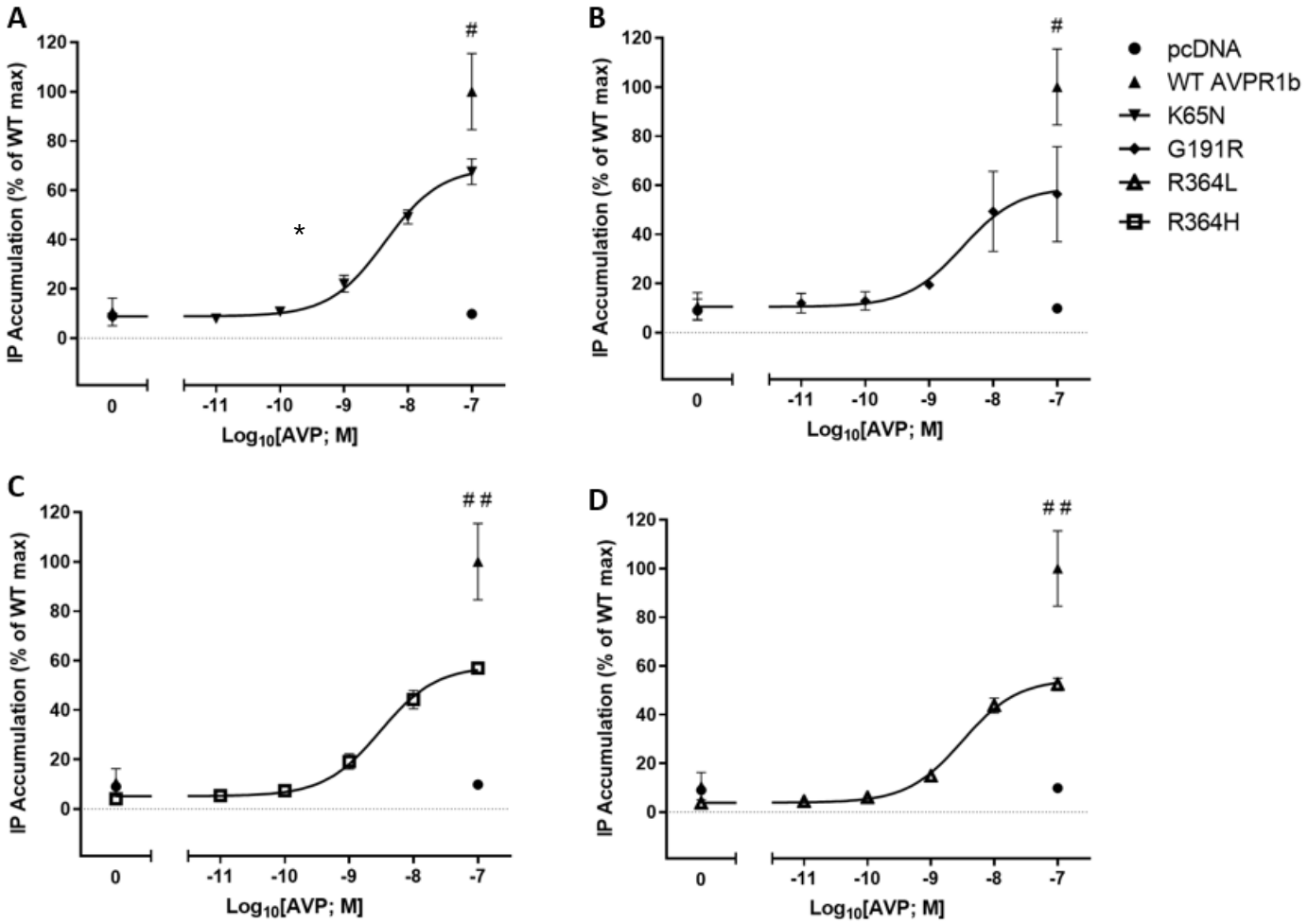


Figure 24: Signalling dose response for AVPR1b variants

*Signalling response in HEK 293T cells transiently transfected with empty vector, WT AVPR1b or **A**: K65N, **B**: G191R, **C**: R364H, and **D**: R364L. Variant AVPRs were measured by radiolabelled inositol phosphate (IP) accumulation assay following stimulation of vehicle (0) or a range of AVP concentrations. Data presented are from three independent experiments (N=3). Data have been fitted to sigmoidal dose response curves from which potencies and Emax were calculated (Table 16). Data are presented as a % maximal activity measured in cells transfected with the WT AVPR1b (stimulated with 0.1 µM AVP) following subtraction of the average basal activity (0) measured in cells expressing the WT receptor and normalisation to cell number. Data are presented as mean ± SEM from three independent experiments.*

Table 16: Statistical analysis of E_{max} and Potency of Dose-response signalling in response to AVP stimulation

Receptor	E_{max} (% of WT)	pEC_{50} (EC_{50} compared to WT)
WT AVPR1b	100 ± 10	8.83 ± 0.04 (1.5 nM)
A: K65N	69 ± 5 #	8.37 ± 0.11 (4.3 nM) *
B: G191R	65 ± 15 #	8.46 ± 0.02 (3.5 nM)
C: R364H	58 ± 3 ##	8.51 ± 0.15 (3.1 nM)
D: R364L	55 ± 3 ##	8.51 ± 0.06 (3.1 nM)

*## p<0.01 and # <0.05 and EC50 (*) where * <0.05, one-way ANOVA followed by Tukey's post-test for comparison with WT.*

6.2.2.3 AVPR1 Variant effects on hormone binding

The signalling capacity analysis of the selected AVPR1 variants showed that F136L had reduced AVP-induced signalling responses compared to the WT AVPR1a and variants K65N, C191R, R364H and R364L had reduced AVP-induced signalling responses compared to the WT AVPR1b (Figure 21 and Figure 20). These reduced responses could be a result of several factors including reduced receptor expression, reduced ligand binding or reduced receptor signalling. Radioligands can be used to measure ligand binding to receptors. Therefore, radiolabelled hormone binding assays were then used to determine the effects of variants on hormone binding.

Radioligands should ideally have high affinity, low non-specific binding, high specific activity to detect low receptor densities, and receptor specificity. Therefore, first, it was important to know the optimal dose of radiolabelled [^{3}H]-AVP to achieve an appropriate signal-to-noise ratio in cells expressing the WT AVPR1a to make examination of the variant receptors feasible (Appendix 1G: Radioligand Binding Assay Optimisation). Based on previous studies, the affinity of AVP was assumed to be 1.1 nM for AVPR1a and 0.7 nM for AVPR1b.[120] Therefore, for this study, it was decided that for the AVPR1a analyses a concentration of 3.3 nM and for the AVPR1b analyses a concentration of 2.1 nM tritiated AVP ([^{3}H]-AVP) (approx. 2-3x the affinity) would be

utilised to ensure that sufficient labelling of the receptors would be achieved.

The binding of AVP to the two AVPR1a variants (G6S and F136L) was compared to WT AVPR1a (Figure 25). The radioligand binding assay revealed that F136L resulted in a significant decrease in radiolabelled ligand binding when compared to WT AVPR1a (33%) where p<0,05; one-way ANOVA followed by Dunnett's post-test), while variant G6S had no effect.

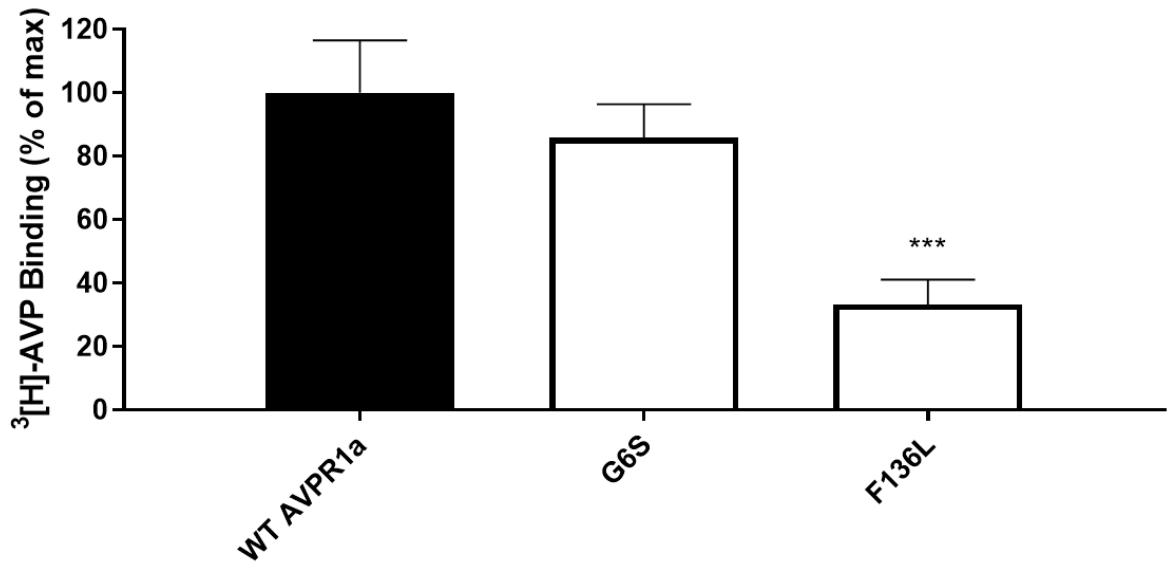


Figure 25: Radioligand binding of WT and variant AVPR1a's

*HEK 293T cells were transiently transfected with either WT AVPR1a, variant AVPR1a's or empty vector. Radioligand binding was measured following incubation with 3.3 nM [H]-AVP for 4 hours at 25 °C. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT AVPR1a after subtraction of non-specific signal measured in cells transfected with empty vector. ***p<0.001, one-way ANOVA followed by Dunnett's post-test for comparison with WT AVPR1a.*

The binding of AVP to the AVPR1b variants was then compared to that of WT AVPR1b (Figure 26). These analyses revealed that variants K65N and R364H resulted in a significant decrease in radiolabelled ligand binding when compared to WT AVPR1b (29% and 54% respectively). Surprisingly, variant G191R resulted in a significant

increase in ligand binding when compared to WT AVPR1b (173%). Variants G191S and R364L had no effect.

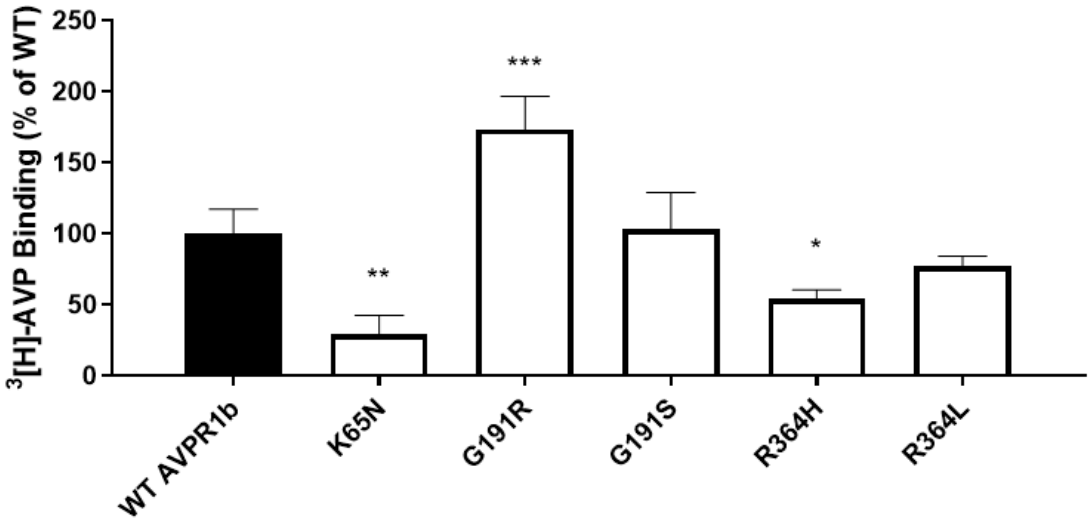


Figure 26: Radioligand binding of WT and AVPR1b variants

*HEK 293T cells were transiently transfected with either WT AVPR1b, variant AVPR1b's or empty vector. Radioligand binding was measured following incubation with 2.1 nM [H]-AVP for 4 hours at 25 °C. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT AVPR1b after subtraction of non-specific signal measured in cells transfected with empty vector. *p<0.05, **p<0.01 and ***p<0.001, one-way ANOVA followed by Dunnett's post-test for comparison with WT AVPR1b.*

6.2.2.4 AVPR Variant effects on receptor expression

In most cases, GPCRs must be expressed at the cell surface in order to be accessible to their ligands. As described in Section 1.4, GPCR variants have been found to often cause receptor conformation complications (misfolding of the receptor protein) and subsequent loss of cell surface expression of the receptor protein due to intracellular retention (in the ER) of nascent receptor proteins by cellular quality control processes. This is a common cause of loss-of-function induced by GPCR variants.

When studying receptor variants, structural changes that result from introduction of the mutations may affect antibody recognition. To circumvent this, an ELISA-based assay exploiting HA-tagged (YPYDVPDYA) variant AVPRs was used to measure and compare total cellular and cell surface expression of each of the selected variant receptors. This epitope tag antigen will remain unaffected by introduction of the mutations to the variant receptors allowing for an unbiased measure of receptor expression. A highly specific anti-HA antibody can then be used to label the receptors, allowing quantification of expression by incubation with HRP-conjugated secondary antibody and colorimetric analysis following application of the HRP substrate. When the receptor is correctly placed in the plasma membrane, the N-terminal HA tag protrudes from the extra cellular surface, so in intact cells, the only receptors accessible for the specific binding of HA antibodies are those expressed at the cell surface. Permeabilization of the cells to allow for penetration of the antibodies into the cells then enables total (internal and cell surface) receptor expression to be measured.

Optimisation was first performed in order to determine the most effective post-transfection incubation period as well as the primary and secondary antibody concentrations for the ELISA to ensure the best signal-to-noise ratio was achieved (Appendix 1E).

The cell surface expression of the WT and variant AVPR1a's were then determined to measure the number of receptors located on the cell surface (Figure 27). Variant F136L showed a significant decrease in cell surface expression when compared to the WT AVPR1a (43%) ($p<0,05$; one-way ANOVA followed by Dunnett's post-test). It should also be noted that G6S showed an increase, although not significant, in cell surface expression when compared WT AVPR1a.

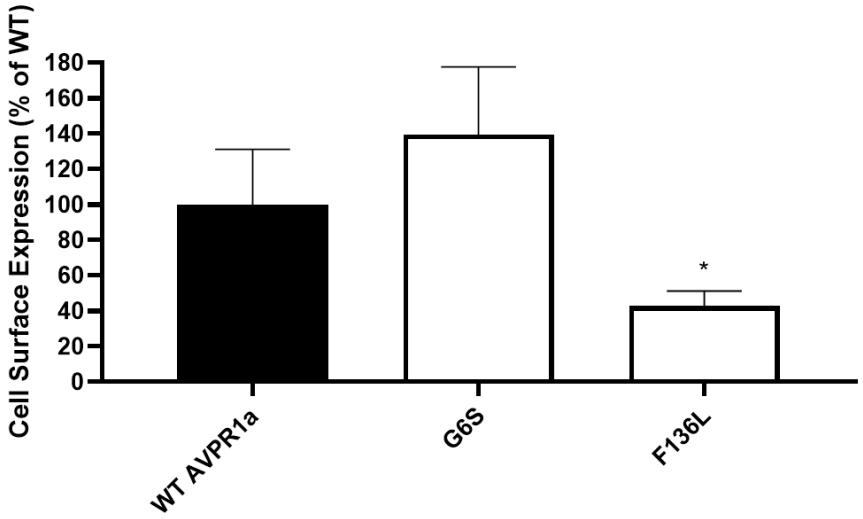


Figure 27: Cell surface expression of WT and variant AVPR1a's

*HEK 293T cells were transiently transfected with either WT AVPR1a, variant AVPR1a or empty vector. Cell surface expression of AVPR1a variants was measured by a receptor ELISA with intact cells. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT AVPR1a after subtraction of non-specific signal measured in cells transfected with empty vector and following normalisation for cell number. *p<0.05, one-way ANOVA followed by Dunnett's post-test for comparison with WT AVPR1a.*

Cell surface expression of the AVPR1b variants were then analysed and it was found that three out of the five variants (K65N, R364H and R364L) showed a significant decrease (49%, 66% and 69%, respectively) in cell surface expression when compared to wild type AVPR1b (p<0,05; one-way ANOVA followed by Dunnett's post-test), while the remaining variants (G191R and G191S) has similar cell surface expression as the WT AVPR1b (Figure 28).

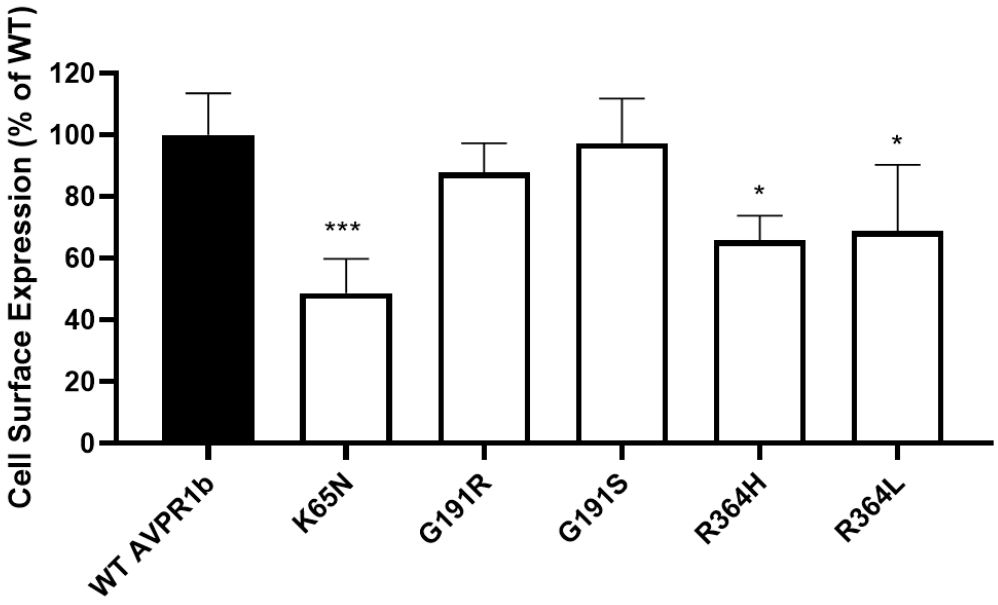


Figure 28: Cell surface expression of WT and variant AVPR1b

*HEK 293T cells were transiently transfected with either WT AVPR1b, variant AVPR1b or empty vector. Cell surface expression of AVPR1b was measured by a receptor ELISA with intact cells. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT AVPR1b after subtraction of non-specific signal measured in cells transfected with empty vector and normalised to the cell number. *p<0.05 and ***<0.001, one-way ANOVA followed by Dunnett's post-test for comparison with WT AVPR1b.*

To determine whether the altered cell surface expression observed was due to altered receptor trafficking to the cell surface or was due to altered receptor biosynthesis and/or degradation, the total number of receptors was then quantified in permeabilised cells. When assessing the total receptor expression of variants of AVPR1a (Figure 29) it was found that both G6S and F136L showed a slight but significant decrease (to 84% and 86% respectively) in total levels of expression compared to the WT receptor (p<0,05; one-way ANOVA followed by Dunnett's post-test).

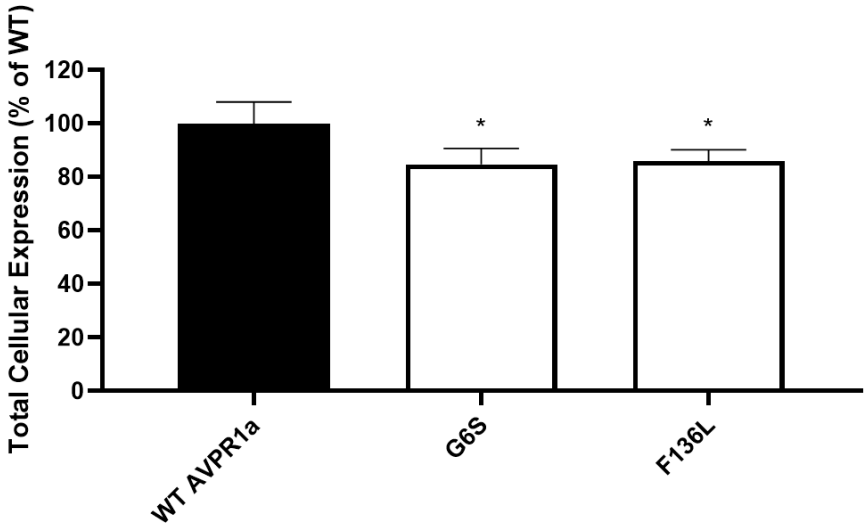


Figure 29: Total cellular expression of WT and variant AVPR1a's

*HEK 293T cells were transiently transfected with either WT AVPR1a, variant AVPR1a's or empty vector. Total expression of AVPR1a variants was measured by a receptor ELISA with permeabilised cells. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT AVPR1a after subtraction of non-specific signal measured in cells transfected with empty vector and following normalisation for cell number. *p<0.05, one-way ANOVA followed by Dunnett's post-test for comparison with WT AVPR1a.*

The total receptor expression of the AVPR1b variants were also examined (Figure 30) and it was found that none of the variants showed a significant decrease in total receptor expression compared to the WT AVPR1b (p<0,05; one-way ANOVA followed by Dunnett's post-test).

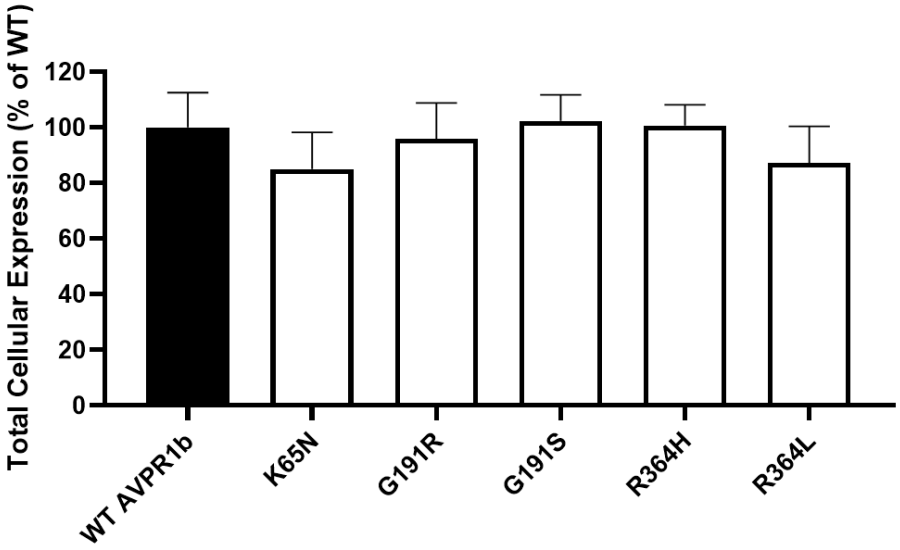


Figure 30: Total cellular expression of WT and AVPR1b variants

*HEK 293T cells were transiently transfected with either WT AVPR1b, variant AVPR1b or empty vector. Total expression of was measured by a receptor ELISA with permeabilised cells. Data are presented as mean ± SEM from three independent experiments (N=3) and have been calculated as percentage of average signal measured for the WT AVPR1b after subtraction of non-specific signal measured in cells transfected with empty vector and normalised to the cell number. *p<0.05, one-way ANOVA followed by Dunnett's post-test for comparison with WT AVPR1b.*

6.3 Discussion

There is a plethora of studies that have linked the dysregulation of AVP and its receptors to behavioural changes seen in humans with psychological disorders. Although the number of SNPs linked to these disorders are, as yet few, this may reflect the fact that the potential links have only relatively recently emerged and therefore extensive genotypic studies have not been undertaken.

Contrary to the OXTR variants (see Chapter 4), in terms of location, it is worthy to note that of the two AVPR1a and five AVPR1b coding region SNP's identified through genetic association studies in conditions such as depression, panic disorder and ASD, only one (F136L) is located in the transmembrane region of the receptor (the site of highest conservation of the GPCR family members and which contains the majority of

residues important for hormone binding and signal transduction as well as many important structural elements). Of all of the psychological disorder-linked AVPR1a and AVPR1b variants examined, the majority (71%; 5/7) were found to have significantly reduced response to AVP stimulation, indicating loss of function. It is important to note that little/no previous functional analysis has been reported for these variants and these are therefore novel findings.

6.3.1 The majority of loss-of-function AVPR1a/b variants exhibit moderately reduced cell surface expression

When cell surface expression of the loss-of-function variants was examined all but two (G191R and G191S) had significantly reduced expression at the cell surface (Figure 27 and Figure 28). However, the reduction in cell surface expression was not severe, with only one variant (AVPR1a F136L) exhibiting cell surface expression <50% of WT levels (43% of WT) and the others exhibiting cell surface expression of approximately 70% of WT levels. All of the variants with reduced cell surface expression had no, or only very slight, reductions in total receptor expression. This indicates that the reductions in cell surface expression are largely due to intracellular receptor retention/impaired cell surface trafficking. This is likely due to disruptions in intramolecular interactions caused by the introduction of the variants that have some effect on receptor folding/conformation. For all the variants with reduced cell surface expression (AVPR1a F136L, and AVPR1b K65N, R364H and R364L), the observed reduction in signalling response was larger than that which would be expected to (<40% of WT responses; Figure 21 and Figure 22)., suggesting that although these variants do appear to affect receptor folding/cell surface trafficking, their major cause of non-functionality is due to disruption in other aspects of receptor function. Thus, based on this limited selection of variants, AVPR1a/b variants may not make good candidates for PC therapies.

6.3.2 AVPR1a Variants F136L and AVPR1b Variants K65N, G191R, R364H and R364L Severely Impair Receptor Signalling/Binding

As discussed above, the relatively moderate reductions in cell surface expression of variants AVPR1a F136L and AVPR1b K65N, R364H and R364L cannot account for the large reductions in signalling responses observed for these variants. Likewise, AVPR1b variant G191R exhibited no reduction in cell surface expression, but a large reduction in AVP-induced signalling response. These observations suggest that either AVP binding, or receptor signalling is impaired or these variant receptors.

For AVPR1a variant F136L, levels of AVP binding was reduced (to 33% of WT levels). However, this is similar to the reduction in cell surface expression observed or this mutant. These data suggest that hormone binding is not being affected by this variant. Conversely, there is a severe (to 8% of WT) reduction in signalling response of this variant, suggesting that there is impairment in its signalling capabilities. Thus, this variant could be classified as a combination Class II/Class IV variant. F136 is located in TM 3 of the AVPR1a. There is high conservation seen in both human neurohypophyseal receptors and mammalian species at this position (Table 11 and Table 13) suggesting that it may be important for receptor function, although it is not very conserved within the Rhodopsin-like GPCR family as a whole. Although this substitution would be considered to be relatively conservative with respect to amino acid properties, six out of the seven VEP programs predicted this variant to be deleterious (Table 21). Conserved residues among GPCRs have indicated a common mechanism for activation and signal transduction to the G protein. Based on model GPCR studies, it is expected that the switching from an inactive to an active conformational change is associated with the relative orientational change of TM 3 and 6, which unmasks G protein binding sites on the intracellular face of the receptor, including the cytoplasmic exposure of buried sequences in ICL 2 and 3 which are important for G protein interactions.[138] The F136 residue is located in the TM 3 core which may play an important role in the switching of the inactive/active states of the GPCR.[135]

For AVPR1b variant R364L, there was only a moderate reduction in cell surface expression (to 69% of WT) and no decrease in AVP binding (a small increase was observed, but this was not statistically significant). These data suggest that hormone binding is also not being affected by this variant. However, there is a large (to 41% of WT) reduction in signalling response of this variant, suggesting that there is impairment in its signalling capabilities. Thus, AVPR1b variant R364L could be classified as predominantly being a Class IV variant. The other variant identified at this position, R364H, also had a similarly moderate reduction in cell surface expression (to 66% of WT levels) but there was also a significant reduction ion AVP binding (to 49% of WT levels), suggesting that, unlike the leucine (L) substitution, introduction of a histidine at this position disrupts hormone binding. This variant also displays a severe reduction in AVP-induced signalling response which cannot fully be accounted for by the reduction in hormone binding. Thus, it appears that this substitution affects receptor trafficking, hormone-binding and hormone-induced signalling (a combination Class II, III and IV variant). The R364 residue is located at the beginning of the C terminus of the AVPR1b. This area is involved in G protein coupling and receptor internalisation.[138] The conservation at this position is only moderate amongst mammalian species (Table 12 and Table 14) and none of the VEP programs predicted either of the variants at this position (R364H or R364L) to be deleterious (Table 21). Interestingly, both variants had similar effects on receptor cell surface expression but the R364H variant had a greater effect on hormone signalling responses and also appeared to affect hormone binding. This is perhaps surprising as replacement of the basic arginine (R) with another basic residue (histidine) would be considered to be more conservative than replacement with hydrophobic leucine. A possible explanation for how these variants are affecting receptor signalling may be that they affect the hydrophilic balance of the C terminal which is very sensitive to the hydrophilic coupling of the $G_{\alpha q/11}$ protein. However, as mentioned above, one would expect a more drastic decrease in R364L as lysine is smaller and more hydrophobic than histidine. It is also perhaps surprising that substitutions in this intracellular region affect hormone interactions (in the case of the R364 variant), but perhaps conformational changes induced by the introduction of this variant are propagated through the receptor and result in alterations to the hormone binding pocket. Further research, perhaps through

molecular modelling and further mutagenesis analyses, is required to investigate how these two different amino acids, at the same residue, impact different functional components of the AVRP1b.

Like AVPR1a variant F136L, AVP binding to AVPR1b variant K65N was also reduced (to 29% of WT levels). However, unlike AVPR1a variant F136L, this large reduction was not reflective of the relatively moderate reduction in cell surface expression (to 69% of WT levels) observed for this variant. As the signalling response (21% of WT) of this variant was similar to the level of binding measured, this suggests that there is impairment in hormone binding. Therefore, hormone binding impairment appears to be the predominant cause of its non-functionality (a Class III variant). This is contradicted by a previous study which reported a significant decrease of signalling in K65N but no change in binding affinity to AVP, and a surprising increase in affinity for desmopressin (AVPR2 agonist). The AVPR2 residue at position 65 is asparagine, which predominantly couples with Gs_{α} and it is therefore hypothesized that K65N increased the coupling preference of the AVPR1b to Gs_{α}. The agonist induced cAMP production was also investigated, however no significant increase in cAMP generation by K65N was observed.[183]

The AVP peptide interacts with a binding pocket on its cognate receptors involving the N-terminus, ECL 1 and TM's 1-2.[90] Invariably conserved residues of the neuropeptides and receptors are important for ligand-receptor interaction and have therefore been protected from evolutionary change. Within the binding pocket, TM 2-5 make up the ligand-accessible interface and are seen to be dominated by a polar network, while the intracellular interface is largely hydrophobic.[184] Residue K65 is located in ICL 1 of the AVPR1b, and it is therefore surprising that this variant affects AVP binding. However, like variant R364H, perhaps introduction of this variant induces a conformational change in the receptor that is propagated thought the receptor to induce an alteration in the hormone binding pocket, but further analysis (such as molecular modelling) would be required to explore this further. There is a high conservation seen in both human neurohypophyseal receptors and mammalian species at this position (Table 12 and Table 14) and this substitution is not a

particularly conservative one, with the replacement of a large charged basic residue with a smaller uncharged residue and the majority of the VEP programs forecasted K65N to induce a deleterious effect on AVPR1b (3/4, Table 21). Previous research poses a contradicting lack of effect of signalling or cellular expression for R364H when compared WT AVPR1b.[183] How the substitution of histidine at this position is affecting receptor signal transduction is not clear and, again, further focused analyses would be required to explore this finding in more detail.

The AVRP1b variant G191R is located within ECL 2. This specific residue is not conserved amongst the mammalian species or human neurohyphophoseal receptors (Table 12 and Table 14) and was not predicted to be deleterious by any of the VEP programs (Table 21). However, the functional analyses presented herein showed this variant to have a significantly reduced signalling response to AVP stimulation (to 41% of WT levels, Figure 22). This variant had no effect on the expression/cell surface localisation of the receptor, but, interestingly, had a significant increase of nearly double that of the WT receptor in AVP binding (Figure 26). Thus, the reduced response of this variant appears to be due to reduced signalling competence (despite increased binding) (a Class IV variant). It is interesting to note that similar disruptions in signalling responses nor increases in AVP binding were observed for the other variant at this position (G191S). This could be explained by the drastic change in amino acid properties seen in G191R, where there is a change from a small, non-polar, hydrophilic amino acid (glycine, G), to a large, polar, basic residue (arginine; R), while the glycine to serine (S) substitution is far more conservative. How variant G191R increases AVP binding is not clear, and it would be interesting in future studies to perform dose response analyses to see how binding affinity is being affected. GPCR 3D modelling has shown that conserved residues responsible for the ligand/receptor recognition process include FQVLPQ in TM 2, GDP in ECL 1, DCWA and PWG in the ELC 2 of the AVPR1b.[185] Position G191 is located between two of these important ligand recognition motifs (DCWA and PWG of ECL 2). Thus, the introduction of a charged arginine at position may either cause altercations of intramolecular interactions in this area that affect the ligand binding pocket, or, indeed, this residue might actually contribute directly to electrochemical interactions with the hormone, which could

account for the altered binding observed. However, further analyses (such as molecular docking or mutagenesis studies) would be required to confirm this.

6.3.3 AVPR1a Variant G6S and AVPR1b Variant G191S Appear to Have No Effect on the Function of AVPR1a/b

When compared to their cognate WT receptors, AVPR1a variant G6S and AVPR1b variant G191S did not appear to show any evidence of non-functionality. Indeed, there was no decrease in AVP-induced signalling (Figure 21 and Figure 22), AVP binding or receptor cell surface expression for either receptor. With regards to conservation amongst species and receptors, neither positions G6 (Table 11 and Table 12) or G191 (Table 13 and Table 14) are highly conserved. The substitution of glycine (G) for serine (S) is also relatively conservative (although there is an alteration in hydrophobicity) and it is noteworthy that all of the VEP programs predicted these two variants to be benign.

Variant G6S is the only variant in this study which is located in the N terminal of the receptor. When comparing this variant to the schematic model of oxytocin/vasopressin receptor indicating amino acid residues that are putatively involved in ligand-binding and associated signal transduction events,[16] it was found that the N terminal regions of the receptor are important for interacting with the linear C-terminal tripeptidic part of the AVP and is therefore vital for ligand binding and selectivity.[186] However, it appears that this particular position does not play an important role in these processes. As previously described, the G191 residue is located near the end of ECL 2. This region is involved in interaction with the cyclic part of AVP and is therefore important for ligand binding,[144,186] but, again, this particular substitution does not appear to affect this process.

6.3.4 Other Noteworthy Observations and Limitations

Initially, it was observed that there was a significant decrease in cell number 48 hours post transfection with WT AVPR1a and 1b when compared to empty vector. This is

very unusual as no previous research has expressed this observation in AVPR1b and no previous research has characterised the functionality of variants in the coding region of AVPR1a (and only a single study has examined a limited number of AVPR1b variants), highlighting the need for further research to confirm these novel results. As previously mentioned, transient transfections induce a stressful state in the cell and may have a direct impact on the proliferation or the cytotoxic state of the cell. It could be argued that the cells remaining were not successfully transfected, however this was disproven with the expression of receptor observed in both cell surface and total ELISA assays (Figure 27, Figure 28, Figure 29, and Figure 30). That there was no difference in total cellular receptor expression levels for the WT or any of the variant AVPR1bs despite there being differential effects on cell growth indicates that these observed effects are not simply due to cellular proliferation being slowed as a result of increased expression of exogenous protein by the cells. Several cell viability assays were included to ensure transfection did not lead to a cell death. A simple experiment to determine the percentage of cell death in cells 24 hours post transfection (Figure 42 and Figure 43) showed that there was no significant decrease in living cell percentage when comparing empty vector transfected cells to the WT and variant AVPR1a/b transfected cells. It is therefore unlikely that the decrease in cell number may be as a result of cytotoxic effects but rather an anti-proliferative effect experienced by the transient transfection of WT AVPR and AVPR variants. Further investigation of the mechanisms behind this observation are of interest for future studies. Also, to prevent this complication in future studies examining AVPR variants *in vitro*, stable clones could be used for functional assays, an option which was not feasible at the time of this study. It is interesting to note that for the AVPR1a/b, there was no correlation between variant functionality and effects on cell number (as both the functional and non-functional variants had similar effects as the WT receptor).

Although differing effects on hormone binding and receptor signalling via IP accumulation were observed in this study, it would be interesting in future studies to examine the effects on these variants on other aspects of receptor function/signalling (including G protein-independent signalling), particularly for those variants that no impairment was found in IP accumulation.

When analysing the location of the variants for AVPR1a/b, it can be seen that the clusters of impairment are more scattered, however, with a limited number of variants, these conclusions are preliminary. It should be noted that certain variants which are located on the ICL 1, TM 3 and C terminal are shown to severely impair the signalling or binding of the receptor (AVPR1a: F136L and AVPR1b: K65N, R364H and R364L) while a variant located on the ECL 2 shows a partial impairment in signalling (AVPR1b: G191R). Variants G6S (AVPR1a) and G191S (AVPR1b) did not show any effect on receptor functionality and can therefore be considered as tolerated. This valuable information provides a clearer understanding of the AVPR and its intricate pathways of binding, activation, and signalling.

7. General Summary and Conclusions

OXT is well known for its classical roles in initiation of myoepithelial contractions during labour and lactation, while AVP plays a role in regulation of blood pressure and osmolality. However, there is also substantial evidence supporting central roles of these neuropeptides in neuronal and cognitive functioning. Indeed, their altered signalling has been implicated in several psychological disorders, social impairments, and behavioural traits.

Oxytocin and AVP elicit their effects through interaction with cognate G protein-coupled receptors (GPCRs). The OXT receptor (OXTR) and two AVP receptor subtypes (AVPR1a and AVPR1b) are expressed in many brain regions. Several studies have highlighted the potential association of genetic variants of these receptors with behavioural/social disorders. Yet, the impacts of these variants on receptor function are largely unknown thus this study aimed to characterise the functional effects of these variants *in vitro*. Understanding the nature of any defects in receptor function can aid in the development of future treatment options to correct/overcome these deficiencies. In some cases, *in silico* tools have been used to predict whether identified single nucleotide polymorphisms (SNPs) will be deleterious to receptor function and be pathogenic. However, these tools have been reported to have limited reliability/accuracy and therefore, the *in vitro* findings were also compared to in silico prediction by a range of different tools in order to assess their reliability in predicting the likeliness of variants identified in these receptors to be causative of/involved in the phenotypes observed.

The overall *in vitro* analysis showed that 6/11 psychological disorder linked variants and 10/15 of the birth disorder linked variants resulted in the impaired function of OXTR. The *in vitro* aspect of this study demonstrates that OXTR variants A63V, A238T, T273R, T273M, R376C and R376G, AVPR1a variant F136L and AVPR1b variants: K65N, G191R, R364H and R364L, result in the severe or partial dysfunction of the OXTR or AVPR and therefor may contribute to the highly complex

pathophysiology of psychological disorders linked in previous genetic association studies. It can also be inferred that the OXTR variants V45L, M133V, H173R, W203R, G221S, I266V and V281M, result in the severe or partial dysfunction of the OXTR and therefore may contribute to the defects experienced in birth disorders linked in previous genetic association studies.

It is also noteworthy to comment on the classification of variants for each receptor, where OXTR variants were mainly Class II and Class IV variants, and AVPR1a/b were largely Class IV variants. This is unusual as most GPCR variants exhibit reduced expression as a result of impaired trafficking to the cell surface. This poses the question that some receptors (i.e. AVPR1a/b) are more susceptible signalling/binding deficiencies. When comparing the more researched OXTR and AVPR2 to the less established AVPR1a/b we see more severe effects on the cell surface expression (Class II) for OXTR and AVPR2 variants, as compared to the more moderate reduction seen in AVPR1/b variants. Perhaps the AVPR1a/b GPCR is inherently more stable and therefore less sensitive to misfolding. Although this study is a very small sample size, so larger numbers of variants would be required to confirm this hypothesis.

It is also interesting to note that overall, AVPR1a/b (4/6) variants were more deleterious than the OXTR psychological disorder variants (6/11) which indicates that the AVPR1 receptors may have a more distinct role in psychological disorders as opposed to OXTR. However, this hypothesis is scarcely credible due to the sample size.

7.1.1 VEP Analysis

Several papers have indicated that the accuracies of contemporary variant effect prediction tools are likely to be considerably lower than reported in their original method publications.[187,188] This has profound implications for how such tools are used in clinical diagnostic and disease-gene discovery programs. Indeed, the predictions generated by such tools should be treated with considerable caution. There is little functional research which has confirmed these predictions, or the accuracy of these

programs and it is therefore imperative to characterise the functional implication of these variants in order to infer a physiological or psychological effect from its receptor. Knowing the accuracy of *in silico* analysis is imperative for modern research if such tools are to be applied to link genotypic with phenotypic observations. Thus, the accuracy of these tools at predicting the effects of the OXTR and AVPR1a/b variant studied here was analysed.

The general precision of some of the VEP programs across all three receptors are seen to be better than others (SIFT: 81%, Polyphen: 69%, FATHMM: 27%, LRT: 76%, PROVEAN: 46%, Mut. assessor: 71% and Mut. test: 81%). It should be noted that programs LRT, Mutation assessor and Mutation test were not able to generate predictions for the AVPR1b receptor. Therefore, the SIFT program is the most accurate while FATHMM is the least accurate of the programs used for *in silico* analysis.

It should be emphasized that certain VEPs conveyed different results amongst certain disorders or variants. When looking at the accuracy of VEP programs just among the OXTR variants, the SIFT program showed a 100% accuracy in predicting variants to be tolerant, and a 95% accuracy in predicting variants to be deleterious. When looking at just the psychological disorder linked OXTR variants, the SIFT program is 100% accurate for both deleterious and tolerant predictions, while programs Mut. Assessor, Mut. Tester, Polyphen, LRT and Provean were revealed to be 65-75% accurate. Interestingly, FATHMM predicted all variants to be tolerated. When focussing on just the birth disorder linked OXTR variants, the prediction accuracy of the VEP programs used were as follows: SIFT and Mut. test program reflected an accuracy of 92%, LRT with 83%, Mut. Assessor and Polyphen with 67%, and FATHMM and Provean with 33%.

The *in silico* analysis of the psychological disorder linked OXTR variants revealed that although the majority of the variants experience a significant change in amino acid, only 4/11 variants (A63, A217, A238 and T273) are seen to be highly conserved across several mammalian species expressing the OXTR gene. The variant effector

prediction programs have only predicted 4/11 of the variants (A63V, A63G, T273R and T273M) to have a deleterious effect. The birth disorder linked OXTR variants included in this study are all predicted to have a deleterious effect. It may be of interest to investigate the accuracy of VEP programs in comparison to the change in amino acid in conserved residues (in GPCR families and/or specific GPCRs etc) to determine non-functionality for different receptors.

Of the AVPR1a variants, only one of the two variants resulted in significant impairment in receptor function, while 80% of the AVPR1b variants resulted in the severe or partial impairment of the signalling or binding of the receptor. With the AVPR variants, it is a bit more difficult to make correlations to locations and accuracies as there are significantly fewer variants which have been reported.

Indeed, it is also challenging to comment on the accuracy of the *in silico* VEP programs when the number of variants is so limited. However, the most accurate programs for the AVPR1a variants, with an accuracy of 57%, are LRT, Mut. assessor and Mut. test. There are no VEP programs which can be considered accurate for AVPR1b variants. The least accurate program seems to consistently be the FATHMM program, yet there may be very little benefit in the elimination of variants from a small sample due to low prediction scores and it is therefore important to complete *in vitro* studies to determine the functionality of AVPR variants.

In conclusion, this study has shown that the use of the SIFT VEP is most reliable for future *in silico* analysis of OXTRs and AVPR1a. It would be interesting in future studies to determine whether this is a general observation for all GPCR variants (as these programs are designed for all protein families and not specifically for GPCR analysis).

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9. Appendices

Appendix 1: Supplemental Data

Appendix 1A: Mutagenesis Primer Sequences

Table 17: Oligonucleotide primer sequences for the introduction of variants into the OXTR, AVPR1a and AVPR1b plasmids

Variant	Primer sequence 5'- 3'		Melting Temp. (Tm)(°C)	Annealing Temp. (Ta)(°C)
OXTR_V45L	sense	GCGTGGAGGTGGCGTTGCTGTGTCTCATC	80.22	65
	antisense	GATGAGACACAGCAACGCCACCTCCACGC		
OXTR_A63V	sense	GTGGTGCGCAGCACCAGCAGCACAC	78.38	58
	antisense	GTGTGCTGCTGGTGCTGCGCACCAC		
OXTR_A63G	sense	GTGGTGCGCAGCCCCAGCAGCACAC	80.02	65
	antisense	GTGTGCTGCTGGGGCTGCGCACCAC		
*OXTR_P108A	sense	ACAGCAGGTCGGCCCCGTAGAAGCG	82.4	60
	antisense	CGCTTCTACGGGGCCGACCTGCTGT		
*OXTR_M133V	sense	CGGTCCAGGGACACGAGCAGCAGCAGG	85.4	64
	antisense	CCTGCTGCTGCTCGTGTCCCTGGACCG		
*OXTR_R150L	sense	GGTCGGTGCGGAGGCGCAGCGAG	84.2	65
	antisense	CTCGCTGCGCCTCCGCACCGACC		
*OXTR_R150S	sense	GTCGGTGCGGCTGCGCAGCGAGC	84.2	58
	antisense	GCTCGCTGCGCAGCCGCACCGAC		
OXTR_V172A	sense	AGAGAAGATGTGCGCCTGCGGCGCGC	78.5	58
	antisense	GCGCGCCGCAGGCGCACATCTTCTCT		
OXTR_H173R	sense	CAGAGAGAAGATGCGCACCTGCGGCGC	78.61	58
	antisense	GCGCCGCAGGTGCGCATCTTCTCTCTG		
OXTR_W203R	sense	TAGCGTGATCCTTGTGATGTAGGCCTTGGGTCC	83.4	64
	antisense	GGACCCAAGGCCTACATCACAAGGATCACGCTA		
OXTR_L206V	sense	CACGATGTAGACAGCTACCGTGATCCATGTGATGT	79.27	59
	antisense	ACATCACATGGATCACGGTAGCTGTCTACATCGTG		
OXTR_A217T	sense	CCGTAGCAGGCAGTGAGCACGATGACC	78.61	58
	antisense	GGTCATCGTGCTCACTGCCTGCTACGG		
OXTR_A218T	sense	CCGTAGCAGGTAGCGAGCACGATGACC	78.61	58

	antisense	GGTCATCGTGCTCGCTACCTGCTACGG		
*OXTR_G221S	sense	TGAAGCTGATAAGGCTGTAGCAGGCAGCGAG	82.2	60
	antisense	CTCGCTGCCTGCTACAGCCTTATCAGCTTCA		
OXTR_A238T	sense	CGCCGCCGTTGCAGCGGTCTTGAG	78,25	58
	antisense	CTCAAGACCGCTGCAACGGCGGCG		
OXTR_E242K	sense	CTCTGGCGCCTTGGCCGCCGCCG	81,67	68
	antisense	CGGCGGCGGCCAAGGCGCCAGAG		
OXTR_G252A	sense	CCACGCGCCCCGCATCGCCAGCC	81,67	68
	antisense	GGCTGGCGATGCGGGGCGCGTGG		
OXTR_I266V	sense	CTTGGCCTTGGAGACGAGCTTGACGCTGC	83.7	68
	antisense	GCAGCGTCAAGCTCGTCTCCAAGGCCAAG		
OXTR_T273R	sense	AAAGTCATCTTGACCCTGCGGATCTTGGCCTTG	79,14	55
	antisense	CAAGGCCAAGATCCGCAGGGTCAAGATGACTTT		
OXTR_T273M	sense	GAAAGTCATCTTGACCATGCGGATCTTGGCCTTGG	80,44	55
	antisense	CCAAGGCCAAGATCCGCATGGTCAAGATGACTTTC		
*OXTR_V281M	sense	ACGATGAAGGCCAGCATGATGATGAAAGTCATCTTG	81	65
	antisense	CAAGATGACTTTCATCATCATGCTGGCCTTCATCGT		
OXTR_R376C	sense	CTGGCTGGAGCTGCAATGGCTCAGGACAAAG	80,31	55
	antisense	CTTTGTCCTGAGCCATTGCAGCTCCAGCCAG		
OXTR_R376G	sense	GGCTGGAGCTGCCATGGCTCAGGACAA	78,61	58
	antisense	TTGTCCTGAGCCATGGCAGCTCCAGCC		
AVPR1a_G6S	sense	CGCGTCGGGACTGGCGGAGAGAC	78,11	65
	antisense	GTCTCTCCGCCAGTCCCGACGCG		
AVPR1a_F136L	sense	GGCCGACGCTAACATGCCGAACACCTG	78,61	60
	antisense	CAGGTGTTCGGCATGTTAGCGTCGGCC		
AVPR1b_K65N	sense	TGCGGGAGCGATTGCGGCCCAGCT	78,25	62
	antisense	AGCTGGGCCGCAATCGCTCCCGCA		
AVPR1b_G191R	sense	CCCCAAGGGAAGCGGAAGTCTGCCCAG	80,13	62
	antisense	CTGGGCAGACTTCCGCTTCCCTTGGGG		
AVPR1b_G191S	sense	CCCCAAGGGAAGCTGAAGTCTGCCCAG	78,61	62
	antisense	CTGGGCAGACTTCAGCTTCCCTTGGGG		
AVPR1b_R364H	sense	AGAGCCGCCGGTGCATCCTGGGC	78,11	65
	antisense	GCCCAGGATGCACCGGCGGCTCT		
AVPR1b_R364L	sense	AGAGCCGCCGGAGCATCCTGGGC	78,11	65
	antisense	GCCCAGGATGCTCCGGCGGCTCT		

** Indicates the primers previously generated by Ms M. Tonikin, Centre for Neuroendocrinology, University of Pretoria*

Table 18: Oligonucleotide Primers used to sequence OXTR, AVPR1a and AVPR1b plasmids

Name of primer	Oligonucleotide sequence 5'- 3'
OXTR forward	AGGTGCACATCTTCTCTCTGC
OXTR reverse	CGTAGCAGGTAGCGAGCAC
AVPR1a forward	TACTTCGTCTTCTCCATGATCG
AVPR1a reverse	GTTGTAGCAGATGAAGCCGTA
AVPR1b forward	ACCCTGGCTATCTTCGTTCTG
AVPR1b reverse	GAGATGGTGTTGATGCTGCTGA
T7 promoter	TAATACGACTCACTATAGGG
BGH reverse	TAGAAGGCACAGTCGAGG

Appendix 1B: Sanger Sequencing to Confirm Introduction of Variants

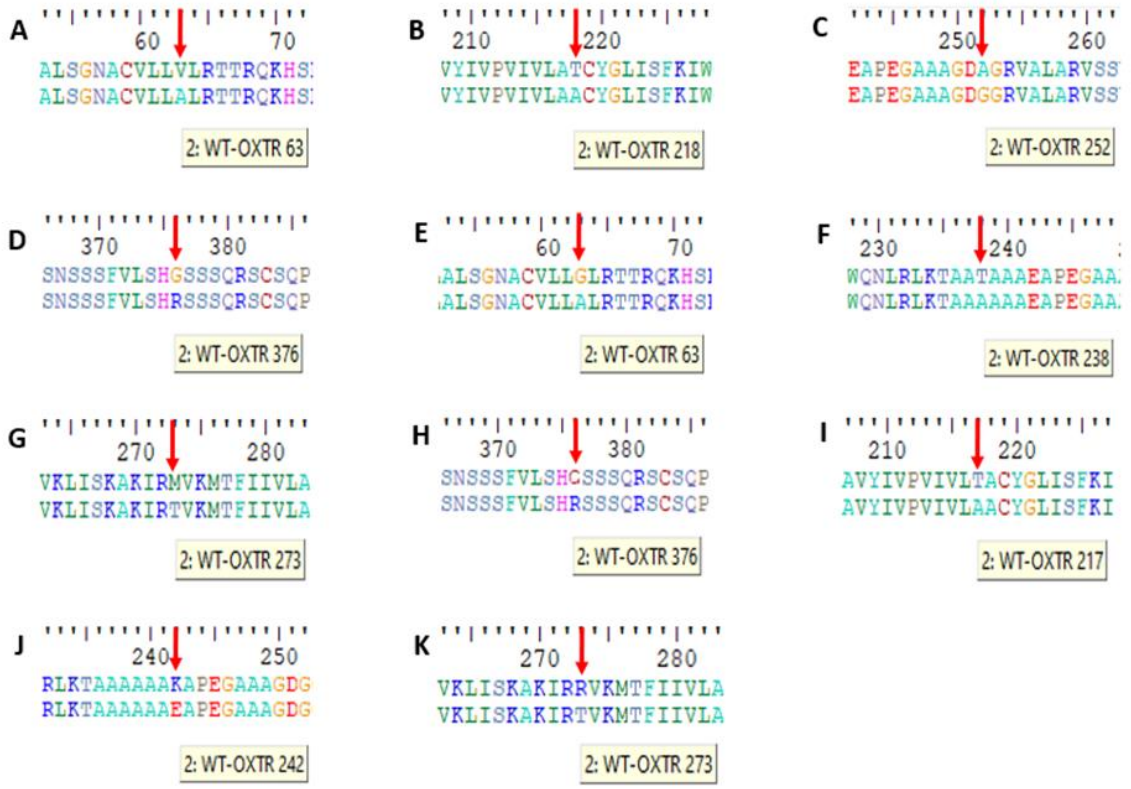


Figure 31: Sanger Sequencing for Psychological disorder linked OXTR variants

Sections of contigs derived from Sanger sequencing confirming introduction of variants (A) A63V, (B) A218T, (C) G252A, (D) R376G, (E) A63G, (F) A238T, (G) T273M, (H) R376C, (I) A217T, (J) E242K and (K) T273R into the HA-tagged WT OXTR open reading frame. Amino acid sequence of the WT OXTR reference sequence is shown on the bottom, and that of the variant contig above.

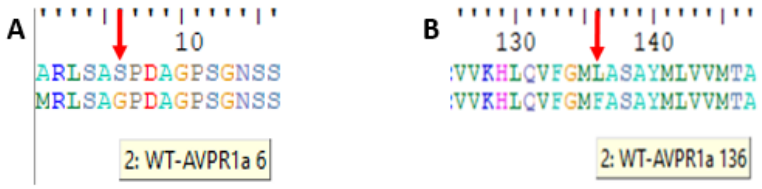


Figure 32: Sanger Sequencing for Psychological disorder linked AVPR1a variants

Sections of contigs derived from Sanger sequencing confirming introduction of variants (A) G6S and (B) F136L into the HA-tagged WT AVPR1a open reading frame. Amino acid sequence of the WT receptor is shown on the bottom, and that of the variant contig above.

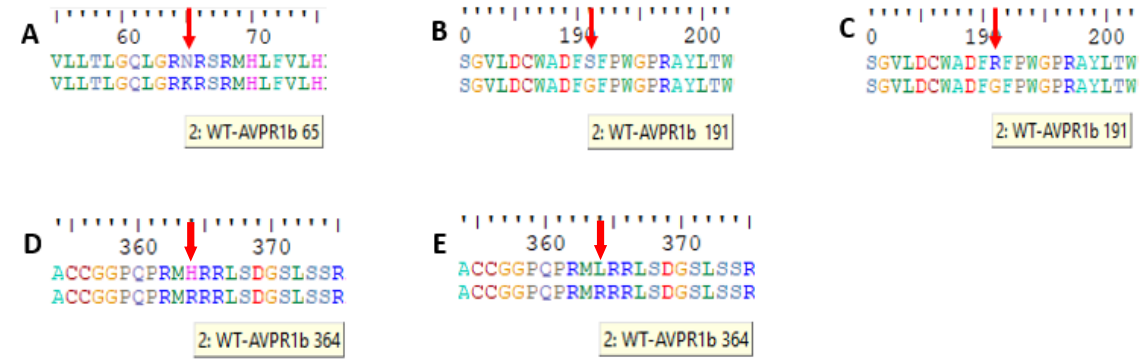


Figure 33: Sanger Sequencing for Psychological disorder linked AVPR1b variants

Sections of contigs derived from Sanger sequencing confirming introduction of variants (A) K65N, (B) G191S, (C) G191R, (D) R364H and (E) R364L into the HA-tagged WT AVPR1b open reading frame. Amino acid sequence of the WT receptor is shown on the bottom, and that of the variant contig above.

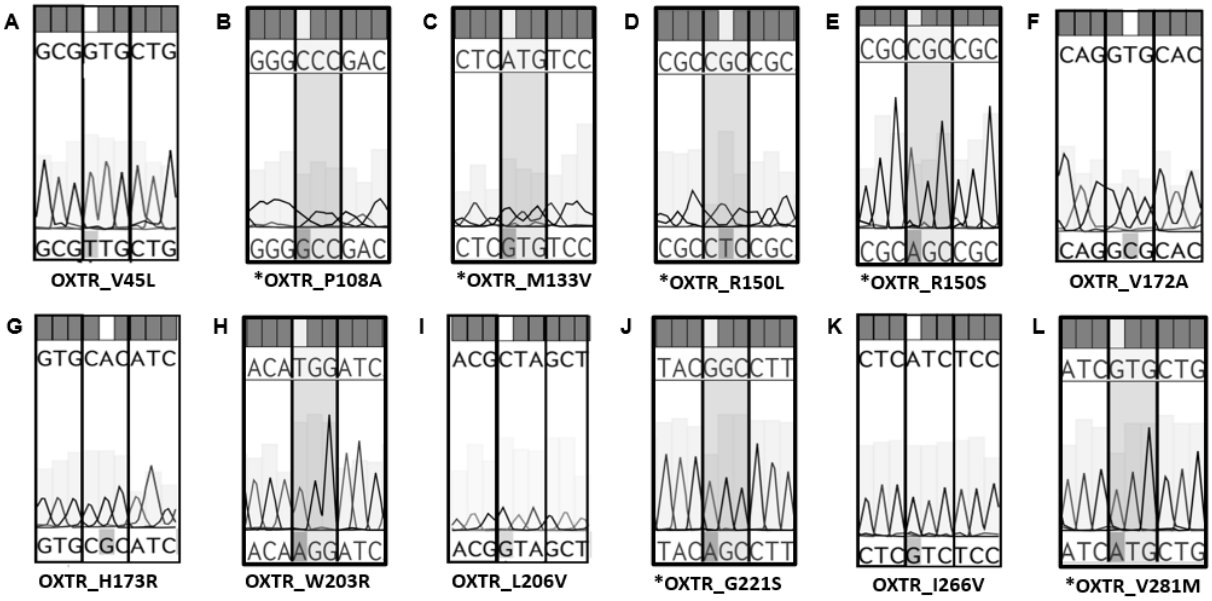


Figure 34: Sanger Sequencing for Birth disorder linked OXTR variants

*Sections of contigs derived from Sanger sequencing confirming introduction of variants (A) V45L, (B) P108A, (C) M133V, (D) R150L, (E) R150S, (F) V172A, (G) H173R, (H) W203R, (I) L206V, (J) G221S, (K) I266V and (L) V281M into the HA-tagged WT OXTR open reading frame. Amino acid sequence of the WT OXTR reference sequence is shown on the bottom, and that of the variant contig above. The * indicates the variants which were previoulsy generated by Ms M. Tonikin, Centre for Neuroendocrinology, University of Pretoria*

Appendix 1C: In silico Analysis

Table 19 : Amino acid property changes for each variant

		WT amino acid				Variant amino acid			
Receptor	**Variant**	**Size**	**Charge**	**Polarity**	**Hydrophobicity**	**Size**	**Charge**	**Polarity**	**Hydrophobicity**
OXTR	V45L	75	0	Non-polar	Hydrophobic	131	0	Non-polar	Hydrophobic
	A63G	89	0	Non-polar	Hydrophobic	75	0	Non-polar	Hydrophobic
	A63V	89	0	Non-polar	Hydrophobic	117	0	Non-polar	Hydrophobic
	P108A	115	0	Non-polar	Hydrophobic	89	0	Non-polar	Hydrophobic
	M133V	149	0	Non-polar	Moderate	117	0	Non-polar	Hydrophobic
	R150L	175	+	Polar	Hydrophilic	131	0	Non-polar	Hydrophobic
	R150S	175	+	Polar	Hydrophilic	105	0	Polar	Hydrophilic
	V172A	117	0	Non-polar	Hydrophobic	89	0	Non-polar	Hydrophobic
	H173R	155	+	Polar	Hydrophilic	175	+	Polar	Hydrophilic
	W203R	204	0	Non-polar	Hydrophobic	175	+	Polar	Hydrophilic
	L206V	131	0	Non-polar	Hydrophobic	117	0	Non-polar	Hydrophobic
	A217T	89	0	Non-polar	Hydrophobic	119	0	Polar	Hydrophilic
	A218T	89	0	Non-polar	Hydrophobic	119	0	Polar	Hydrophilic
	G221S	75	0	Non-polar	Hydrophobic	105	0	Polar	Hydrophilic
	A238T	89	0	Non-polar	Hydrophobic	119	0	Polar	Hydrophilic
	E242K	146	-	Polar	Hydrophilic	147	+	Polar	Hydrophilic
	G252A	75	0	Non-polar	Hydrophobic	89	0	Non-polar	Hydrophobic
	I266V	131	0	Non-polar	Hydrophobic	117	0	Non-polar	Hydrophobic
	T273R	119	0	Polar	Hydrophilic	175	+	Polar	Hydrophilic
	T273M	119	0	Polar	Hydrophilic	149	0	Non-polar	Moderate
	V281M	117	0	Non-polar	Hydrophobic	149	0	Non-polar	Moderate
	R376C	175	+	Polar	Hydrophilic	121	0	Polar	Moderate
	R376G	175	+	Polar	Hydrophilic	75	0	Non-polar	Hydrophobic
AVPR1a	G6S	75	0	Non-polar	Hydrophobic	105	0	Polar	Hydrophilic
	F136L	165	0	Non-polar	Hydrophobic	131	0	Non-polar	Hydrophobic
AVPR1b	K65N	147	+	Polar	Hydrophilic	132	0	Polar	Hydrophilic
	G191R	75	0	Non-polar	Hydrophobic	175	+	Polar	Hydrophilic
	G191S	75	0	Non-polar	Hydrophobic	105	0	Polar	Hydrophilic
	R364H	175	+	Polar	Hydrophilic	155	+	Polar	Moderate
	R364L	175	+	Polar	Hydrophilic	131	0	Non-polar	Hydrophobic

Analysing the size (molar mass), charge, polarity and hydrophobity for both the original and variant amino acids.

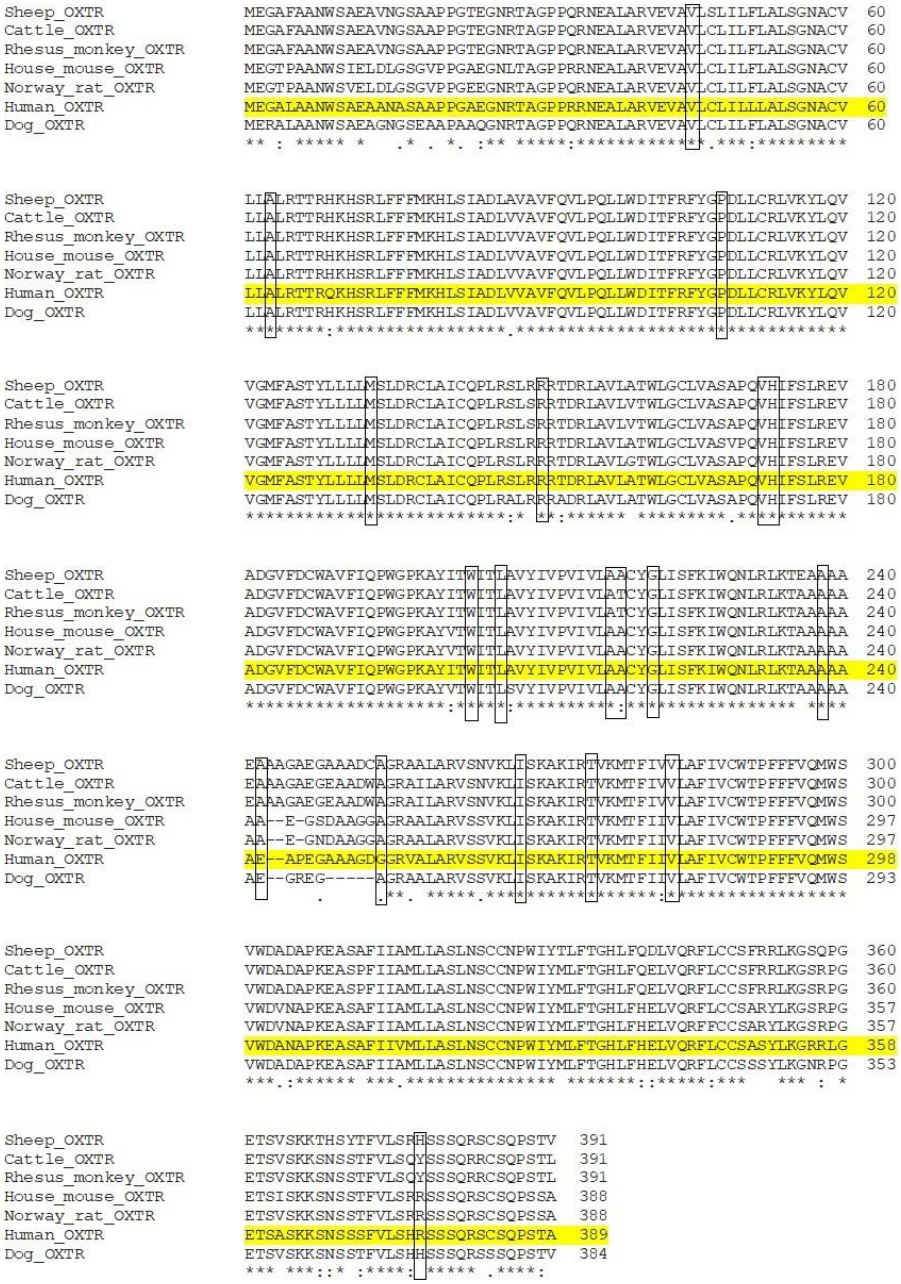


Figure 35: Multiple sequence alignment of mammalian oxytocin receptor amino acid sequences

*Reference sequences (Human OXTR: NM_000916, House mouse OXTR: NM_001081147.2, Norway rat OXTR: NM_012871.4, Rhesus monkey OXTR: NM_001044732.1, Cattle OXTR: NM_174134.2, Sheep OXTR: NM_001009752.1 and Dog OXTR: NM_001198659.1) were aligned using Clustal Omega. The human OXTR sequence is highlighted in yellow. The residues noted to be different between species are shown below the aligned sequence where: * indicates the same residue, one dot indicates similar residues, two dots indicate different residues, and a space indicates very different residues. The residues selected for this study (psychological and birth disorders) are indicated with the black block.*

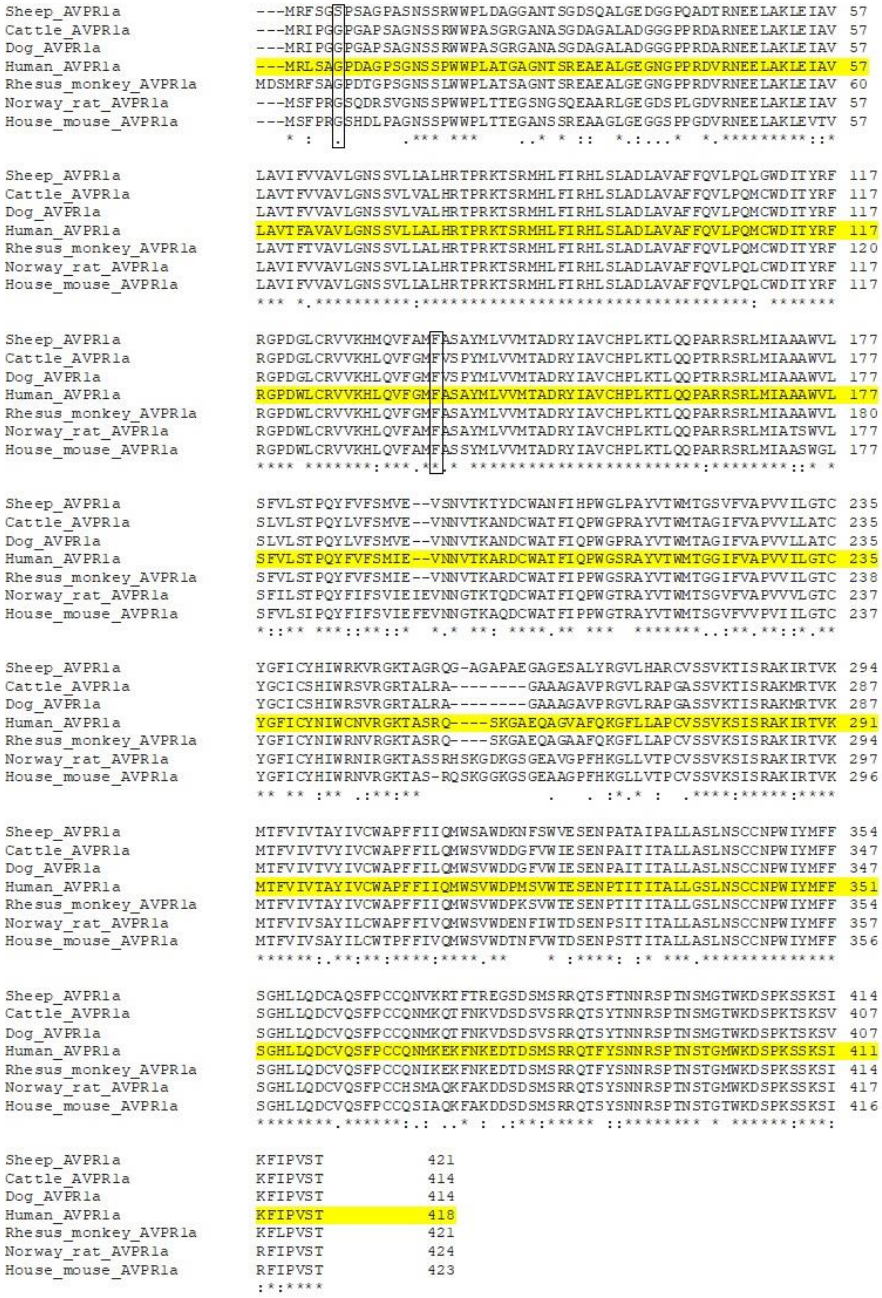


Figure 36: Multiple sequence alignment of mammalian arginine vasopressin receptor 1a amino acid sequences

*Reference sequences (Human AVPR1a: NM_000706.5, House mouse AVPR1a: NM_016847.2, Norway rat AVPR1a: NM_053019.2, Rhesus monkey AVPR1a: XM_001116798.4, Cattle AVPR1a: NM_001104990.1, Sheep AVPR1a: NM_001199792.1, and Dog AVPR1a: NM_001198658.1) were aligned using Clustal Omega. The human AVPR1a sequence is highlighted in yellow. The residues noted to be different between species are shown below the aligned sequence where: * indicates the same residue, one dot indicates similar residues, two dots indicate different residues, and a space indicates very different residues. The residues selected for this study are indicated with the black block.*

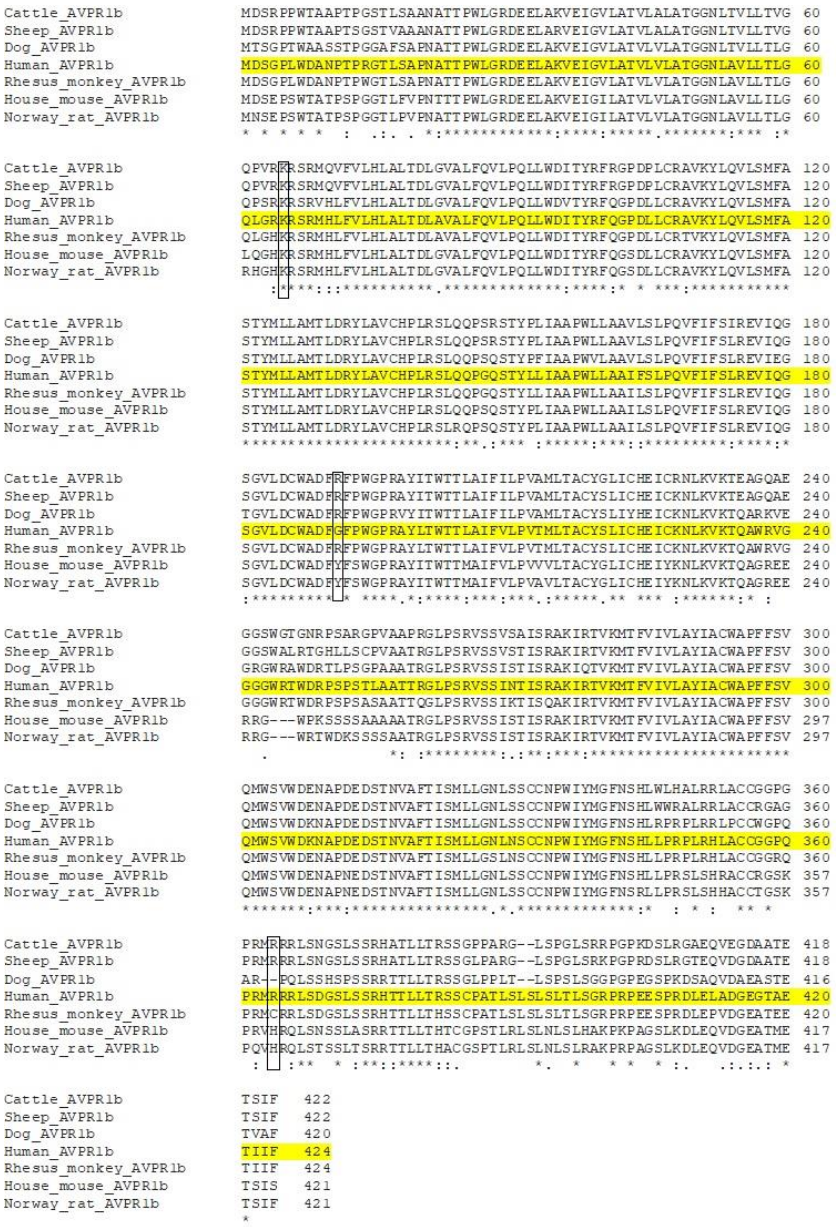


Figure 37: Multiple sequence alignment of mammalian arginine vasopressin receptor 1b amino acid sequences

*Reference sequences (Human AVPR1b: NM_000707.5, House mouse AVPR1b: NM_011924.2, Norway rat AVPR1b: NM_001289800.1, Rhesus monkey AVPR1b: NM_001246222.1, Cattle AVPR1b: NM_001192142.1, Sheep AVPR1b: NM_001246237.1, and Dog AVPR1b: XM_545695.4) were aligned using Clustal Omega. The human AVPR1b sequence is highlighted in yellow. The residues noted to be different between species are shown below the aligned sequence where: * indicates the same residue, one dot indicates similar residues, two dots indicate different residues, and a space indicates very different residues. The residues selected for this study are indicated with the black block.*

Analysis of Conservation of Residues in OXTR/AVPR's in Human Gene

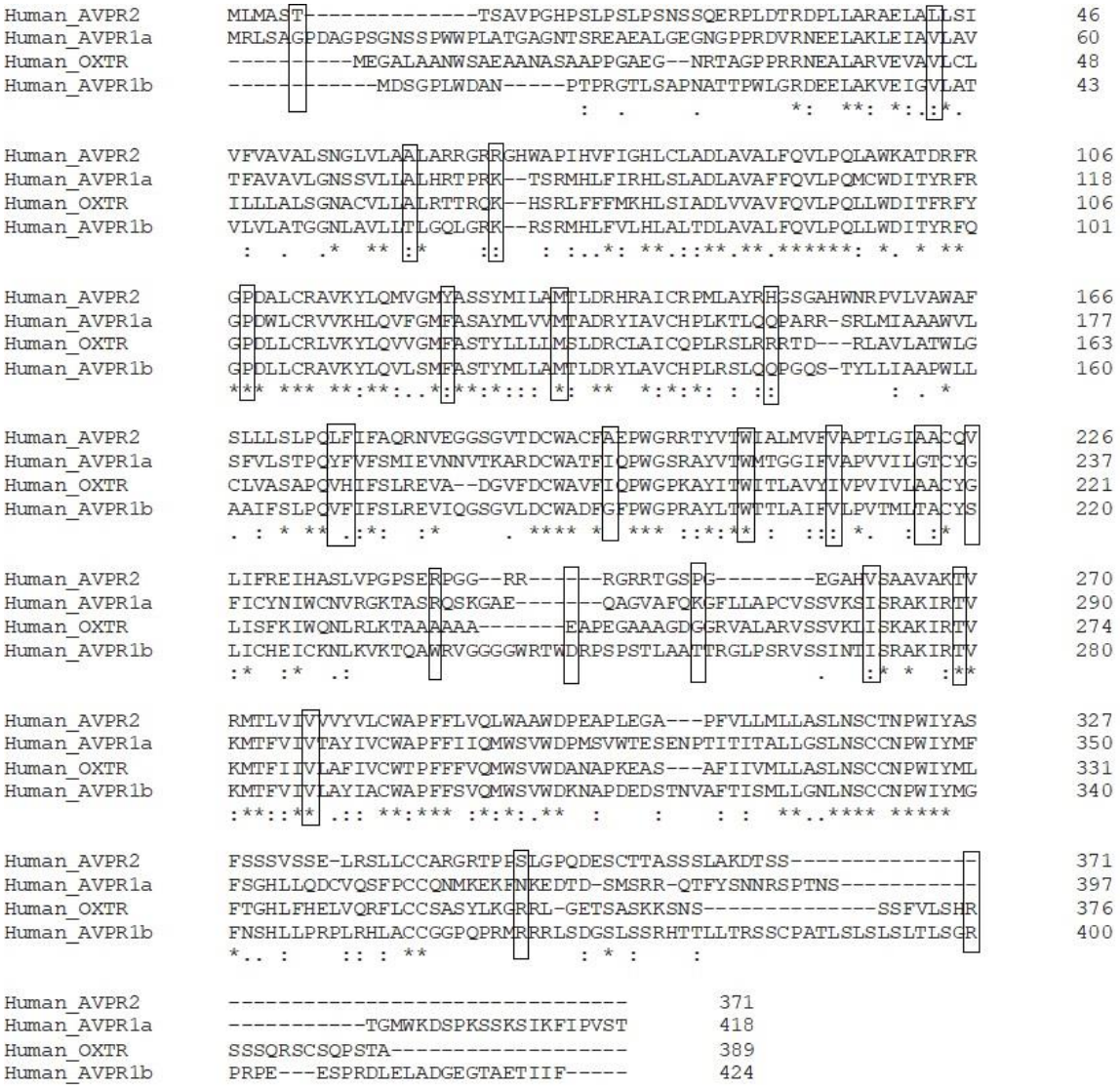


Figure 38: Multiple sequence alignment of human neurohypophysis hormone receptor amino acid sequences

*Reference sequences (Human AVPR2: NM_000054.7. Human AVPR1a: NM_000706.5, Human OXTR: NM_000916, and Human AVPR1b: NM_000707.5) were aligned using Clustal Omega. The residues noted to be different between species are shown below the aligned sequence where: * indicates the same residue, one dot indicates similar residues, two dots indicate different residues, and a space indicates very different residues.*

Table 20: Summary of GMoS analysis of residue conservation for transmembrane-located variant residues

	Variant	BW	Location	Most common residues (%)				Other residues (%)	
OXTR	V45L	1.38	TM 1	**L (31)**	**V (25)**	F (10)	I (9)		
	A63G	1.56	TM 1	F (20)	V (16)	I (15)	L (12)	**A (6)**	**G (4)**
	A63V	1.56	TM 1	F (20)	**V (16)**	I (15)	L (12)	**A (6)**	
	M133V	3.46	TM 3	I (57)	L (17)	**M (13)**	**V (10)**		
	V172A	4.61	TM 4	L (33)	I (13)	F (12)	**A (12)**	**V (7)**	
	H173R	4.62	TM 4	L (23)	V (17)	I (16)	F (15)	**R (1)**	**H (1)**
	W203R	5.41	TM 5	L (16)	V (11)	F (10)	Y (10)	**W (4)**	**R (1)**
	L206V	5.44	TM 5	**L (26)**	**V (20)**	I (18)	F (10)		
	A217T	5.55	TM 5	L (18)	V (15)	**A (1**5)	I (13)	**T (9)**	
	A218T	5.56	TM 5	F (20)	V (16)	I (15)	L (11)	**A (6)**	**T (6)**
	G221S	5.59	TM 5	**G (14)**	L (12)	T (11)	**S (10)**		
	T273R	6.33	TM 6	A (31)	V (19)	L (10)	**T (10)**	**R (1)**	
	T273M	6.33	TM 6	A (31)	V (19)	L (10)	**T (10)**	**M (3)**	
	V281M	6.41	TM 6	**V (39)**	L (23)	I (11)	**M (8)**		
AVPR1a	F136L	3.37	TM 3	Y (21)	T (17)	**F (16)**	**L (10)**		

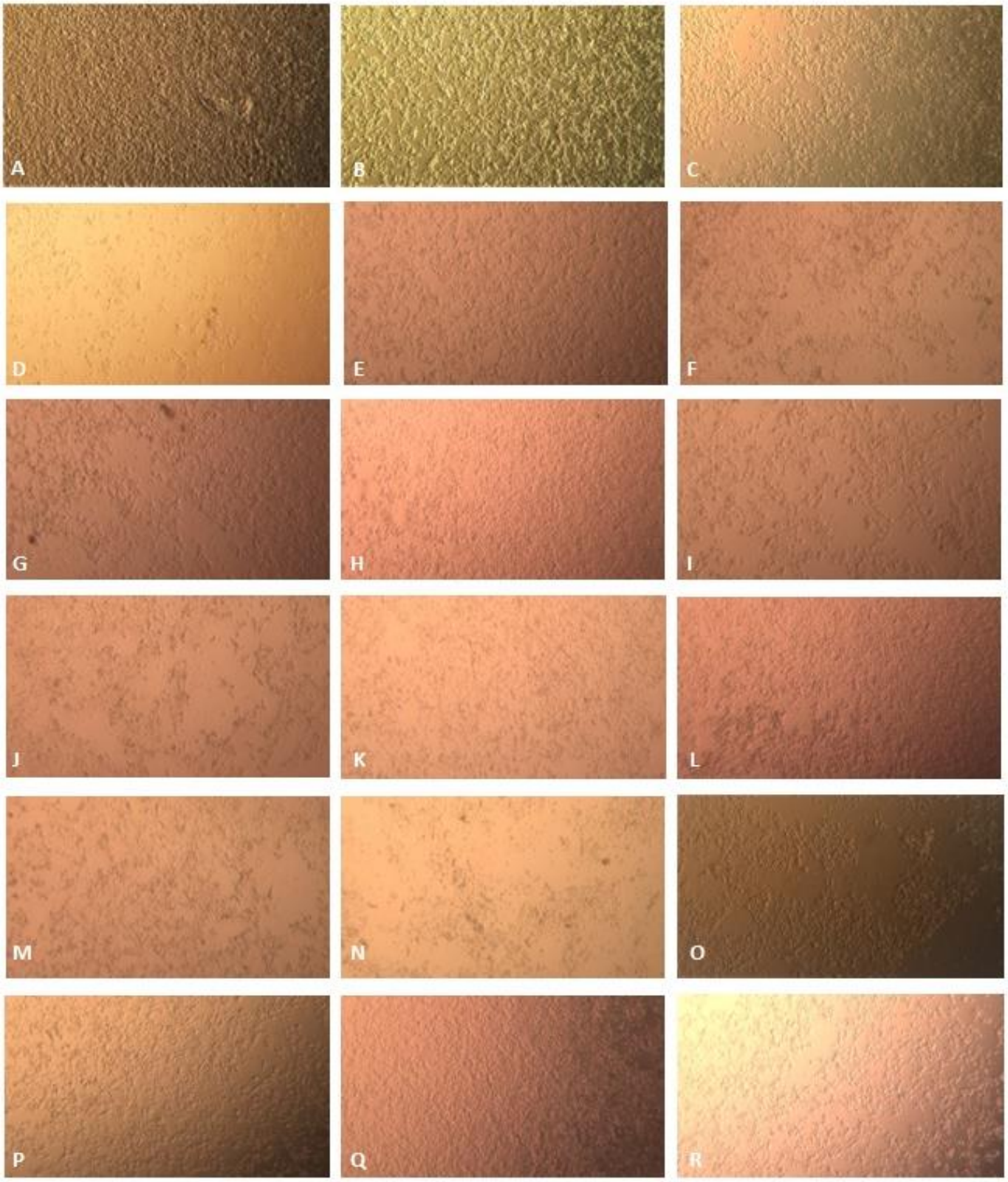
The text in bold identifies the residues which are relevant to analysed mutation. The number in brackets are the percentage of occurrence within the GPCR family. The last column comments on the specific change in residue in relation to the common residues in GPCRs. Note that this program can only mutations found in the transmembrane domain of the GPCR.

Table 21 : Variant effect prediction program predictions for identified variants.

Variant	Rs number	SIFT	Polyphen	FATHMM	LRT	Provean	Mut. Asses	Mut. Test	Total Deleterious
OXTR									
V45L	rs201689053	0	0.99	T, T	D	M	D	D	5/7
A63G	rs171114	0,03	0,4	T, T	D	D	M	D	5/7
A63V	rs171114	0,04	0,66	T, T	D	D	L	D	5/7
P108A	rs202138705	0	0.93	T	D	M	D	D	5/7
M133V	rs201782300	0.01	1	T	D	L	D	D	5/7
R150L		0	0.93	T	D	D	D	D	6/7
R150S	rs547238576	0.01	0.91	T	D	M	D	N	4/7
V172A	rs115324487	0.3	0.03	T	N	N	D	N	1/7
H173R		0,04	0,42	T	D	D	D	D	5/7
W203R		0	0.93	T	D	T	D	D	5/7
L206V	rs150746704	0.45	0.09	T	D	L	D	N	2/7
A217T		1	0,01	T	N	N	N	D	1/7
A218T	rs4686302	0,4	0,01	T	N	N	N	P	0/7
G221S	rs143908202	0.35	0.585	T	N	L	D	D	4/7
A238T	rs61740241	0,53	0,02	T	U	N	N	N	0/7
E242K		0,79	0,01	T	N	N	M	N	1/7
G252A		0,91	0	T	N	N	N	N	0/7
I266V	rs770798571	0	0.99	T	D	M	D	D	5/7
T273R	rs237901	0	1	T	D	D	H	D	6/7
T273M	rs237901	0	1	T	D	D	H	D	6/7
V281M	rs144814761	0	1	T	D	M	D	D	5/7
R376C	rs35062132	0,01	0	T	N	N	L	N	1/7
R376G	rs35062132	0,03	0,01	T	N	N	L	N	1/7
AVPR1a									
G6S	rs2228154	0,31	0,01	T	N	N	N	P	0/7
F136L	rs1042615	0	1	T	D	D	M	P	6/7
AVPR1b									
K65N	rs35369693	0,01	0,63	T		D			3/4
G191R	rs33990840	0,66	0	T		N			0/4
G191S	rs33990840	0,28	0,11	T		N			0/4
R364H	rs28632197	0,27	0	T		N			0/4
R364L	rs28632197	0,07	0	T		D			0/4

For the SIFT and Polyphen (red : deleterious, yellow: possibly damaging, green: benign, blue: tolerated low confidence) FATHMM (T : tolerant), LRT and Provean (D: deleterious, N: neutral, U: unkown), Mutation tester (D: disease causing, N: neutral polymorphism, P: automatic polymorphism), Mutation Assessor (H: high functional impact, M: medium functional impact, L: low functional impact). Blank spaces indicate that the AVPR1b sequence did not meet the requirements for the nonsynonymous mutation to be scored in the excluded programs.

Appendix 1D: Cell Images 48 hours post-transfection with OXTR variants



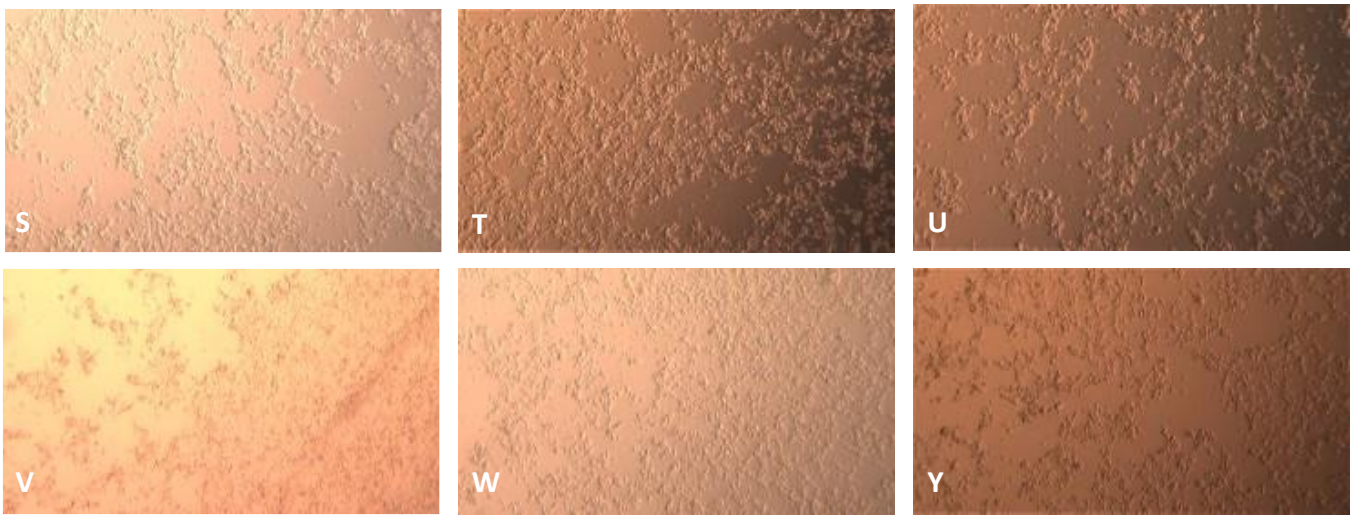


Figure 39: Cell images 48 hours post transfection of OXTR variants

HEK 293T cells were transiently transfected with either ***A:*** *empty vector,* ***B:*** *WT OXTR, or OXTR variant:* ***C:*** *A63G,* ***D:*** *A63V,* ***E:*** *A217T,* ***F:*** *A218T,* ***G:*** *A238T,* ***H:*** *E242K,* ***I:*** *G221S* ***J:*** *G252A,* ***K:*** *H173R,* ***L:*** *I266V,* ***M:*** *L206V,* ***N:*** *M133V,* ***O:*** *P108A,* ***P:*** *R150L,* ***Q:*** *R150S,* ***R:*** *R376C,* ***S:*** *R376G,* ***T:*** *T273M,* ***U:*** *T273R,* ***V:*** *V45L,* ***W:*** *V172A,* ***X:*** *V281M and* ***Y:*** *W203R. Microscopic images were taken 48 hours post transfection at 4x Magnification.*

Appendix 1E: Cell Viability

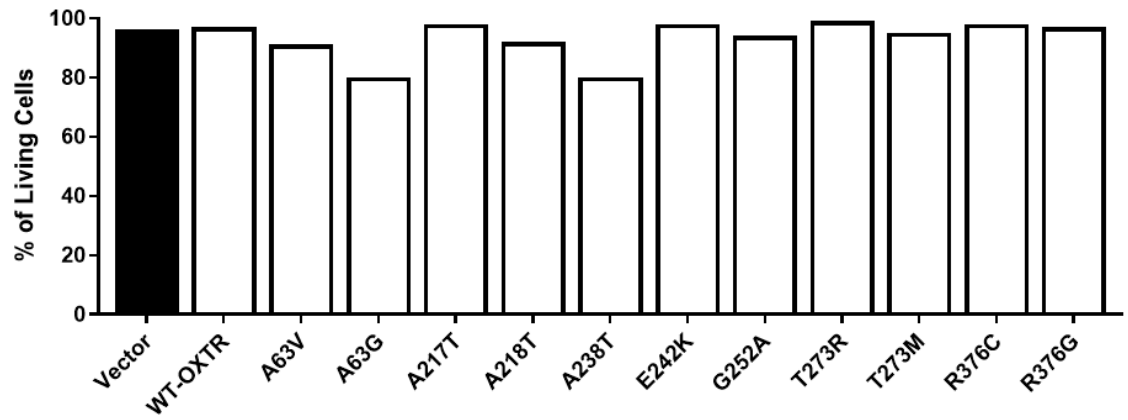


Figure 40: Percentage of living cells 24 hours post-transfection in psychological disorder linked OXTR variants.

HEK 293T cells were transiently transfected with either empty vector, WT OXTR, or OXTR variant linked to psychological disorders. The percentage of viable cells was measured using the Cell Countess II following the dying of the cells using trypan blue. Data are presented from one independent experiment (N=1). No Condition resulted in a decrease in cell viability when compared to vector.

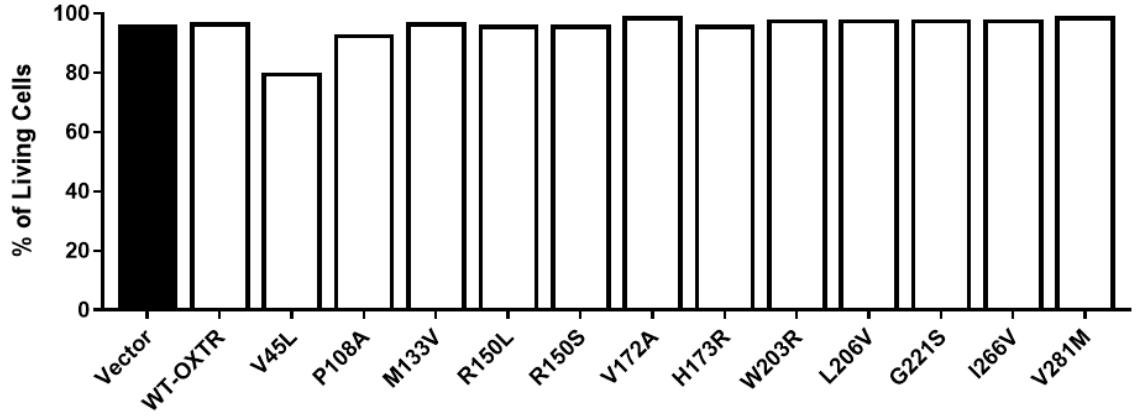


Figure 41: Percentage of living cells 24 hours post-transfection in preterm birth disorder linked OXTR variants.

HEK 293T cells were transiently transfected with either empty vector, WT OXTR, or OXTR variant linked to preterm birth disorders. The percentage of viable cells was measured using the Cell Countess II following the dying of the cells using trypan blue. Data are presented from one independent experiment (N=1). No condition resulted in a decrease in cell viability when compared to vector.

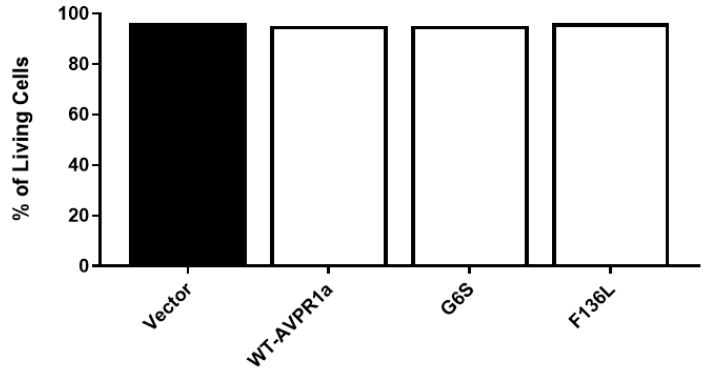


Figure 42: Percentage of living cells 24 hours post-transfection in psychological linked AVPR1a variants.

HEK 293T cells were transiently transfected with either empty vector, WT AVPR1a, or AVPR1a variant linked to psychological disorders. The percentage of viable cells was measured using the Cell Countess II following the dying of the cells using Trypan Blue. Data are presented from one independent experiment (N=1). No condition resulted in a decrease in cell viability when compared to vector.

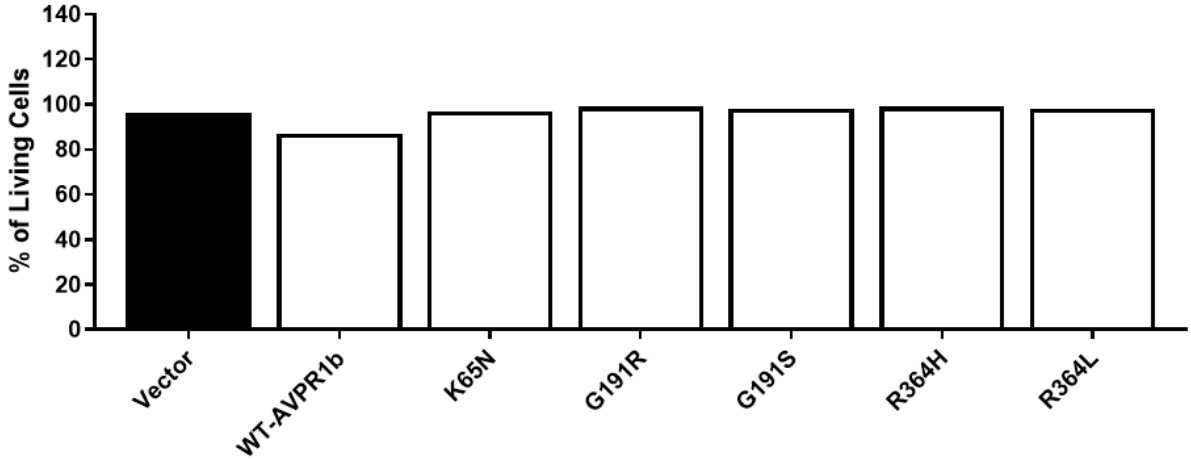


Figure 43: Percentage of living cells 24 hours post-transfection in psychological linked AVPR1a variants.

HEK 293T cells were transiently transfected with either empty vector, WT AVPR1b, or AVPR1b variant linked to psychological disorders. The percentage of viable cells was measured using the Cell Countess II following the dying of the cells using trypan blue. Data are presented from one independent experiment (N=1). No condition resulted in a decrease in cell viability when compared to vector.

Appendix 1F: Receptor ELISA Optimisation

Extensive optimisation was performed in order to determine the most efficient transfection period as well as the primary and secondary antibody concentration for the total and cell surface ELISA to ensure the best signal-to-noise ratio was achieved. Total receptor expression was measured in cells transfected with the WT OXTR, AVPR1a or AVPR1b (for measurement of 'signal') and was compared to that of cells transfected with empty vector (for measurement of background/'noise'). Two different transfection periods were tested, 24h and 48h and it was observed that at 48h post-transfection, the greatest signal-to noise (fold-over vector-transfected cells) was observed for all of the WT receptors (Figure 44).

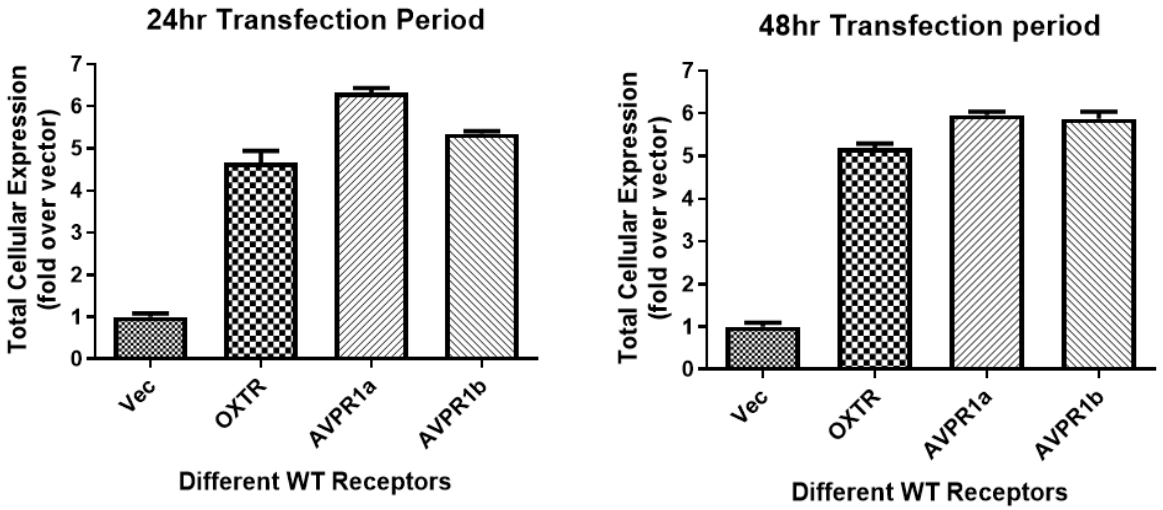


Figure 44: Total cellular expression of WT OXTR, AVPR1a and AVPR1b after a 24- or 48-hour transfection period.

HEK 293T cells were transiently transfected with either WT OXTR, AVPR1a, AVPR1b or empty vector (Vec). Total expression of WT receptors was measured by a receptor ELISA following permeabilisation of the cells. Data are presented from one independent experiment (N=1) and are calculated as fold-over vector transfected cells to determine signal-to-noise ratios.

Using different concentrations of primary and secondary antibodies, it was seen that the combination of 1:5000 for both primary and secondary antibody achieved the highest signal-to-noise ratio (Figure 45).

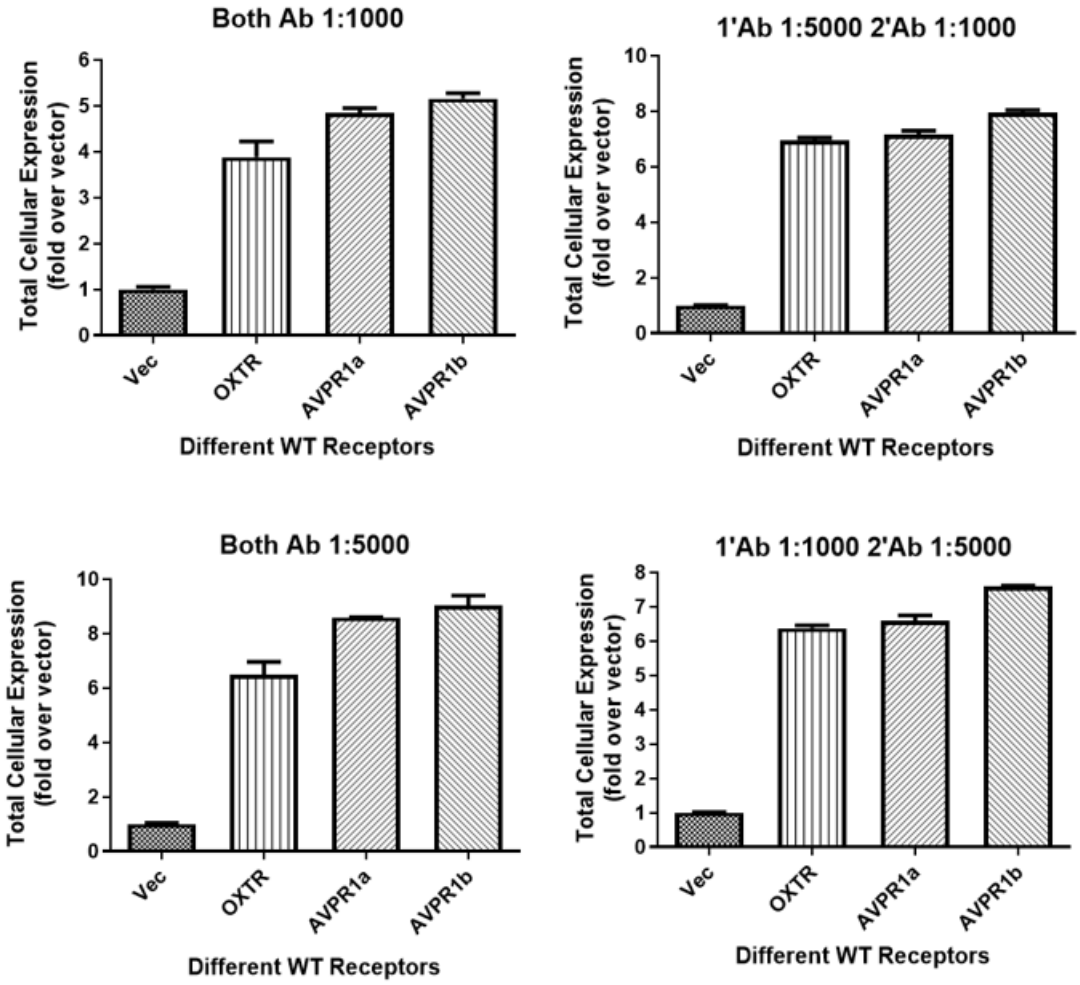


Figure 45: Total cellular expression of WT OXTR, AVPR1a and AVPR1b measured using different antibody concentrations.

HEK 293T cells were transiently transfected with either WT OXTR, AVPR1a, AVPR1b or empty vector (Vec). Total expression of WT receptors was measured by a receptor ELISA following permeabilisation of the cells and using different combination of primary and secondary antibody concentrations as indicated. Data are presented from one independent experiment (N=1) and are calculated as fold-over vector transfected cells to determine signal-to-noise ratios.

Appendix 1G: Radioligand Binding Assay Optimisation

Extensive optimisation was performed in order to determine the conditions to ensure the best signal-to-noise ratio was achieved. Radioligand binding was measured in membranes prepared from cells transfected with the WT OXTR (for measurement of 'signal') and was compared to that of membranes prepared from cells transfected with empty vector (for measurement of background/'noise'). The published affinity of AVP for the OXTR is 1.7 nM.[120] In our initial pilot radioligand binding assay, [^{3}H]-AVP was used at 2 nM, which resulted in no difference in measurable binding between vector and WT OXTR membranes (data not shown). Therefore, a range of concentrations of [^{3}H]-AVP (3 nM to 7 nM) was tested to determine the non-specific and specific binding (Figure 46). However, in all of the conditions tested, the background binding was too high in order to measure accurate specific ligand binding to the WT-OXTR. Due to limitations in radioligand availability, which prevented further optimisation, it was therefore decided to not proceed with radioligand binding assays with the OXTR variants.

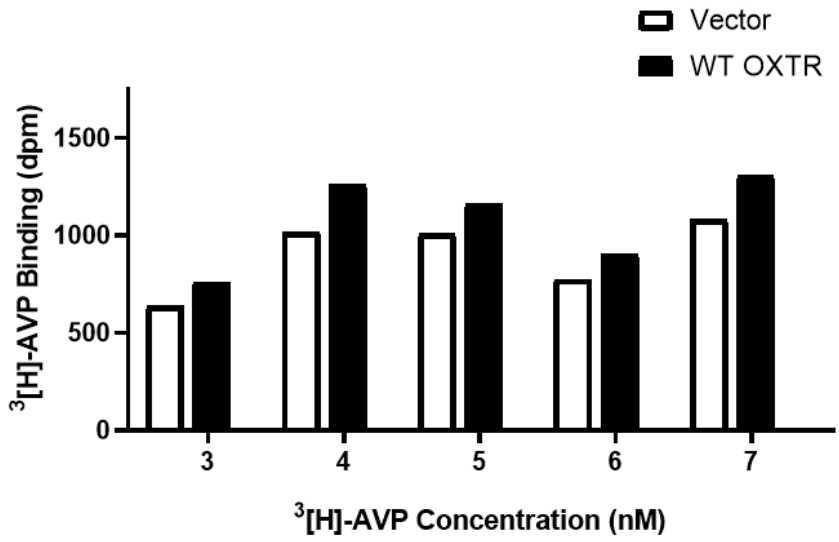


Figure 46: [^{3}H]-AVP binding to wild type OXTR.

Binding response (dpm) of [^{3}H]-AVP to membranes prepared from HEK 293T cells transiently transfected with vector or WT OXTR (WT OXTR) was measured by radiolabelled ligand binding assay following incubation with a range of concentrations of [3H]-AVP (3 nM to 7 nM). Data are presented as mean ± SEM from one experiment (N=1).

Appendix 2: MSc Protocol Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MSc Committee
School of Medicine
Faculty of Health Sciences

20 April 2022

Dr C Newton
Department of Physiology
Faculty of Health Sciences

Dear Dr,

Ms MA Steven, Student no 17004455

Please receive the following comments with reference to the MSc Committee submission of the abovementioned student:

Student name	Ms MA Steven	**Student number**	17004455
Name of study leader	Dr C Newton		
Department	Physiology		
Title of MSc	**Revised approved title: Functional characterisation of gene variants of oxytocin and vasopressin receptors implicated in psychological disorders** Functional characterisation of gene variants of oxytocin and vasopressin receptors implicated in psychiatric disorders		
Date of first submission	March 2021		
Comments to study leader March 2021	• Please revise the title – decide to focus either on psychological disorders or only on psychiatric disorders. Revise the aim to align with the title. • Please include a List of Abbreviations, List of Tables and List of Figures. • Methodology lacks detail – please expand the description of the different procedures. Include instrument settings to be used. • Please include manufacturer's details of all instruments and reagents. • Please correct all typographical errors. • Include a section describing the project outputs. • Include a data capturing sheet. • Revise the reference list as it has several inconsistencies; please include all authors – do not use et al. in reference list.		
	• Thank you for submitting the revised protocol and documents requested.		
May 2021	• Thank you for submitting the ethics approval letter.		
March 2022	• Thank you for nominating the examiners.		
Decision	This protocol has been approved. Ethics approval has been obtained. The examiners have been approved.		

Yours sincerely

Prof Marleen Kock (Chair: MSc Committee)

MSc Committee, School of Medicine
Faculty of Health Sciences
University of Pretoria,
Private Bag X323
Pretoria 0001, South Africa
Tel +27 (0)12 319 2325
Fax +27 (0)12 323 0732

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix 3: MSc Ethics Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

14 April 2022

Approval Certificate
Annual Renewal

Dear Miss MA Steven,

Ethics Reference No.: 168/2021 – Line 1
Title: Functional characterisation of gene variants of oxytocin and vasopressin receptors implicated in psychological disorders

The **Annual Renewal** as supported by documents received between 2022-03-16 and 2022-04-13 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-04-13 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2023-04-14.
- Please remember to use your protocol number (168/2021) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
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University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

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Appendix 4: Letter of Statistical Clearance

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences
Department of Immunology

Letter of Statistical Clearance

Thursday, March 25, 2021

This letter is to confirm that the MSc student with the Name: **Michelle Stevens**, Student No: **17004455** studying at the University of Pretoria discussed the project with the title; **Functional characterisation of gene variants of oxytocin and vasopressin receptors implicated in psychological disorders** with me.

I hereby confirm that I am aware of the project and the statistical analysis of the data generated for the project is appropriate for achieving the research aims. Statistics will be calculated on Graphpad Prisms 7.

Yours sincerely

Prof Pieter WA Meyer

Ass. Professor and HoD

Prof PWA Meyer
Head of Department: Immunology
ResCom appointed Biostatistician
University Pretoria

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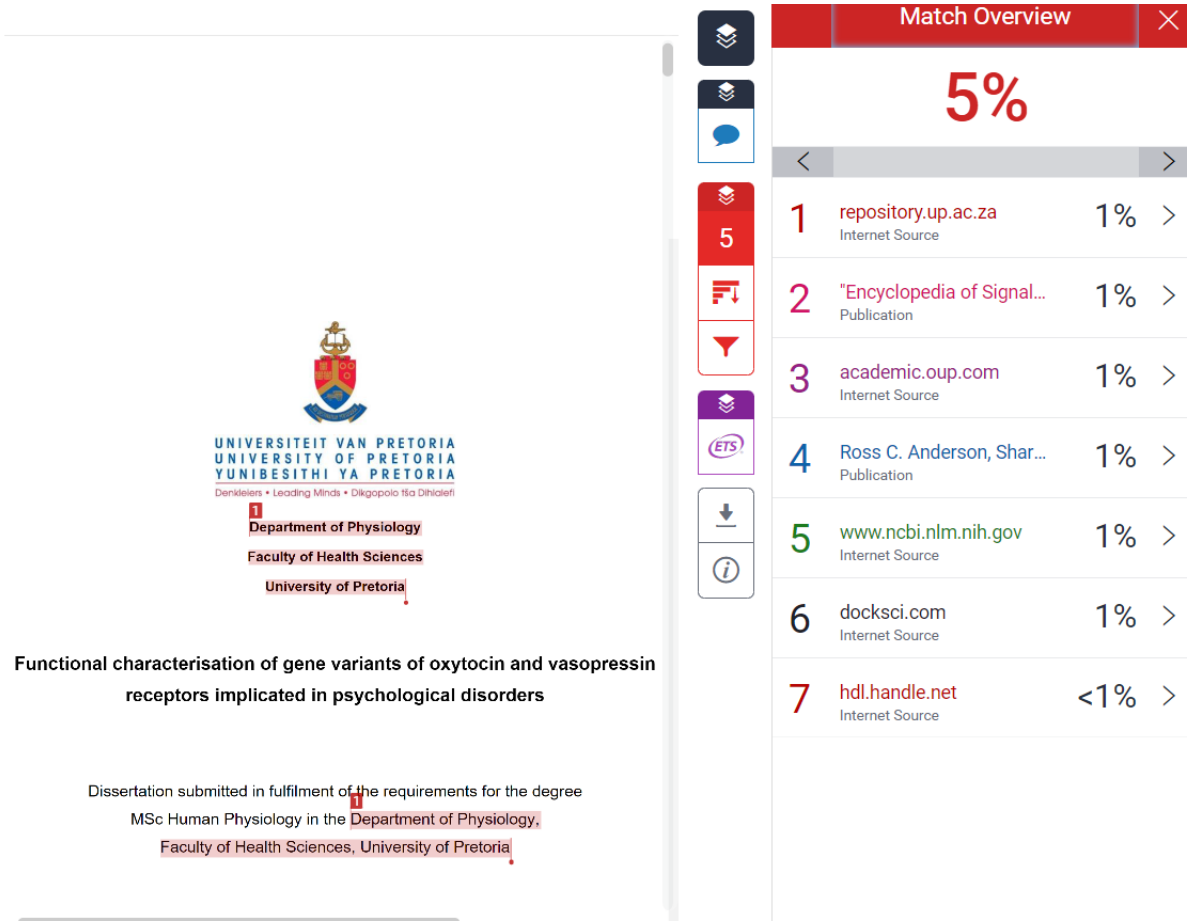
Departement Immunologie
Lefapha la Disaense tša Maphelo
Kgoro ya Immunolotši

Appendix 5: Turnitin Originality Report



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Department of Physiology
Faculty of Health Sciences
University of Pretoria

Functional characterisation of gene variants of oxytocin and vasopressin receptors implicated in psychological disorders

Dissertation submitted in fulfilment of the requirements for the degree
MSc Human Physiology in the Department of Physiology,
Faculty of Health Sciences, University of Pretoria

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