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THE IMPACT OF OXYTOCIN AND $GLYT_1$ INHIBITORS ON SOCIAL BEHAVIOUR

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Abstract

Schizophrenia is a complex mental disorder characterised by various symptoms which fall into three categories of positive, negative and cognitive. In particular, negative symptoms are poorly treated by current medications although several adjunctive therapies are under investigation including Glycine Transporter ($GlyT_1$) inhibitors and neuropeptides such as oxytocin. Despite accumulating preclinical and clinical evidence that such compounds can influence social behaviour and improve negative symptoms in patients, there is little information as to the precise mechanisms by which they work. Therefore, the aims of this thesis were to ultimately determine some of the key regions and potential signalling pathways activated following administration of these compounds in Lister-hooded rats.

Firstly, a functional map of $GlyT_1$ inhibitor RO4993850, an analogue to Bitopertin, identified the selective activation of neurons within the rostral and caudal prefrontal cortex (PFC), suggesting potential NMDA receptor activation in brain areas involved in motivation and goal-directed behaviour. This was further assessed in a novel 'dual-hit' neonatal-PCP isolation-rearing rodent model for schizophrenia which was shown herein to induce locomotor hyperactivity and social deficits including reduced social interaction (an index for negative symptoms) and increased communication (as assessed by ultrasonic vocalisations (USVs)). Interestingly neonatal-PCP isolation-reared rats emitted more pro-social 50 kHz USVs which were also longer in duration

and had a greater change in call bandwidth compared to controls. Neonatal-PCP isolation-rearing was also shown to selectively decrease parvalbumin expression (a calcium binding protein present in GABAergic interneurons) in the hippocampus but not in the rostral PFC sub-regions assessed, producing similar changes to other rodent models. Microdialysis studies however revealed no change to basal PFC and striatal dopamine levels in these rats. Chronic treatment with the $GlyT_1$ inhibitor RO4993850 improved social deficits in the social interaction test and altered both USV emissions and call characteristics but showed no effect on locomotor hyperactivity, parvalbumin expression in either the PFC or hippocampus, nor dopamine overflow in the PFC or striatum. Finally, an established dose of the neuropeptide oxytocin which did not influence core body temperature, was shown to attenuate PCP-induced hyperactivity, increase pro-social behaviour and selectively enhance dopamine release in the nucleus accumbens (NAc) in group-housed Lister-hooded rats. Thereby providing supporting evidence for regionally-specific oxytocin-dopaminergic interactions within the mesocorticolimbic circuits responsible for regulating associative and rewarding behaviour.

There are therefore several potential mechanisms by which both $GlyT_1$ inhibitors and oxytocin can influence social behaviour, most likely via activation of key brain loci involved in motivation. Although further work is required, results herein indicate the potential of $GlyT_1$ inhibitors and oxytocin as adjunctive therapies to treat predominant negative symptoms in schizophrenia.

Publications

Papers:

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Abbreviations

ACC	Anteriocingulate Cortex
aCSF	Artificial Cerebrospinal Fluid
ANOVA	Analysis of Variance
ASD	Autism Spectrum Disorders
ASST	Attentional Set-Shifting
AVP	Arginine Vasopressin
BBB	Blood Brain Barrier
BLA	Basolateral Amygdala
BMA	Basomedial Amygdala
BNSS	Brief Negative Symptom Scale
BOLD	Blood-Oxygen-Level-Dependent
BPRS	Brief Psychiatric Rating Scale
CA1/3	Cornu Ammonis 1/3
CAINS	Clinical Assessment Interview for Negative Symptoms
CB	Cannabinoid
CER	Conditioned Emotion Response
CG1/2	Cingulate Cortex ½

CNS	Central Nervous System
CNVs	Copy Number Variants
CSF	Cerebrospinal Fluid
D_{1-5}	Dopamine Receptors D_{1-5}
DAB	3, 3'- Diaminobenzidine
DG	Dentate Gyrus
DISC1	Disrupted In Schizophrenia 1
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, Edition 5
fMRI	Functional Magnetic Resonance Spectroscopy
GABA	Gamma-aminobutyric Acid
GAD_{67}	Glutamate Decarboxylase, 67kDa isoform
GAT-1	GABA Transporter 1
GD	Gestational Day
$GlyT_1$	Glycine Transporter 1 Inhibitor
GPCR	G-protein Coupled Receptor
GPi	Globus Pallidus internal segment
GSH	Glutathione
GWAS	Genome Wide Association Studies

HPA	Hypothalamic-Pituitary-Adrenal
HPLC	High Performance Liquid Chromatography
I.C.V.	Intracerebral route of administration
I.P.	Intraperitoneal route of administration
I.V.	Intravenous route of administration
IEG	Immediate Early Gene
IHC	Immunohistochemistry
IL	Infralimbic Cortex
kHz	Kilohertz
LMA	Locomotor Activity
MAM	Methyloxymethanol Acetate
mGluRs	Metabotropic Glutamate Receptors
MHC	Major Histocompatibility Complex
MRI	Magnetic Resonance Imaging
NAc	Nucleus Accumbens
NFPS	N[3-(40-fluorophenyl)-3-(40-phenylphenoxy)-propyl]-sarcosine
NMDA	N-Methyl-D-Aspartate
NOD	Novel Object Discrimination

NRG1	Neuregulin 1
OFC	Orbital Frontal Cortex
P.O.	Oral route of administration
PANSS	Positive and Negative Syndrome Scale
PBS	Phosphate Buffered Saline
PCP	Phencyclidine
PCP-SI	Neonatal Phencyclidine combined with Social Isolation
PET	Position-emission Tomography
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
PGC Consortium	Psychiatric Genomics Consortium
PND	Postnatal Day
PolyI:C	Polyinosinic:polycytidylic Acid
PPI	Pre-Pulse Inhibition
PRL	Prelimbic Cortex
PVN	Paraventricular Nucleus
REDOX	Oxidation-Reduction Reaction
RNS	Reactive Nitrogen Species

ROS	Reactive Oxygen Species
S.C.	Subcutaneous route of administration
SAL-GH	Neonatal Saline combined with Group Housing
SANS	Scale for Assessment of Negative Symptoms
SEM	Standard Error of the Mean
SN	Substantia Nigra
SNPs	Single Nucleotide Polymorphisms
SOD	Superoxide Dismutase
SON	Supraoptic Nucleus
SPECT	Single-photon Emission Computed Tomography
SR49059	(2S)-1-[[(2R,3S)-5-Chloro-3-(2-chlorophenyl)-1-[(3,4-dimethoxyphenyl)sulfonyl]-2,3-dihydro-3-hydroxy-1H-indol-2-yl]carbon-yl]-2-pyrrolidinecarboxamide
TNFα	Tumour Necrosis Factor Alpha
USV	Ultrasonic Vocalisations
VTA	Ventral Tegmental Area
5-HT	Serotonin

Table of Contents

Chapter 1: General Introduction..... 1

1.1: Thesis overview 1

1.2: Background of schizophrenia 1

1.3: Symptoms of schizophrenia 2

1.4: Genetic risk factors for schizophrenia 4

1.4.1: Pre-GWAS 4

1.4.2: Post-GWAS.....7

1.4.3: Genetics and the environment.....9

1.5: The neurodevelopmental hypothesis.....14

1.6: Neurobiology of schizophrenia.....15

1.6.1: Dopamine hypothesis.....15

1.6.2: Glutamate hypothesis 17

1.7: Morphological changes 20

1.8: Treating schizophrenia 21

1.8.1: Typical antipsychotics.....21

1.8.2: Atypical antipsychotics 22

1.8.3: Third generation antipsychotics.....23

1.8.4: Modulating glutamate.....24

1.8.5: Adjunctive therapies....28

1.9: Animal models of schizophrenia....35

1.9.1: Drug-induced models....36

1.9.2: Environmental models....40

1.9.3: 'Two-hit' models....47

1.10: Assessing 'schizophrenia' in rodents....52

1.10.1: Assessing positive symptoms....52

1.10.2: Assessing negative symptoms....53

1.11: Concluding remarks....58

1.12: Thesis aims....59

Chapter 2: Assessing neuronal activation induced by $GlyT_1$ inhibitor RO4993850 in the rat brain....60

2.1: Introduction....60

2.1.1: C-fos as a neuronal marker....60

2.1.2: Glycine Transporter Inhibitors....65

2.2: Aims....68

2.3: Methods....69

2.3.1: $GlyT_1$ inhibitor induced c-fos activation....69

2.3.2: PCP induced c-fos activation....72

2.4: Results....74

2.4.1: $GlyT_1$ inhibitor induced c-fos activation 74

2.4.2: PCP induced c-fos activation 78

2.5: Discussion 82

2.5.1: $GlyT_1$ inhibitor induced c-fos activation 82

2.5.2: Antipsychotic-induced c-fos activation 83

2.5.3: The role of the OFC in schizophrenia 84

2.5.4: Impaired signalling within the OFC 86

2.5.5: Morphological changes in the OFC 87

2.5.6: PCP-induced c-fos activation 89

2.5.7: Summary 90

Chapter 3: Effects of chronic $GlyT_1$ inhibitor treatment on social behaviours in a neurodevelopmental rat model 92

3.1: Introduction 92

3.1.1: Neonatal-PCP isolation-rearing 92

3.1.2: $GlyT_1$ inhibitor RO4993850 93

3.1.3: Aims 95

3.2: Methods 96

3.2.1: Neonatal-PCP Isolation-Rearing 96

3.2.2: Experimental Design 97

3.3: Study 1: 98

Part 1: Effects of neonatal-PCP isolation-rearing on behaviour.....98

3.3.1: Locomotor Activity (LMA).....98

3.3.2: Chronic Injections.....99

3.3.3: Social Interaction.....99

3.3.4: Ultrasonic Vocalisations (USVs).....100

3.4: Study 1:.....103

Part 2: Cortical effects in neonatal-PCP isolation-reared rats.....103

3.4.1: C-fos immunoreactivity following social interaction.....103

3.4.2: Parvalbumin immunoreactivity following social interaction 103

3.5: Study 2:.....106

Effects on dopamine overflow following an acute dose of RO4993850..106

3.5.1: Stereotaxic surgery.....106

3.5.2: Collection of Dialysate Samples.....106

3.5.3: Confirmation of Probe Placement.....107

3.5.4: Analysis of Monoamines.....108

3.6: Results:.....109

Study 1:.....109

Part 1: Effects of neonatal-PCP isolation-rearing on behaviour.....109

3.6.1: Novel-arena induced drug-free LMA.....109

3.6.2: LMA following acute RO4993850.....112

3.6.3: Social interaction following chronic treatment with $GlyT_1$ inhibitor RO4993850.....114

3.6.4: USVs during social interaction.....123

3.6.5: Comparing behaviour to USV emissions 133

3.6.6: Study 1:.....135

Part 2: Cortical effects in neonatal-PCP isolation-reared rats.....135

3.6.7: Study 2:.....142

Effects on dopamine overflow following an acute dose of $GlyT_1$ inhibitor RO4993850.....142

3.7: Discussion 147

3.7.1: Study 1:.....147

Part 1: Effects of the $GlyT_1$ inhibitor RO4993850 on behaviour 147

3.7.2: Social Interaction 152

3.7.3: USVs during social interaction.....156

Part 2: Effects of the $GlyT_1$ inhibitor RO4993850 on cortical connectivity 162

3.7.4: C-fos immunoreactivity 162

3.7.5: Parvalbumin immunoreactivity 165

Study 2:.....170

3.7.6: Effects of $GlyT_1$ inhibitor RO3994850 on monoamines.....170

3.8: Future Work.....171

3.9: Summary.....172

Chapter 4: Effects of oxytocin on social behaviours in drug-naïve rats 174

4.1: Introduction.....174

4.1.1: Effects of oxytocin on social behaviour.....174

4.1.2: Oxytocin: route of administration.....176

4.1.3: Oxytocin: mechanism of action.....177

4.1.4: Therapeutic potential of oxytocin in neuropsychiatric disorders 182

4.2: Aims.....187

4.3: Methods.....188

4.3.1: Animals.....188

4.3.2: Experiment Design.....188

4.3.3: Drugs.....190

4.3.4: Measuring body temperature.....190

4.3.5: Locomotor activity.....191

4.3.6: Social interaction.....191

4.3.7: USVs.....192

4.3.8: Stereotaxic surgery.....192

4.3.9: Microdialysis probe implantation.....192

4.3.10: Collection of dialysate samples 193

4.3.11: Analysis of monoamines.....193

4.4: Results.....194

Experiment 1: Oxytocin dose-response on LMA and body temperature 194

4.4.1: Effect of oxytocin on LMA 194

4.4.2: Effect of oxytocin on body temperature 194

Experiment 2: Oxytocin, behaviour and oxytocin-dopamine interactions.....197

4.4.3: Effect of oxytocin on PCP-induced hyperactivity and temperature.....197

4.4.4: Effect of oxytocin on social interaction in drug-naïve rats....199

4.4.5: Effect of oxytocin on USVs.....202

4.4.6: Effect of oxytocin on monoamines.....204

4.5: Discussion 206

Experiment 1: Oxytocin dose-response on LMA and body temperature 206

Experiment 2: Oxytocin, behaviour and oxytocin-dopamine interactions.....208

4.5.1: Effect of oxytocin on PCP-induced hyperactivity and temperature.....208

4.5.2: Effect of oxytocin on social interaction 211

4.5.3: Effect of oxytocin on USVs.....213

4.5.4: Effect of oxytocin on monoamines.....214

4.5.5: Future Studies.....216

4.5.6: Summary.....217

Chapter 5: General Discussion..... 219

5.1: Summary of findings.....219

5.2: Altered signalling within schizophrenia.....222

5.3: Translational relevance of the 'dual-hit' neonatal-PCP isolation-rearing model.....226

5.4: Future studies.....228

5.5: Conclusions.....229

References..... 231

List of Figures

Figure 1.1: Diagrammatic representation of oxytocin transport in the brain..34

Figure 2.1: Intracellular signalling pathways that lead to c-fos production.....64

Figure 2.2. Diagrammatic representation of rat brain areas selected for c-fos immunohistochemistry:.....75

Figure 2.3: Density of c-fos counts (counts/μm^2) in various brain sub-regions: 77

Figure 2.4. Example images taken from the ventral OFC. 79

Figure 2.5. Density of c-fos counts (counts/μm^2) in various brain sub-regions: 81

Figure 3.1: Novel-arena drug-free locomotor activity (LMA).....111

Figure 3.2: Locomotor activity (LMA) following treatment with a $GlyT_1$ inhibitor. 113

Figure 3.3: Total time spent in social interaction. 115

Figure 3.4: Time spent in individual interactive behaviours during social interaction. 117

Figure 3.5: Latency to engage in interactive behaviours.....121

Figure 3.6: Total distance moved during social interaction. 122

Figure 3.7: Number of ultrasonic vocalisations emitted between each rat pair during social interaction. 125

Figure 3.8: Example spectrograph images of ultrasonic vocalisations.....126

Figure 3.9: Duration of ultrasonic vocalisations emitted between each rat pair during social interaction. 129

Figure 3.10: Changes in call bandwidth for each call sub-type emitted between each rat pair during social interaction. 132

Figure 3.11: Density of c-fos counts (counts/μm^2) in various brain sub-regions. 138

Figure 3.12: Mean cell counts for parvalbumin immunoreactivity in various brain sub-regions. 140

Figure 3.13: Parvalbumin staining of hippocampal sections. 141

Figure 3.14: Changes to dopamine overflow in the PFC and striatum.....145

Figure 3.15: Probe placement for stereotaxic surgery for microdialysis. 146

Figure 4.1: Oxytocin receptor expression in the social decision-making network across four vertebrate classes. 181

Figure 4.2: Effects of oxytocin on locomotor activity and core body temperature.....196

Figure 4.3: Effects of oxytocin on PCP-induced locomotor activity and core body temperature.....198

Figure 4.4: Effects of oxytocin on social interaction 201

Figure 4.5: Effects of oxytocin on pro-social 50 kHz ultrasonic vocalisations. 203

Figure 4.6: Effects of oxytocin on monoamines. 205

Figure 4.7: Mechanism by which oxytocin can inhibit locomotor activity. 210

Figure 5.1: Altered signalling within schizophrenia. 225

List of Tables

Table 1.1: Summary of select behavioural and neurochemical changes observed in various animal models for schizophrenia. 51

Table 3.1: Behaviour observed between rat pairs during first USV call.....133

Table 3.2: C-fos immunoreactivity in selective brain regions 136

Table 3.3: Basal Levels (pmol/ml) of dopamine and metabolites 144

Chapter 1: General Introduction

1.1: Thesis overview

Schizophrenia is a complex mental disorder characterised by various symptoms which fall into three categories of positive, negative and cognitive. Current medications can rather effectively treat positive symptoms of the disease but not negative or cognitive symptoms. The overall aims of this thesis were to (1) investigate the effects of two pharmacological compounds; a novel glycine transporter ($GlyT_1$) inhibitor and oxytocin on rat behaviour and to (2) gain further insight into the behavioural and neurochemical changes induced in a neurodevelopmental 'two-hit' neonatal-PCP isolation-reared rat model, with particular focus on behaviour changes which have relevance to the negative symptoms of schizophrenia.

1.2: Background of schizophrenia

Schizophrenia is a chronic and severe mental disorder that affects approximately 1% of the population (NIMH). The first detailed description of the disorder, originally named dementia praecox, was laid down by Emil Kraeplin in 1893 (Ion and Beer, 2002) but the term schizophrenia i.e. splitting of the mind, was coined by Eugen Bleuler in 1908 and was intended to describe the separation of function between personality, thinking, memory, and perception (Ashok et al., 2012, Lawrie, 2013). Schizophrenia is conceptualised as a psychotic disorder, as indicated in the Diagnostic and Statistical Manual

(DSM-V) of Mental Disorders, but in addition to a psychotic pathology, the heterogeneity of the disorder means patients can often present with diverse symptom combinations (Tandon et al., 2013).

1.3: Symptoms of schizophrenia

Typically, schizophrenia manifests in adolescence and young adulthood, with men classically developing the disorder at an earlier age than women. The symptoms of schizophrenia fall into three main categories of positive, negative and cognitive. Core positive symptoms include delusions, hallucinations, and disorganized speech, which are diagnosed with a high reliability and are considered necessary for a reliable diagnosis of schizophrenia (Tandon et al., 2013).

Negative symptoms can be harder to recognise but the current consensus definition includes symptoms of affective flattening (poverty of speech), alogia, avolition, asociality, and anhedonia. These symptoms over the last few decades have been further sub-divided into two separate but inter-linked sub-domains; diminished expression (i.e. affective flattening and poverty of speech) and amotivation (i.e. avolition, apathy, asociality, anhedonic deficits; (Foussias and Remington, 2010, Messinger et al., 2011)). Current evidence does however suggest that anhedonia in the strictest sense of experiencing pleasure is not a characteristic feature of schizophrenia, but there may be a possible anticipatory pleasure deficit, beliefs of low pleasure and/or reduced pleasure-seeking behaviour (Foussias et al., 2014).

Negative symptom severity has been consistently linked to worse functional outcomes in schizophrenia, including specific relationships with impaired occupational functioning, household integration, social functioning, engagement in recreational activities and quality of life (Foussias et al., 2014). The amotivation subdomain of negative symptoms has demonstrated a significant relationship to functional outcomes in schizophrenia (Green et al., 2012). Since these symptoms are poorly addressed by current treatments, these patients are heavily dependent on others for day-to-day life. In 2012, the Schizophrenia Commission claimed that the cost of schizophrenia on society is approximately £12 billion a year in England alone (report available at https://www.rethink.org/about-us/the-schizophrenia-commission), reinforcing the current unmet clinical need for effective treatments which improve social behaviour and functioning.

Cognitive symptoms include deficits in learning and memory, with impairments seen in tasks using working memory, attention flexibility and executive function (Haier, 1980, Weinberger, 1988, Bukovskaya and Shmukler, 2016, Sahu et al., 2016, Mier and Kirsch, 2017, Zai et al., 2017). Treatments for this symptom domain, like negative symptoms, remain elusive with currently available antipsychotics showing little therapeutic efficacy. The lack of available treatments however, is largely due to poor understanding of the underlying mechanisms causing development and disease progression. At the same time, the relevance of both negative and cognitive symptoms in terms of functional

recovery has become central to rapidly expanding efforts focused on better understanding these domains and to establish effective treatments.

1.4: Genetic risk factors for schizophrenia

1.4.1: Pre-GWAS

The hereditary risk associated with schizophrenia was first determined in epidemiology studies, with incidence of the disease higher (6-17%) between parents and siblings, whilst monozygotic twins showed an approximate 50-80% risk of developing the disorder (Lewis and Lieberman, 2000b, Sullivan et al., 2003).

Original genetic studies highlighted several candidate genes that are thought to play a role in schizophrenia. Although recent genome-wide association studies (GWAS) have somewhat questioned the relevance of these candidate genes, as discussed later, their roles in synaptic development and maturation means they are still of interest. Although impossible to list and discuss all candidate genes implicated in schizophrenia aetiology, a select few are discussed further.

1.4.1.1: Disrupted In Schizophrenia 1 (DISC1)

Based on familial studies, one of the first genes to be associated with the aetiology of schizophrenia was Disrupted In Schizophrenia 1 (DISC1), resulting from an abnormal translocation on Chromosome 1. Although recent GWAS

studies have failed to identify DISC1 as a candidate risk gene for schizophrenia (Sullivan, 2013), DISC1, which encodes a multifunctional scaffold protein, plays a crucial role in neuronal development, particularly synaptogenesis, cell migration and synaptic plasticity. It is also associated with several cytoskeletal proteins and membrane receptor trafficking. Therefore the potential role of DISC1 as a molecular lead to understand how altered intracellular signalling and trafficking can contribute to the symptomatology of schizophrenia is of key interest (Harrison and Weinberger, 2005, Jaaro-Peled, 2009, Atkin et al., 2011, Johnstone et al., 2011a, Dahoun et al., 2017, Murphy and Millar, 2017, Norkett et al., 2017, Tomoda et al., 2017).

1.4.1.2: Neuregulin 1 (NRG1)

Neuregulin1 (NRG1) and its primary receptor target ErbB4 have also been implicated (Harrison and Law, 2006, Mei and Xiong, 2008b). The NRG1 gene encodes a number of proteins with diverse isoforms and expression patterns that have epidermal growth factor (EGF)-like domains, allowing interaction with tyrosine kinase ErbB receptors (Mei and Xiong, 2008b). The additional involvement of NRG1 in synaptogenesis, neuronal migration, glial cell formation, myelination as well as neuron-glial interactions means the gene can affect both pre- and post-natal development, potentially leading to aberrations in signalling pathways and cortical connectivity, which contribute to schizophrenia pathophysiology.

1.4.1.3: Dysbindin

The synaptic protein Dysbindin is thought to influence excitatory neurotransmission via its role in receptor trafficking and exocytosis (Karlsgodt et al., 2011, Papaleo et al., 2012b). Reduced dysbindin expression in the prefrontal cortex (PFC) and hippocampus was found in post-mortem tissue from schizophrenia patients and has been linked to negative symptoms (Weickert et al., 2004, Weickert et al., 2008, Papaleo et al., 2012b). Furthermore, the protein is coded by Dystobrevin-Binding Protein 1 (DTNBP1) and mutations in this gene show strong susceptibility to the onset of schizophrenia (Williams et al., 2005).

1.4.1.4: Oxytocin

There is accumulating evidence for inter-individual variations in oxytocin signalling in schizophrenia aetiology. Two genetic variants of the oxytocin gene have been identified, along with four genetic variants of the oxytocin receptor gene. In particular, the oxytocin receptor single nucleotide polymorphisms (SNPs) rs53576 and rs237885 have been associated with schizophrenia and rs237902 is associated with negative symptom scores (Montag et al., 2013). In particular, the rs53576 SNP results in an AA as opposed to AG/GG genotype, which is associated with higher striatal dopamine transporter availability (Wang et al., 2013, Chang et al., 2014), decreased amygdala volume, decreased hypothalamic functional connectivity and reduced functional coupling between the PFC and amygdala (Wang et al., 2013, Wang et al., 2014).

Although a functional change is yet to be determined as a result of these SNPs, as well as an understanding as to how they may contribute to disease manifestation, it does appear that they may play a role in individual susceptibility to altered anxiety-related and social behaviour. For example, genetic variations of the oxytocin receptor have been shown to modulate the effectiveness of positive social interaction against a stressful experience; individuals with the AG/GG genotype have reduced cortisol release in response to stress after receiving social support compared to individuals with the same genotype who received no support (Chen et al., 2011).

1.4.2: Post-GWAS

Recent advances in genetics have highlighted key DNA regions via GWAS. The latest and largest GWAS on schizophrenia was published in 2014 by the schizophrenia Psychiatric Genome Wide Association Study Consortium (PGC) team featuring a multi-stage GWAS of 36,989 cases and 113,075 controls. A total of 128 associations in 108 independent loci were identified, and it is estimated that approximately 8300 independent SNPs at 22 genetic loci can contribute to the risk of developing schizophrenia (Ripke et al., 2013, Consortium, 2014). However, as a lot of these associations are to genomic regions (loci) that often encompass more than one gene, it is difficult to determine which specific genes are affected. In addition, many of the SNPs identified lie within non-coding DNA regions, presenting a challenge when

determining the biological relevance of these genome-wide associations (Harrison, 2015).

However, some of the key regions identified by these studies that have garnered interest include the SNPs found in the α_{1c} and β_2 subunits of voltage-gated L-type calcium channels due to the role of calcium signalling in learning, memory and neuronal plasticity (Murray et al., 1991) as well as the SNPs that converge on NMDA signalling. For example, a genome-wide significant SNP was found within the NMDA receptor GRIN2A subunit gene, and at loci for GRIA1, GRM3 and SRR (Pocklington et al., 2014, Harrison, 2015). In addition, various rare copy number variants (CNVs) have been identified (Perkins DO, 2017) such as the CNV located on chromosome 16 (16p11.2) which encompasses approximately 29 genes. Although the majority of genes located within this 600kB genomic region have not been individually associated with schizophrenia, some of the genes are thought to play a role in synaptic and dendritic function/development, and microduplication of this genomic region is significantly associated with an increased genetic risk of developing the disease (McCarthy et al., 2009, Chang et al., 2017). However, the strongest associated locus found by the PGC team lies on an extended region on chromosome 6 and the MHC locus which encodes a number of genes related to immune function (Patterson, 2009, Consortium, 2014, Corvin and Morris, 2014), also thought to play a role in schizophrenia aetiology, as discussed later.

The contribution these SNPs and CNVs have on the aetiology of schizophrenia remains unclear but it is proposed that CNVs are more penetrant and

constitute a significantly increased risk of illness (Kirov et al., 2014). The general consensus is that a number of key changes within DNA ultimately result in a build-up of 'susceptibility' genes, collectively producing enough additive effect to constitute onset of schizophrenia (Giusti-Rodriguez and Sullivan, 2013). Therefore, schizophrenia is largely considered to be a highly polygenic disorder (Wray et al., 2014) with susceptibility resulting predominantly from numerous common variants of low penetrance (Gatt et al., 2015, Kendler, 2015, Giegling et al., 2017). There may also be gene-gene interactions i.e. epistasis, which have additional effects (Mackay and Moore, 2014). However, the exact involvement these genes have on disease pathophysiology remains unknown, largely due to a lack of understanding as to their exact functional and biological impact. Although impossible to list all the gene variants thought to contribute to the risk of schizophrenia, some of those identified are involved in the modulation of dopamine, glutamate, serotonin and neuronal development and function, as reviewed extensively in (Giegling et al., 2017).

1.4.3: Genetics and the environment

There is also growing consensus that the environment can have effects on genetic susceptibility to schizophrenia, in particular the time and impact of the environmental stressors on brain development (Dempster et al., 2013). Various environmental factors have been extensively studied to determine the risk they may contribute to schizophrenia onset. Overall, these external influences can be placed into three broad categories of prenatal factors, such as gestational

complications prior to birth; social stressors, including trauma and adversity in life, particularly during early development and childhood; as well as substance abuse.

1.4.3.1: Prenatal factors

Increasing evidence has implicated the importance of gestational complications during foetal development. For example, hypoxia caused by oxygen starvation to the brain produces reactive oxygen species (ROS), mitochondrial dysfunction and cellular apoptosis (Thornton et al., 2012), mediated through altered calcium signalling. The impact of neuronal toxicity at this crucial development stage has shown strong epidemiological links to developing schizophrenia later in life (Schmidt-Kastner et al., 2006) and has been reproduced in preclinical models (as discussed later). Additionally, maternal infection during foetal development has shown relatively consistent clinical linkage to schizophrenia. The exact and extensive list of which particular infections contribute are not all known, but likely candidates include toxoplasmosis gondii and herpes simplex virus 2, as extensively reviewed elsewhere (Brown and Derkits, 2010).

A plausible causal link between maternal infection and the increased risk of schizophrenia may be through cytokines, a family of soluble polypeptides that form an essential part of the immune response to infection. For example, an increase in maternal levels of pro-inflammatory cytokines such as TNF_{α} during the second trimester and early third trimester has been associated with

increased incidence of schizophrenia (Buka et al., 2001). Although the mechanisms by which this occurs remains unclear, the activation of these inflammatory mediators may contribute to some of the morphological changes observed in schizophrenia patients such as oligodendrocyte maturation, myelination and function (Davis et al., 2003).

Environmental stressors are also thought to influence oxytocin signalling, which has received considerable interest over the last decade due to its potential role on aspects of social behaviours, particularly maternal care. For example, high levels of maternal care of rat pups (such as increased grooming or licking behaviour) is correlated with increased oxytocin receptor binding within the central nucleus of the amygdala and bed nucleus of the stria terminalis of recipient adult rats (Francis et al., 2002) and maternal separation has been shown to decrease oxytocin immunoreactivity within the paraventricular nucleus (PVN) of female C57BL/6 mice (Veenema et al., 2007) and in the amygdala of male Wistar rats (Oreland et al., 2010). Additionally, rat pups exposed to long periods of maternal separation (3h daily PND 1-25) show decreased pup grooming behaviour and decreased maternal aggression in adulthood (Boccia and Pedersen, 2001). Furthermore, early-life stress pre-weaning has been shown to decrease social interaction in adult Sprague-Dawley rats tested PND 56, effects which are ameliorated following oxytocin infusion into the central amygdala (Lee et al., 2007). Therefore, environmental adversity during neonatal development can potentially result in altered oxytocin neurotransmission, which can translate into impaired social

behaviours in adulthood. Although the majority of evidence is based on preclinical models, the same influences may affect human behaviours and contribute to their susceptibility to psychiatric illness since oxytocin function appears highly conserved across species. For example, women susceptible to postnatal stress have reduced plasma oxytocin levels (Feldman et al., 2007, Kim et al., 2014), which could potentially influence offspring care and development, making it a possible environmental risk factor for schizophrenia susceptibility.

1.4.3.2: Social stressors

Early-life adversity such as maternal separation and/or childhood abuse, is another major factor thought to contribute to disease onset (Schenkel et al., 2005, Wicks et al., 2005). These traumatic events in childhood may then subsequently impact upon the trajectory of normal development as various studies have reported an association between experience of emotional, physical and/or sexual abuse with the prevalence of three or more psychotic symptoms (Janssen et al., 2004, Spauwen et al., 2006). However, it must be noted that the measurement of psychotic symptoms in these studies was not part of a diagnosis of schizophrenia (preventing identification of a possible causal relationship). Nonetheless the occurrence of these experiences during the important developmental time period from childhood to adolescence suggests a plausible risk factor. Moreover, such events are naturally stressful and various groups have examined correlations between the impact of stress and the development of schizophrenia, with particular focus on hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Kudielka et al., 2004, de Kloet et al.,

2005, Jones and Fernyhough, 2007), and higher brain centres such as the PFC and anteriocingulate cortex (ACC) which integrate social cues to determine the appropriate response to local events and surroundings (Pruessner et al., 2010). However, some also argue that activation of the HPA axis and its subsequent sensitisation due to stress or altered dopaminergic neurotransmission can only contribute to the onset of psychosis when an individual is already genetically predisposed (Wray et al., 2014, Zai et al., 2017).

In addition to adverse events in childhood, the socioeconomic status of an individual is thought to have substantial influence. In studies focussing on urbanisation and the social impact of various neighbourhoods, being raised in an urban neighbourhood from birth up to the age of 15 proposed a greater risk of developing schizophrenia compared to living in a rural area (Pedersen and Mortensen, 2001). This may be due to increased pollution exposure common to urbanised areas, which can contribute to aberrant signalling in development and thus an increased risk to schizophrenia (Brown, 2011).

1.4.3.3: Substance abuse

With regards to substance abuse and schizophrenia, cannabis use is reported to constitute a six fold increase in the risk of developing the disease (Zammit et al., 2002) and the continued use of cannabis has been shown to increase relapse rates and result in more severe positive symptoms compared to both former cannabis-users and cannabis-naïve individuals (Schoeler et al., 2016). Negative symptoms can also be induced including blunting affect and

amotivation (Rovai et al., 2013), as well as effects on learning and memory (Lichtman et al., 2002, Wilson and Nicoll, 2002), as reviewed in (Murray et al., 2017).

The use of cannabis in adolescence is thought to constitute a greater risk for psychosis when combined with a high genetic risk score for schizophrenia (French et al., 2015) suggesting cannabis use itself may only contribute to or be an underlying factor for schizophrenia development in patients with a pre-existing increased familial risk for the disease (Proal et al., 2014). It is proposed that cannabis use during a critical time of brain circuit development and maturation can cause aberrant PFC development, and may contribute to some of the morphological changes observed in the global pallidus, striatum and thalamus, although this remains controversial (Smith et al., 2014). This is likely due to significant changes in dopaminergic signalling as cannabis can evoke dopamine release within the associative striatum (Voruganti et al., 2001, Bossong et al., 2015). Therefore, the exact extent to which drugs of abuse contribute to disease onset remains controversial, but an individual's genetic predisposition appears to be an important underlying factor for all environmental risks.

1.5: The neurodevelopmental hypothesis

Therefore the heterogeneity and complexity of schizophrenia development cannot be explained by any single factor alone, but it is instead a result of numerous risk factors and a complex neurobiology that can build from foetal

development, childhood, up until the age of onset. There is increasing evidence associating the effects of both genetic and environmental influences in schizophrenia patients but it is generally proposed that there is a genetic predisposition to the disease due to the presence of multiple gene variants (Sawa and Snyder, 2002, Harrison and Weinberger, 2005, Farrell et al., 2015, Zai et al., 2017). Exposure to environmental stressors at critical time points in development such as early-life exposure to infections, social class, substance abuse and/or complications during pregnancy (Marcelis et al., 1998, Bromet and Fennig, 1999), see (Brown, 2011) for a review), further contribute to disease susceptibility.

1.6: Neurobiology of schizophrenia

1.6.1: Dopamine hypothesis

The original hypothesis for the neurobiology of schizophrenia largely focusses on dysfunction of the dopaminergic system, particularly hyperactivity of the mesolimbic pathway contributing to positive symptoms (Meltzer and Stahl, 1976). This was based on evidence that both typical antipsychotics such as chlorpromazine, and atypical second generation antipsychotics such as risperidone, are potent dopamine D_2 receptor antagonists and therefore reduce dopaminergic neurotransmission (Seeman, 2006). The efficacy of these compounds in the treatment of schizophrenia also uniquely correlates with their affinity for the dopamine D_2 receptor (Kapur and Seeman, 2001, Takeuchi et al., 2013). Furthermore, psychotic-like symptoms (similar to the positive

symptoms seen in schizophrenic patients) can be induced in healthy individuals following administration of stimulants such as amphetamine (Janowsky and Risch, 1979), which enhance dopamine release and prevent its reuptake (Seiden et al., 1993).

However, recent imaging studies have since highlighted that instead of mesolimbic dopaminergic pathway hyperactivity, there is hyperdopaminergic activity within the associative and sensorimotor striatum (Howes et al., 2009, Egerton et al., 2013). For example, PET imaging studies have shown significantly increased striatal dopamine release in schizophrenia patients administered 0.2mg/kg amphetamine (Kegeles et al., 2000). PET and SPECT imaging also report an increase in striatal $D_{2/3}$ receptor density, specifically in the precommisural dorsal caudate of the associative striatum (Kegeles et al., 2010b). In contrast to striatal hyperactivity however, hypo-dopaminergic activity within the PFC is thought to contribute to the negative and cognitive symptoms of schizophrenia (Abi-Dargham et al., 2012). Consequently links between the striatum and dorsolateral PFC is of key interest in understanding symptom onset (Lodge and Grace, 2011, Dandash et al., 2017).

Despite this, current antipsychotics fail to significantly improve cognitive and negative symptoms, suggesting a more complex neurochemical aetiology than altered dopaminergic neurotransmission alone. The proposed alterations to dopaminergic signalling in different brain regions i.e. striatal hyper- and mesocortical hypo-activity occur through unknown mechanisms. However, one key relationship extensively studied and still of keen interest is the interaction

between glutamate and dopamine, and there remains an ongoing debate as to whether disrupted glutamatergic or dopaminergic signalling comes first in disease progression (Eyles et al., 2012, Gao and Snyder, 2013).

1.6.2: Glutamate hypothesis

The role of altered glutamatergic signalling on schizophrenia aetiology has been extensively studied (Olney and Farber, 1995, Egerton et al., 2012, Dauvermann et al., 2017), although the mechanism by which glutamate dysfunction occurs remains unclear. Glutamate is the major excitatory neurotransmitter in the brain and acts on two major receptor classes; three subtypes of ionotropic receptors (AMPA, kainate and NMDA) and eight subtypes of metabotropic receptors (mGluRs, (Masu et al., 1993)), many of which have been found to be altered in post-mortem studies of schizophrenia patients (Eastwood et al., 1997, Nudmamud and Reynolds, 2001, Weickert et al., 2013). Glutamate levels were decreased in frontal brain regions of chronic schizophrenic patients compared to those at high risk or in first-episode psychosis, suggesting the involvement of abnormal glutamate regulation in disease progression (Natsubori et al., 2014), although long-term antipsychotic use may also contribute to these observations.

In particular, NMDA receptor hypofunction is believed to play a key role as the administration of NMDA receptor antagonists such as PCP and ketamine induce positive, negative and cognitive symptoms in healthy individuals, whilst triggering psychosis and worsening symptoms in schizophrenics (Allen and

Young, 1978, Ghoneim et al., 1985, Javitt, 1987, Krystal et al., 1994, Lahti et al., 1995). The NMDA receptor hypofunction model has been proposed to link the natural development of schizophrenia and the 'schizophrenia-like' side-effects induced by NMDA receptor antagonists (Javitt and Zukin, 1991, Krystal et al., 1994, Lahti et al., 1995, Olney et al., 1999). It is suggested that NMDA receptor dysfunction may cause excess synaptic glutamate release resulting in stimulation of ionotropic AMPA receptors, and/or the overstimulation of postsynaptic cells leading to excitotoxicity and cell death (Shenton et al., 2001, Kreczmanski et al., 2007), contributing to some of the pathological changes seen in schizophrenia patients.

NMDA receptor hypofunction is also believed to impact the signal transduction of other neurotransmitter pathways including dopamine and GABA. The interaction between pyramidal glutamatergic neurons and fast-spiking GABAergic interneurons is of key interest; GABAergic interneurons regulate activity in the corticolimbic circuit and are activated via pyramidal neurons and so disruptions in this circuit alter glutamatergic neurotransmission. A decrease in GABAergic function leads to a decrease in the functional connectivity or integrative signalling neurotransmission capacity within cortical networks. For example, disinhibition of pyramidal neurons increases glutamate release, excessively stimulating ionotropic glutamate receptors to result in excitotoxic effects (Lewis et al., 2005, Nakazawa et al., 2012). The resulting changes in cortical neurotransmission may contribute to the complicated neurobiology underlying schizophrenia. The fact that fast-spiking interneurons in the PFC

undergo dramatic changes in glutamatergic receptors during the adolescent period, with the majority becoming lost during cortical development and with few exhibiting NMDA receptor-mediated currents in adulthood (Wang and Gao, 2009) supports this theory.

In addition, post-mortem analysis of schizophrenia patients found decreased numbers of GABAergic parvalbumin interneurons (Gao and Snyder, 2013). A reduction in these neurons may contribute to cortical disinhibition (Beasley et al., 2002, Zhang and Reynolds, 2002, Sakai et al., 2008) and contribute to the resulting cognitive impairments and negative symptoms seen within schizophrenia. For example, administration of the NMDA receptor antagonist MK-801 in rats was shown to increase randomised spike activity within the PFC. This may account for disrupted frontal lobe function, or even enhance cortical noise through reduced signal neurotransmission efficiency of PFC neurons, leading to the impaired filtering of irrelevant information i.e. disinformation (Jackson et al., 2004). Furthermore, drugs which act as positive GABAergic allosteric modulators, such as benzodiazepines, can improve psychotic symptoms in some schizophrenia patients (Wolkowitz and Pickar, 1991). Although the therapeutic efficacy of these compounds have shown varying results, it supports the link between disrupted glutamatergic and GABAergic signalling and schizophrenia. Furthermore, ketamine-treated patients show increased striatal dopamine release in response to intravenous (i.v.) amphetamine (0.25mg/kg) highlighting the potential for disrupted

glutamatergic signalling to influence dopaminergic activity (Kegeles et al., 2000).

The pathophysiology of schizophrenia however, cannot be explained by these simple changes in neurotransmitters alone. Complex alterations in signalling can impact upon brain circuitry and therefore affect sensory, cognitive and emotional processing, resulting in the onset of symptoms seen (Lisman et al., 2008, Marek et al., 2010). For example, activation of D_1 receptors can result in the activation of divergent second messenger systems such as enhanced Na^+/K^+ ATPase activity, enhanced NMDA-gated currents and altered calcium signalling (Cepeda et al., 1993, Surmeier et al., 1995, Cepeda et al., 1998). As D_1 receptors are believed to be involved in cortical processing, are localised adjacent to glutamatergic synapses and found in greater abundance in frontal brain regions (Goldman-Rakic et al., 2000), understanding how neurotransmitter systems impact upon one another is of clear importance.

1.7: Morphological changes

Comparisons of brain structures between normal individuals and schizophrenics has also highlighted key differences in specific sub-regions and neuronal circuitry, particularly within the PFC, hippocampus, amygdala and basal ganglia, as extensively reviewed by (Shenton et al., 2001). For example, reduced grey matter volume was found in the temporal gyrus and cerebellum in patients presenting with hallucinations (Neckelmann et al., 2006). There are also abnormalities in brain connectivity between the mediodorsal thalamic

nucleus and dorsal PFC, with reduced white matter volume found in the left parietal, bilateral temporo-occipital cortex and genu of the corpus callosum (Tibbo et al., 1998, Lewis and Lieberman, 2000b, Foong et al., 2001), which is thought to contribute to negative symptoms.

Comparisons between schizophrenic patients and their unaffected siblings however, also suggest that some morphological changes such as lateral ventricle size, have strong heritability which does not always result in manifestation of the disease. For example, enlarged ventricles can be seen in unaffected twins of schizophrenia patients (Styner et al., 2005), consistent with the proposal that development of schizophrenia is a consequence of both genetic and early-life environmental influences. The consensus remains however, that disease progression over time has a greater role for structural abnormalities than genetic factors alone (Boos et al., 2012), which can advance those at a prodromal stage of the illness (high risk) into transition towards developing schizophrenia (van Haren et al., 2012, Moran et al., 2013).

1.8: Treating schizophrenia

Current available treatments for schizophrenia broadly fall into two categories; typical and atypical antipsychotics, although these can be further sub-divided.

1.8.1: Typical antipsychotics

Typical antipsychotics were discovered in the early 1950s and include the compounds chlorpromazine and haloperidol which were shown to alleviate

positive symptoms in patients with schizophrenia via dopaminergic D_2 receptor antagonism. The effectiveness of these compounds was also shown to correlate to their selective affinities for this receptor subtype (Nordstrom et al., 1993, Nyberg et al., 1995). Depending on the pharmacology of the chemical class, these compounds have several side effects with the most common being constipation and dry mouth (via muscarinic acetylcholine $M_{1/2}$ receptors), weight gain, sedation (via histamine H_1 receptors) as well as extrapyramidal side-effects leading to adverse motor control such as dyskinesia, Parkinsonism and ataxia (via additional dopamine $D_{1\text{-}4}$ receptor affinity; (Kasper et al., 1999, Strange, 2001).

1.8.2: Atypical antipsychotics

The first atypical antipsychotic, clozapine, was found to have high efficacy while devoid of extrapyramidal side effects. Because of the risk of agranulocytosis associated with its use, clozapine had to firstly demonstrate its unique capacity to also be effective in treatment-resistant patients before reaching the market. Since clozapine has similar affinity as other antagonists for dopamine D_2 and the serotonin $5HT_{2A}$ receptor, it was postulated that high 5-HT_{2A} receptor affinity might be responsible for the reduced extrapyramidal side-effects due to attenuation of serotonergic input to the basal ganglia, potentially mediating inhibition of dopamine release. Basal ganglia circuitry signals through a number of brain regions including the striatum, globus pallidus, substantia nigra and the thalamus, contributing to the activation of key cortical regions and

therefore control of cortical function (Middleton and Strick, 2000). The effect serotonergic control has on basal ganglia circuitry has been extensively reviewed by (Di Matteo et al., 2008, Miguelez et al., 2014), and so compounds which influence these receptors could lead to downstream effects on basal ganglia and ultimately motor function, cognition and behaviour (Kapur and Remington, 1996, Kapur et al., 1999).

Additional atypical antipsychotics with affinity for $5HT_{2A}$ and other receptors include olanzapine, ziprasidone and risperidone, all of which improve positive symptoms in schizophrenia patients with reduced motor control side-effects. However, the rich pharmacology of these compounds still induce some side effects including weight gain, as well as a risk of metabolic syndrome (due to H_1 receptor antagonism), which are still of concern. In addition, atypical antipsychotics fail to improve negative and cognitive symptoms in patients.

1.8.3: Third generation antipsychotics

Aripiprazole and cariprazine both differ in their pharmacology in that they act as $D_{2/3}$ receptor partial agonists as opposed to D_2 and 5-HT_{2A} receptor antagonists, and can arguably be classed as third generation antipsychotics (Citrome, 2016, Topolov and Getova, 2016). Cariprazine has an approximately 10-fold greater affinity for the D_3 as opposed to D_2 receptor subtype (Agai-Csongor et al., 2012, Caccia et al., 2013, Citrome, 2016). Its properties as a partial agonist should lead to inhibition of overstimulated dopaminergic

receptors when endogenous dopamine levels are high, and should increase dopaminergic activity when levels are low.

1.8.4: Modulating glutamate

Due to clear evidence of altered glutamate neurotransmission in schizophrenia (Natsubori et al., 2014), considerable research has investigated the effect of glutamatergic modulators on disrupted brain circuits. This includes ionotropic AMPA and Kainate receptors, mGluRs, in particular $mGluR_2$ and $mGluR_5$ which are considered key targets (Homayoun et al., 2004, Homayoun et al., 2005, Niswender and Conn, 2010), and NMDA receptors. Discussing each of these approaches in detail is beyond the scope of this thesis however, novel compounds acting on these receptor subtypes have been extensively reviewed elsewhere (Hashimoto et al., 2013).

1.8.4.1: NMDA receptors

NMDA receptors are composed of various subunits, each of which play a biochemical and pharmacological role (Paoletti and Neyton, 2007). NMDA receptors are activated by co-agonists glutamate and glycine which act on different binding sites of the receptor. Excessive NMDA receptor activation at the glutamatergic site induces cytotoxic effects, including neuronal cell death and epileptic seizures (Hirose and Chan, 1993), therefore several compounds were developed to target the glycine modulatory site with the intent of avoiding such toxicity.

NMDA receptors bind glycine with a 100-fold higher affinity than glycine receptors, and administration of 0.5µM-20µM glycine potentiates NMDA receptor currents (Eulenburg et al., 2005). The glycine binding site became a popular target for drug development when glycine (0.8g/kg) reversed PCP-induced hyperactivity in rodents (Heresco-Levy et al., 1996) and following pilot clinical studies where exogenous glycine induced a 15-30% improvement in negative symptoms (Heresco-Levy et al., 1996). In addition, 14 patients suffering from chronic schizophrenia showed significant improvement in negative symptoms compared to controls following glycine intake (Javitt et al., 1994). Although high doses of glycine are required due to poor blood-brain barrier (BBB) penetration, no serious side-effects were observed over an eight-week dosing regimen (Leiderman et al., 1996). Longer term studies in animals using high doses of glycine (1-5g/kg/day) over a 1-5 month period also showed little sign of neurotoxicity (Shoham et al., 2001).

Alternative ligands for the glycine binding site have been examined including endogenous amino acid agonist D-serine. Given adjunctively at 30mg/kg/d with either olanzapine or risperidone, D-serine improved all three symptom classes in schizophrenic patients (Heresco-Levy et al., 2005), however little effect was seen in patients receiving clozapine (Tsai et al., 1999, Evins et al., 2000), suggesting that clozapine can modulate NMDA receptors even if not directly.

1.8.4.2: Emergence of $GlyT_1$ inhibitors

Two types of glycine transporters have been identified to date; $GlyT_1$ and $GlyT_2$ (Guastella et al., 1992, Liu et al., 1992). $GlyT_1$ are considered to be a viable therapeutic target to regulate NMDA receptor function as affinity studies showed these transporters to be responsible for ~70% of glycine transport, with the remaining 30% transported by $GlyT_2$ (Aroeira et al., 2013). Localisation studies also showed $GlyT_2$ are exclusive to glycinergic neurons whereas $GlyT_1$ are expressed at pre- and post-synaptic sites on glutamatergic synapses in various brain regions including the thalamus, hypothalamus, periaqueductal gray, subthalamic nucleus and hippocampus (Cubelos et al., 2005), suggesting $GlyT_1$ association with NMDA receptors *in vivo.* For example, early studies showed both glycine and $GlyT_1$ inhibitors to reverse PCP-induced hyperactivity (Javitt et al., 1999). In addition, NMDA receptor currents in $GlyT_1$ (+/-) CA1 pyramidal cells were enhanced *in vitro* (Martina et al., 2005) and mice heterozygous for $GlyT_1$ (expressing ~50% less $GlyT_1$ compared to wildtype) showed improved memory retention during a Morris-water maze spatial learning task (Tsai et al., 2004). Furthermore, inhibition of $GlyT_1$ can enhance NMDA-dependent long-term potentiation (Alberati et al., 2012).

One of the first $GlyT_1$ inhibitors to be tested clinically for schizophrenia was sarcosine (N-methylglycine), a naturally occurring amino acid. In a six-week randomised double-blind clinical trial comparing the effects of sarcosine and D-serine in chronic schizophrenia patients, sarcosine was shown to improve clinical measures compared to placebo (Lane et al., 2010). Although it reduced

positive and negative symptoms in schizophrenia patients, in a similar way to D-serine, fewer significant effects were seen in patients already receiving clozapine (Lane et al., 2006), supporting further the ability of clozapine to modulate NMDA receptor function.

Pharmaceutical industries have therefore tried to identify new potent and specific $GlyT_1$ inhibitors. Example compounds include NFPS (N[3-(40-fluorophenyl)-3-(40-phenylphenoxy)-propyl]-sarcosine) and ORG 24461 (R,S-(+/-)N-methyl-N-[(4-trifluoromethyl)-phenoxy]-3-phenyl-propylglycine), both of which are derivatives of sarcosine (Harsing, 2003), and ORG 25935 (cis-N-methyl-N-(6-methoxy-1-phenyl-1,2,3,4-tetrahydronaphthalen-2-ylmethyl)-aminomethylcarboxylic acid hydrochloride) and RG1678 (Bitopertin) which are both non-amino acid derivatives (Chue, 2013). Bitopertin is thus far the only compound to reach phase III clinical trials, after successful Phase II trials in patients with predominant negative symptoms (Umbricht et al., 2014), as reviewed in (Singer et al., 2015). In a large multisite Phase III clinical trial however, Bitopertin failed to reach the primary end point in the same population of patients, but did significantly improve sub-optimally controlled positive symptoms in one of the three Phase III clinical studies (Bugarski-Kirola et al., 2016). Furthermore, in a phase II study, in patients with acute exacerbation versus olanzapine as a positive control, Bitopertin significantly improved positive symptoms (the secondary endpoint; (Bugarski-Kirola et al., 2014).

Despite disheartening results, $GlyT_1$ inhibitors remain of interest as potential therapeutics since the indirect modulation of NMDA receptors showed evidence for improvement in negative symptoms. Further understanding how these compounds work may help direct research into novel compounds that act as better targets. In light of this, chapter 2 and 3 of this PhD thesis will focus on further characterisation of RO3994850, an analogue of Bitopertin developed by F. Hoffmann-La Roche Ltd., where a dose of 1-3mg/kg RO3994850 was shown to attenuate social deficits in a neonatal-PCP isolation-rearing 'two-hit' neurodevelopmental rat model, as discussed later (McIntosh, 2014).

1.8.5: Adjunctive therapies

Additional physiological mechanisms thought to underlie and/or contribute to schizophrenia include inflammatory-pathway activation and oxidative stress. For example, studies have shown dysregulation of the immune system such as increased plasma and artificial cerebrospinal fluid (aCSF) levels of both interleukins and cytokines (pro-inflammatory markers), as well as increased microglia activation (markers of neuroinflammation) in schizophrenia patients compared to controls, even prior to treatment with antipsychotics (van Berckel et al., 2008, Miller et al., 2011, Kirkpatrick and Miller, 2013, Garcia-Bueno et al., 2014). Furthermore, GWAS studies have associated schizophrenia with genetic loci thought to play a role in overall immune function (Consortium, 2014), as discussed earlier.

As a result, novel adjunctive therapies currently under investigation include anti-oxidants and nutritional interventions, as reviewed in (Dodd et al., 2013, Arroll et al., 2014, Brown and Roffman, 2016). For example, schizophrenia patients show decreased levels of polyunsaturated fatty acids (PUFAs), essential components of cell membrane phospholipids, particularly within caudate regions (Horrobin et al., 1991, Yao et al., 2000) and supplementation of PUFAs such as OMEGA-3 combined with vitamin C and E, was associated with improved psychopathology following four months treatment (Arvindakshan et al., 2003). Furthermore, a 12 week supplementation program of OMEGA-3 combined with vitamin E reduced the incidence of psychosis in young high-risk adults (Amminger et al., 2010). However, a meta-analysis also found that OMEGA-3 supplementation showed no significant improvement in symptoms in chronic schizophrenia patients (Fusar-Poli and Berger, 2012), suggesting that different schizophrenia sub-groups may respond differently to these add-on therapies.

A further example is the use of sodium nitroprusside, a vasodilator which is metabolised *in vivo* to produce nitric oxide, an anti-oxidant. Like a number of anti-oxidants, nitric oxide abnormalities have been observed in schizophrenia, including low serum levels of nitric oxide metabolites (Das et al., 1998, Ramirez et al., 2004, Maia-de-Oliveira et al., 2016), altered nitrergic neurons in the frontal cortex (Akbarian et al., 1993) and a correlation between plasma nitric oxide metabolites and negative symptom severity (Nakano et al., 2010), reviewed in (Bernstein et al., 2011). Furthermore, the supplementation of

sodium nitroprusside (0.5µg/kg/min, 4h, i.v.) improved BPRS and PANSS negative symptom scores in patients during a single-centre randomised double-blind placebo-controlled trial for up to 4 weeks following treatment (Hallak et al., 2013). The potential therapeutic efficacy of this anti-oxidant against negative symptoms has also been replicated in animal models. For example, 1mg/kg sodium nitroprusside reversed ketamine-induced impairments in novel object discrimination (NOD) and social interaction tests (Trevlopoulou et al., 2016). However, the clinical benefit of sodium nitroprusside may be selective to certain schizophrenia sub-groups, and it may have no significant efficacy towards cognitive symptoms. For example, sodium nitroprusside (0.5µg/kg/min, 4h) showed no improvement in a spatial working memory task compared to schizophrenia patients receiving placebo (Stone et al., 2016).

Discussion of all possible anti-oxidants and supplements currently under investigation is beyond the remit of this thesis. They do however suggest an eventual progression towards combined therapy when treating schizophrenia, particularly in treatment-resistant patients although an understanding as to how such physiological pathways are altered in schizophrenia and how they contribute to disease onset must first be achieved.

1.8.5.1: Oxytocin

The neuropeptide oxytocin has also gained interest for the treatment of schizophrenia based on evidence that oxytocinergic system activation could

target all three symptom domains following its synthesis in the PVN and supraoptic nucleus (SON) of the hypothalamus, as shown in Figure 1.1. Possibly due to downstream effects on dopamine, 5-HT, glutamate and GABA signalling pathways (Dolen, 2015, Feifel et al., 2016). In particular, oxytocin and 5-HT have been shown to coordinate responses to social behaviour and mood. For example, oxytocin infusions (10µg/2µl) facilitated 5-HT release in the median raphe nucleus and decreased anxiety-like behaviour in an open field in mice, whilst co-infusion with the 5-HT_{2A} receptor antagonist ritanserin (5mg/kg) ameliorated these effects (Yoshida et al., 2009). Therefore, oxytocin may exert its anxiolytic effects via oxytocin receptor activation in 5-HT neurons. This is supported *in vivo* where oxytocin labelled cells and 5-HT transporter labelled fibres have been shown to overlap in the PVN of the primate hypothalamus (Emiliano et al., 2007). Additionally, 5-HT axon length and density is increased in the anterior hypothalamus, cortical amygdala and ventromedial hypothalamus in juvenile male prairie voles administered 3µg oxytocin in the neonatal period (PND 1; (Eaton et al., 2012)). In particular, studies in prairie voles have highlighted the potential role of oxytocin in social attachments and bond formation due to their unique tendency to form monogamous relationships compared to montane or meadow voles, both polygamous species (Fisher et al., 2005). This appears to be influenced by neuropeptides as prairie voles show an increased density of oxytocin receptors in the NAc compared to montane or meadow voles. (Young and Wang, 2004).

Oxytocin also has several effects on cognition. For example, oxytocin knockout mice have impaired reversal learning in a T-maze task (Sala et al., 2011) and oxytocin (2ng into the lateral ventricles) in female mice improved performance in the 8 arm radial maze (Tomizawa et al., 2003). Oxytocin can also potentially modulate glutamatergic function involved in sensorimotor gating. For example, oxytocin (0.04-1mg/kg) dose-dependently restored sensorimotor deficits in an acoustic startle paradigm in male Sprague-Dawley rats (Feifel and Reza, 1999), whilst oxytocin knockout mice show a larger pre-pulse inhibition (PPI) deficit compared to wildtypes (Caldwell et al., 2009). However, oxytocin does not always improve cognitive deficits. For example, oxytocin infusions into the nucleus basalis of Meynert (NBM) impaired spatial memory (increased latency to learn) in a Morris Water Maze task, which was reversed by oxytocin receptor antagonist Atosiban (Wu and Yu, 2004). Therefore despite controversial results, both animal and human studies suggest a functional oxytocin system is required for normal sensorimotor gating and cognitive flexibility, although effects on memory are less well understood, reviewed in (Bakermans-Kranenburg and van, 2013).

Despite some promising results in the clinic however, the benefits of adjunctive oxytocin therapy remains controversial. In particular, it is thought that those with impaired or altered oxytocin signalling have sensitised responses to oxytocin administration which can confound any therapeutic potential. For example, healthy individuals with adverse childhood experiences such as parental separation or divorce have decreased cortisol production following

intranasal oxytocin (24IU) administration compared to controls (Meinlschmidt and Heim, 2007). A number of studies have since indicated that the positive effects of oxytocin on behaviour or neurobiology are decreased or absent in patients with negative caregiving experiences, therefore suggesting that early-life adversity can potentially alter basal oxytocin signalling and oxytocin receptor function (Bakermans-Kranenburg and van, 2013, Wang et al., 2013).

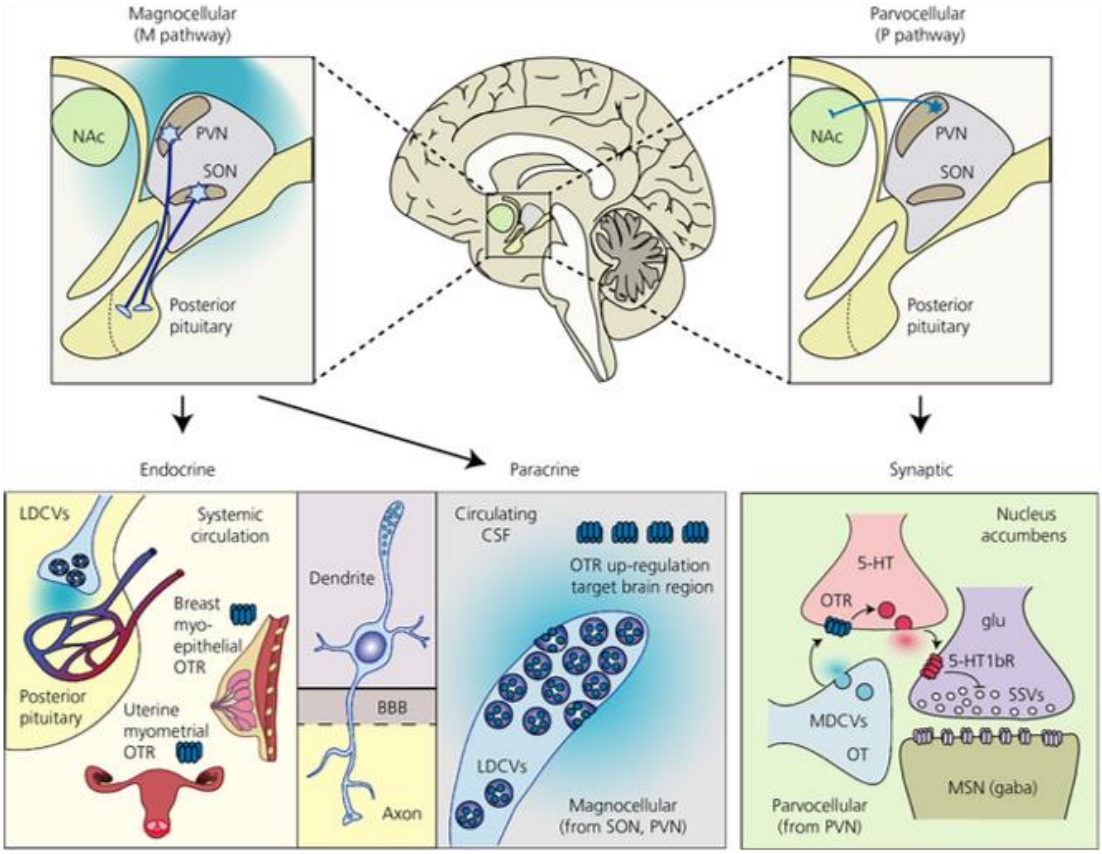


Figure 1.1: Diagrammatic representation of oxytocin transport in the brain. Top; Localisation of magnocellular (left) and parvocellular (right) neuronal subtypes in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. Bottom: Endocrine (left) and paracrine (centre) release mechanisms of the magnocellular pathway, and synaptic release mechanism (right) of the parvocellular pathway. NAc, nucleus accumbens; LDCV, large dense core vesicle; OTR, oxytocin receptor; BBB, blood–brain barrier; CSF, cerebrospinal fluid; 5-HT, serotonin; MDCV, medium dense core vesicle; OT, oxytocin; OTR, oxytocin receptor; MSN, medium spiny neurone; SSV, small synaptic vesicle; 5-HT1bR, serotonin receptor subtype 1b; glu, glutamate. Figure taken from (Dolen, 2015).

1.9: Animal models of schizophrenia

Animal models have been used extensively in an attempt to understand schizophrenia and improve therapy. They are valuable tools to investigate the neurobiological basis of neuropsychiatric disorders, not only allowing the structural and cellular changes to be seen over time, but also provide the opportunity for novel therapies to be tested and validated for their effects on disease symptoms. Animal models need to be translationally relevant; simple and reproducible to the extent that basic behavioural traits can be monitored and likened to appropriate corresponding behavioural changes in humans i.e. must have good validity. The model should either mimic core behavioural symptoms of the disease (face validity), replicate the neurochemical and structural effects associated with the onset of disease such as genetic alterations and neurotransmitter hyper/hypo-activity (construct validity), and/or have good predictive validity such that administration of a compound or combination of compounds results in the expected pharmacological response.

In a broad-sense there are four main categories of animal models for schizophrenia: genetic, lesion- or drug-induced and developmental, which can induce 'schizophrenia-like' symptoms in rodents. Some of the newer animal models in development aim to combine two of these approaches to better replicate the chronological symptomatology of schizophrenia and to improve predictive and face validity to the disease. Each type of model induces particular behavioural, neurochemical and/or structural changes that are akin

to those seen in schizophrenic patients (reviewed in (Jones et al., 2011c) and summarised in Table 1.1.). Due to the complexity of schizophrenia, it is difficult to replicate all aspects of the disease in animals however specific components can be studied dependent on the type of model used. Although impractical to discuss all types of animal models within this thesis, some drug-induced and environmental models are explored further, along with the rationale behind newer 'two-hit' models which combine elements from pre-existing models to produce more robust effects in rodents that better reflect schizophrenia symptomatology.

1.9.1: Drug-induced models

1.9.1.1: Amphetamine

Based on evidence that antipsychotics act as potent dopamine D_2 receptor antagonists, original drug-induced models for schizophrenia used compounds able to influence dopaminergic neurotransmission. For example, chronic administration of amphetamine (d-AMPH sulphate; 1-5mg/kg; 6 days; 3 injections per day) dose-dependently induced hyperactivity in rats and altered dopaminergic regulation, originally proposed to be characteristic of the positive symptoms seen within schizophrenia (Russig et al., 2003, Featherstone et al., 2007). However, few behavioural changes were seen with amphetamine that also reflected other symptom domains; repeated administration did not produce deficits in social interaction in rats (Sams-Dodd, 1998) and cognitive impairments were restricted to PFC-dependent tasks such as the attentional

set-shifting task (ASST; (Featherstone et al., 2008)). Thus, current models typically administer sub-chronic or chronic NMDA antagonists to mimic aspects of all symptoms classes. The most commonly used NMDA receptor antagonist is PCP, which often results in more robust behavioural changes within rodents.

1.9.1.2: PCP

Acute systemic administration of PCP (0.32-10mg/kg) induces increased activity and stereotypy compared to controls (Segal et al., 1981), reduces social interaction (Sams-Dodd, 1997), increases immobility in the forced swim test (Noda et al., 1995), impairs PPI (Geyer et al., 2001) and induces deficits in learning and memory tasks such as set shifting (Egerton et al., 2005) and reversal learning (Abdul-Monim et al., 2003). Thus overall, PCP induces behavioural symptoms in rodents that mimic positive, negative and cognitive symptoms in patients (Neill et al., 2014). However, one flaw with acute pharmacological challenge is that effects are short-lived, questioning its administration as a true 'model' for psychiatric disease. As a result, repeated PCP dosing is often used, varying from daily PCP injections (2-5mg/kg) for 5-14 days followed by a washout period, twice daily PCP injections (2-5mg/kg) for seven days followed by a seven-day washout period, or chronic intermittent paradigms with PCP administered consecutively for 5 days followed by 3 injections a week for 3 weeks (Egerton et al., 2008). Repeated PCP administration induces deficits in NOD, PPI, reversal learning, attentional set-shifting, social withdrawal and causes locomotor sensitisation to an acute PCP challenge (Sams-Dodd, 1996, 1998, Abdul-Monim et al., 2007, Snigdha and

Neill, 2008, McLean et al., 2012, Redrobe et al., 2012a). Neurochemical changes are also produced. For example, reduced basal PFC dopamine and glutamate levels as shown in microdialysis studies (Jentsch et al., 1997, Jenkins et al., 2010), reduced expression of striatial D_1 receptors (Choi et al., 2009) and loss of hippocampal parvalbumin-positive GABAergic interneurons (Abdul-Monim et al., 2007, Jenkins et al., 2010, McKibben et al., 2010).

Atypical antipsychotics such as clozapine, olanzapine and risperidone show an ability to reverse some of these PCP-induced deficits in reversal learning, attentional set-shifting, locomotor hyperactivity and social interaction (Sams-Dodd, 1997, Grayson et al., 2007, Rodefer et al., 2008, Snigdha and Neill, 2008, Goetghebeur and Dias, 2009), which is inconsistent with clinical findings. Another criticism of PCP-induced models is that behavioural alterations are induced in adult rats and so they do not reflect the neurodevelopment aspect of schizophrenia.

1.9.1.3: Neonatal PCP

To increase the predictive and face validity of these models therefore, NMDA receptor antagonists have been administered in the early post-natal period to better mimic symptomatology progression and to provide greater translatability to clinical pathophysiology. A single acute dose of 10mg/kg PCP at PND 7 is not sufficient to induce robust behavioural changes (Wang et al., 2001). However the repeated administration of PCP 10mg/kg PND 7, 9 and 11 induces a range of behavioural changes in the adult rat including hyperactivity,

locomotor sensitisation following acute PCP (Anastasio and Johnson, 2008a, Boctor and Ferguson, 2010), and impairments in social interaction (Nakatani-Pawlak et al., 2009), social novelty recognition (Harich et al., 2007), PPI (Anastasio and Johnson, 2008a), and in learning and memory tasks including NOD, reversal learning, set-shifting and spatial working memory (Sircar, 2003, Andersen and Pouzet, 2004, Boctor and Ferguson, 2010, Redrobe et al., 2012a). Therefore, chronic administration of PCP over at least 3 days seems necessary for these behavioural deficits to be observed, although the extent of behaviour deficit can be inconsistent (Rasmussen et al., 2007, Boctor and Ferguson, 2009).

Repeated administration of neonatal PCP on PND 7, 9 and 11 also induces molecular and neurochemical changes in the rat brain. Noted changes include upregulated expression of NMDA and $GABA_A$ receptors within the PFC, hippocampus and thalamus (du Bois et al., 2008), upregulation of NMDA receptor subunits NR1 and NR2 (Anastasio and Johnson, 2008b) and a decreased number of parvalbumin-positive GABAergic interneurons, specifically within the PFC (Redrobe et al., 2012b, Kaalund et al., 2013a). Neonatal PCP is also able to target apoptopic genes, specifically upregulating Txnip, Dnmt3a, Tieg and Ddit4 (thought to be pro-apoptopic) and downregulating Pcp4, Igf2, Ptgs2 and Cited 2 (thought to be anti-apoptopic) by at least PND 12 (Liu et al., 2011), leading to subsequent apoptosis, particularly within the PFC (Wang et al., 2003). Overall, these alterations in both excitatory and inhibitory neurotransmission, as well as the pathological changes induced by apoptopic cell death likely contribute to the behavioural deficits induced in

adult rats, and suggests that neurotoxicity during the crucial stages of development can underlie some of the long-term changes observed within this model (Wang et al., 2001, Gaskin et al., 2016, Grayson et al., 2016).

1.9.2: Environmental models

Increasing evidence, as discussed earlier, implicates neonatal exposure to environmental insults such as maternal stress, malnutrition, infection/immune system activation or obstetric complications to contribute to the risk of developing schizophrenia. Therefore, a number of models aim to replicate symptom chronology by use of environmental manipulations such as post-weaning social isolation or administration of compounds such as the mitotoxin methylazoxymethanol (MAM) during gestation, to induce long-term behavioural changes akin to those seen in schizophrenia (Fone and Porkess, 2008a, Lodge and Grace, 2009). Use of these types of models therefore often offers an advantage when determining the ability of pharmacological agents to reverse behavioural changes in rodents.

1.9.2.1: Gestational MAM

The antimitotic and antiproliferative agent MAM, when administered to pregnant dams, selectively targets developing neuronal cells and reduces DNA synthesis via DNA methylation to result in impaired brain development in rats and mice (Cattabeni and Di Luca, 1997). However the gestational day (GD) that MAM is administered can differentially effect the extent of anatomical,

molecular and behaviour changes in resultant offspring. For example, administration of MAM GD 17 results in subtle reductions to cortical thickness throughout the medial PFC, parahippocampal cortices, thalamus and hippocampus (Flagstad et al., 2004, Le Pen et al., 2006, Matricon et al., 2010, Chin et al., 2011) as opposed to administration on GD 15 which results in gross neuronal histopathology, microencephaly and a reduced total cortical mass by up to 67% (Moore et al., 2006), most likely due to this time point being the peak of neurogenesis in developing rat pups (Bayer et al., 1995).

The severe cortical changes induced by earlier MAM treatment however do not always correlate to a more severe behavioural phenotype in adult rats. For example, MAM administration GD 9-15 did not impair PPI, latent inhibition, conditioned emotional response (CER) or NOD responses (Fiore et al., 1999, Jongen-Relo et al., 2004) and only produced mild deficits in reversal learning (Leng et al., 2005). However, GD 17 MAM administration resulted in novel arena-induced locomotor hyperactivity, impaired performance in spatial learning and memory tasks (radial arm maze, Y-maze, Morris water maze and attention set-shifting), as well as deficits in NOD, PPI and social interaction (Flagstad et al., 2005, Le Pen et al., 2006, Moore et al., 2006, Featherstone et al., 2009, Snyder et al., 2013). Therefore use of MAM administration on GD 17 is often preferred due to its ability to produce subtle morphological changes and impair behaviour in a way that has better resemblance to the changes seen in schizophrenia.

Very few studies have determined the pharmacological reversal of these deficits but $mGluR_5$ positive allosteric modulator L5N2463359 (2.5mg/kg i.p.) was shown to attenuate reversal learning deficits in GD 17 MAM treated adult rats (Gastambide et al., 2012). It is worth noting however, that hyperdopaminergic tone is predominantly observed in the nucleus accumbens (NAc) within this model. For example, electrophysiological studies have shown GD 17 MAM-treated rats to have increased dopaminergic neuron firing in the PFC-VTA pathway and increased dopamine release in the NAc in response to amphetamine treatment (Breier et al., 1997, Lodge and Grace, 2007), somewhat questioning the validity of the model as in humans there is believed to be hyperdopaminergic activity within the associative and sensorimotor striatum (Howes et al., 2009, Egerton et al., 2013), as discussed in section 1.6.1.

1.9.2.2: Maternal immune activation

Exposure to a bacterial or viral infection during pregnancy can elevate pro-inflammatory cytokines within the blood circulation and subsequently affect offspring brain development, as discussed earlier (Brown, 2011). The systemic administration of immune-activating agents such as polyriboinosinic-polyribocytidilic acid (Poly I:C; 4mg/kg, GD 15), a by-product of viral replication, can result in acute cytokine elevation in pregnant dams and resultant offspring show sensitisation to hyperlocomotor activity following exposure to amphetamine (1mg/kg) and MK-801 (0.3mg/kg), impaired NOD, latent inhibition and reversal learning in T-maze and Morris water maze tasks, and reduced social interaction and consummatory anhedonia as assessed by the

sucrose-preference test (Zuckerman et al., 2003, Zuckerman and Weiner, 2005, Meyer et al., 2006a, Bitanihirwe et al., 2010, Wolff et al., 2011).

Maternal immune activation by Poly I:C has also been shown to decrease parvalbumin expression in the hippocampus and medial PFC, and GAD-67 expression within the hippocampus. Furthermore, lower dopamine and glutamate levels have been reported within these brain regions of resultant offspring (Nouel et al., 2012). Some of the deficits induced by Poly I:C can be restored by treatment with existing antipsychotics. For example, acute treatment with haloperidol (0.1mg/kg) and clozapine (5mg/kg) restored latent inhibition in adult offspring of female Wistar rats treated with 4mg/kg i.v. Poly I:C (Zuckerman et al., 2003). However, resultant deficits in offspring following exposure to other bacterial or viral agents are less well documented compared to other pre-clinical models and so further work is required to fully determine their reproducibility, reviewed in (Meyer et al., 2006b, Meyer and Feldon, 2010a).

1.9.2.3: Post-weaning social isolation-rearing

Another rodent model is the isolation-rearing of rat pups from weaning. This social isolation from conspecifics acts as an early-life adversity and the deprivation of rats from their normal behaviours such as social play during development causes behavioural, neuroanatomical and neurochemical changes in adult rats (Fone and Porkess, 2008a, Jones et al., 2011c), often classed as the 'isolation syndrome.'

One of the first behavioural changes observed is spontaneous and novelty-induced hyperactivity, often characterised as a lack of, or reduced acclimatisation to a novel environment which may be due to hyperactivity of the mesolimbic dopaminergic system (Jones et al., 1990a, b, Fabricius et al., 2010a, Fabricius et al., 2010b, Han et al., 2011). This change in locomotor activity (LMA) has been observed just two weeks post-isolation in rats and so has a very early onset (Weiss et al., 2000, Levine et al., 2007, Watson et al., 2012a) although the extent of hyperactivity is rat strain-dependent. For example, Wistar and Lister-hooded rat strains show significantly increased locomotor hyperactivity compared to Sprague-Dawley rats (Weiss et al., 2000). LMA is also influenced by external stimuli, such as increased handling or housing conditions, which can ameliorate this behaviour. For example, Wistar rats reared in grid-floor cages showed reduced LMA compared to animals reared in sawdust cages (Weiss et al., 1999).

With regards to other symptom domains, isolated rats show deficits in social interaction, increased aggressive behaviour (Wongwitdecha and Marsden, 1996, Meng et al., 2010, Moller et al., 2011) and numerous cognitive deficits, including impaired performance in NOD (Bianchi et al., 2006, McLean et al., 2010b, Jones et al., 2011b, Meffre et al., 2012, Watson et al., 2012b, McIntosh et al., 2013), set-shifting (McLean et al., 2010a), reversal learning (Jones et al., 1991, Quan et al., 2010) and PPI, with the latter deficit appearing 8 weeks post-weaning (Geyer et al., 1993, Bakshi and Geyer, 1999, Cilia et al., 2001, Schubert

et al., 2009), although the extent of impairment has not always been consistent (Cilia et al., 2005, Fone and Porkess, 2008a, Jones et al., 2011c).

In a similar way to other animal models, post-weaning social isolation is thought to induce neurochemical and molecular changes in the rat brain that contribute to the behavioural changes seen in the adult rat. For example, there is increased dopamine overflow in the NAc and amygdala (contributing to the increased hyper-locomotor activity) and decreased dopamine turnover in the PFC, suggested to be akin to the hypofrontality observed in schizophrenia patients (Jones et al., 1992, Fone and Porkess, 2008a). This hypofrontality is partially reversed following antipsychotic treatment with clozapine and olanzapine but not haloperidol (Heidbreder et al., 2001). Changes to glutamatergic signalling and glutamate receptors have also been observed in isolated rats; NMDA receptor density is increased in the frontal cortex, and mRNA and protein expression of the NR2A subunit is increased within the medial PFC (Turnock-Jones et al., 2009, Toua et al., 2010). There is also upregulated expression of AMPA receptor $GluR_1$ and $GluR_2$ subunits within the hippocampus, and $mGluR_6$ and $AMPA_3$ receptor subunits are upregulated within the medial PFC (Levine et al., 2007, Sestito et al., 2011). Microdialysis studies have also shown a decrease in basal glutamate overflow within the PFC (Melendez et al., 2004), suggesting that in addition to dopamine, glutamate function also plays an important role in the 'isolation syndrome' phenotype.

Isolation-reared rats also show significant volume loss in the medial PFC (Day-Wilson et al., 2006, Schubert et al., 2009) and decreased dendritic spine density

of hippocampal and PFC neurons (Silva-Gomez et al., 2003, Pascual et al., 2007, Wang et al., 2012). Loss of inhibitory neurotransmission is also apparent, with decreased hippocampal parvalbumin- and calbindin- but not calretinin-positive GABAergic interneurons (Harte et al., 2007a) and reduced GABA-transporter 1 (GAT-1) expressing chandelier cartridges, a subset of parvalbumin containing GABA interneurons that can synapse to the axon initial segment of pyramidal neurons (Bloomfield et al., 2008). Post-weaning social isolation also alters gene expression, with the down-regulation of several genes associated with glutamate signalling, apoptosis, synaptic function and some immediate early genes (IEGs) including Egr1, Egr2, c-Fos and Arc (Levine et al., 2007, Murphy et al., 2010, Gaskin et al., 2016).

A number of studies have shown acute treatment with atypical antipsychotic drugs such as clozapine, risperidone and olanzapine as well as the $mGluR_{2/3}$ agonist LY279368 to reverse locomotor hyperactivity, deficits in NOD, social interaction and PPI (Bakshi et al., 1998, Fabricius et al., 2011a, Jones et al., 2011b, Khan et al., 2011, Moller et al., 2012, Watson et al., 2012b, McIntosh et al., 2013), indicating isolation-rearing to have a degree of predictive validity. The model does however have some limitations. For example, minimal repeated handling prevents induction of some behavioural changes, including hyperlocomotion to novelty (Holson et al., 1991) and PPI deficits (Krebs-Thomson et al., 2001, Rosa et al., 2005). Although a distinct advantage is that it requires no physical intervention, making it a pure environmental model

which can easily be combined with other interventions to form a 'two-hit' model that results in a potentially more robust phenotype in adult rats.

1.9.3: 'Two-hit' models

The combination of two existing models as a novel 'dual-hit' aims to produce behavioural and neurochemical changes in adult rats that are more representative of schizophrenia symptomatology. The combination of these manipulations also enables further investigation into the genetic and environmental interactions that influence disease onset and symptom manifestation. One such model is a genetic and environmental model using DISC1 knockout mice combined with Poly I:C. The combination of these two manipulations in mice results in locomotor hypersensitivity to MK-801, reduced social interaction and social recognition as well as impaired performance in NOD (Nagai et al., 2011). However the lack of PPI impairment in this model suggests it produces a better mimicry of affective disorders rather than schizophrenia (Perry et al., 2004, Ibi et al., 2010).

The majority of 'dual-hit' models however, combine post-weaning isolation-rearing with another intervention. For example, MK-801 treatment PND 7-10 combined with isolation-rearing induces deficits in PPI and NOD in Sprague-Dawley rats (Lim et al., 2012). A common model used routinely within our laboratory is the combination of neonatal PCP (10mg/kg s.c. PND 7, 9 and 11) with isolation-rearing PND 21-25. Gaskin et al. (2014) combined these approaches to find robust behavioural changes in the adult rat, including

locomotor hyperactivity, deficits in NOD, PPI, CER and social deficits as measured in social interaction, which has since been replicated (McIntosh, 2014, Watson et al., 2016).

Regarding treatments with antipsychotics, although clozapine has only some efficacy towards negative symptoms in humans, it nearly always shows improvements in social deficits in the majority of animal models (Breier et al., 1994, Buchanan et al., 1998, Llorca et al., 2000, Harich et al., 2007, Nakatani-Pawlak et al., 2009). However social deficits in neonatal-PCP isolation-reared rats were not attenuated with acute clozapine (2.5mg/kg i.p.; (McIntosh, 2014)), suggesting the model to have better predictive validity for negative symptoms in treatment-resistant patients. Despite this, acute administration of RO4993850, a $GlyT_1$ inhibitor analogue of Bitopertin, also attenuated social deficits in this same model, reflecting results from Phase II, but not Phase III clinical trials (Hofmann et al., 2012, Umbricht et al., 2014, Hofmann et al., 2016). Although this highlights some of the limitations of assessing negative symptoms in rodents (as discussed later), it does suggest that the robust behavioural deficits produced in adult rats by such combined approaches, offers a better predictive validity than other traditionally used animal models.

Type of Animal Model		Locomotor Activity	Prepulse Inhibition	Cognitive Deficits	Social Interaction	Neurobiology/ Neuropathology	Effect of antipsychotics
GENETIC	DISC-1 knockout (Clapcote et al., 2007, Hikida et al., 2007, Shen et al., 2008, Jaaro-Peled et al., 2009, Jaaro-Peled et al., 2010, Niwa et al., 2010, Johnstone et al., 2011b, Papaleo et al., 2012a, Walsh et al., 2012)	Inconsistent; some studies report hyper-LMA e.g. CamK-ΔC strain. Others report no change.	Inconsistent; some studies report PPI deficits, others report no change.	Deficits in spatial memory (T-maze) and NOD	Inconsistent; decreases in social interaction in some strains e.g. CamK-ΔC.	Enlarged lateral ventricles, ↓ reduced cortical thickness, ↓ Pv-positive neurons, ↓ dendritic density in PFC and HIPP.	Deficits in NOD reversed by CLZ. PPI deficits in L100P mice reversed by CLZ and HLP.
	Neuregulin knockout (Mei and Xiong, 2008a, Jaaro-Peled et al., 2009, Kato et al., 2010, Shamir et al., 2012, Yin et al., 2013)	Spontaneous hyperactivity	Deficits in PPI	Impaired CER and ↓ social recognition memory	↓ Social interaction. Reported ↑ aggression	Enlarged lateral ventricles and ↓ functional forebrain NMDA receptors	Spontaneous and psychostimulant-induced hyper-LMA reversed by CLZ.
DRUG-INDUCED	Amphetamine (Sams-Dodd, 1998, Russig et al., 2003, Featherstone et al., 2007, Featherstone et al., 2008)	Locomotor sensitisation to AMPH	Deficits in PPI (dose-dependent)	Deficits in PFC-dependent tasks e.g. ASST	No reduction in social interaction	Enhanced mesolimbic dopamine response	CLZ and HLP attenuate locomotor sensitisation
	Phencyclidine (chronic and neonatal) (Sams-Dodd, 1996, 1998, Abdul-Monim et al., 2007, Snigdha and Neill, 2008, Jenkins et al., 2010, McKibben et al., 2010, McLean et al., 2012, Redrobe et al., 2012a, Neill et al., 2014)	Locomotor sensitisation to PCP	Reports of deficits in PPI but not sustained	Deficits in NOD, reversal learning set-shifting (ASST)	↓ Social interaction	↓ Basal dopamine and glutamate, ↓ PFC and HIPP Pv immunoreactivity, ↓ expression of striatial D_1 receptors	Atypical antipsychotics (excl. HLP) attenuate deficits in NOD and reversal learning. CLZ reverses social interaction deficits.

LESION-INDUCED	<u>Neonatal ventral hippocampal lesions (induced by 3µg/µl excitotoxin ibotenic acid)</u> (Chambers et al., 1996, Sams-Dodd, 1997, Lipska et al., 2003, Flores et al., 2005, Levin and Christopher, 2006, Endo et al., 2007)	Novel-arena induced hyper-LMA and ↑enhanced sensitivity to drugs inc. AMPH, cocaine or PCP.	Deficits in PPI in adulthood.	Impaired spatial learning and memory e.g. impaired radial arm maze choice accuracy.	Deficits in social interaction with ↑ aggression.	↓ GAD67 mRNA expression. ↓ Pyramidal neuron spine density and dendritic length in the PFC. ↑ $GABA_A$ receptor expression.	Antipsychotics can reverse locomotor hyperactivity but not social interaction deficits. CLZ can further impair spatial memory.
ENVIRONMENTAL	<u>Maternal immune activation</u> (Meyer et al., 2006b, Meyer et al., 2009, Bitanihirwe et al., 2010, Meyer and Feldon, 2010b, a, Wolff and Bilkey, 2010, Wolff et al., 2011, Nouel et al., 2012).	↑ Sensitivity to amphetamine and MK-801.	Deficits in PPI	Impaired NOD, latent inhibition and reversal learning in the T-maze and MWM.	↓ Social interaction	Lower basal dopamine and glutamate, and ↓ Pv expression in the PFC and HIPP.	Acute CLZ and HLP reverse PPI and reversal learning deficits.
	<u>Post-weaning social isolation</u> (Harte et al., 2007a, Levine et al., 2007, Fone and Porkess, 2008b, Fabricius et al., 2010a, McLean et al., 2010b, Murphy et al., 2010, Fabricius et al., 2011a, Jones et al., 2011b, Jones et al., 2011d, Wang et al., 2012, Watson et al., 2012b, McIntosh et al., 2013, Moeller et al., 2013).	Novel-arena induced locomotor hyperactivity after 2-3 weeks isolation.	Deficits in PPI following 6 weeks isolation but effects can be inconsistent.	Deficits in NOD, ASST, reversal learning and CER.	Deficits in social interaction and an increase in aggressive behaviours.	↓ PFC volume. ↓ Dendritic spine density of HIPP and PFC neurons. ↓ HIPP Pv positive neurons, ↓ basal glutamate.	Atypical antipsychotics, 5-HT_6 antagonists and $mGluR_{2/3}$ agonists reverse hyper-LMA and attenuate deficits in NOD, social interaction and PPI

TWO-HIT	Neonatal-PCP combined with Isolation-rearing (Gaskin et al., 2014, McIntosh, 2014, Gaskin et al., 2016, Watson et al., 2016)	Novel-arena induced hyper-LMA.	Deficits in PPI	Impaired NOD and CER	Deficits in social interaction	Under investigation. Down-regulation of scz-linked genes, including Pv and GAD67	CLZ failed to restore deficits in social interaction.

Table 1.1: Summary of select behavioural and neurochemical changes observed in various animal models for schizophrenia. AMPH; Amphetamine, ASST; Attentional Set-Shifting Task, CER; Conditioned Emotion Response, CLZ; Clozapine, DISC-1; Disrupted In Schizophrenia 1, D1; Dopamine D1 receptor, GABA; Gamma Amino Butyric Acid, GAD67; Glutamic Acid Decarboxylase Enzyme (67 kDa isoform), HIPP; Hippocampus, HLP; Haloperidol, 5-HT6; 5-Hydroxytrypatmine (serotonin) 6 receptor, LMA; Locomotor Activity, mGluR2/3; Metabotropic Glutamate 2/3 receptor, MWM; Morris Water Maze, NMDA; N-Methyl-D-aspartic acid, NOD; Novel Object Discrimination, PCP; Phencyclidine, PFC; Prefrontal Cortex, PPI; Pre-pulse Inhibition of acoustic startle, Pv; Parvalbumin, scz; schizophrenia.

1.10: Assessing 'schizophrenia' in rodents

1.10.1: Assessing positive symptoms

The positive symptoms of schizophrenia such as hallucinations are difficult to model in rodents however hyperlocomotor activity, and to some extent neophobia, are still considered to be a relevant index. In this long established paradigm, LMA is assessed in an open field following placement into a novel arena. Typically, rats placed in a novel environment will show elevated levels of activity which decreases over time as rats become habituated to their surroundings. However rats with a 'schizophrenia-like' phenotype habituate at a slower rate (Wang et al., 2001, Fone and Porkess, 2008a, Nakatani-Pawlak et al., 2009, Boctor and Ferguson, 2010, Jones et al., 2011c, Le Pen et al., 2011, Zhang et al., 2012), potentially reflecting an increased desire to escape and therefore a neophobic response (Fone et al., 1996).

The hyperactivity is believed to be primarily due to hyper-dopaminergic activity within the NAc, and antipsychotics can antagonise dopamine D_2 receptors within this region to attenuate the hyperlocomotion observed (Fabricius et al., 2011b, Le Pen et al., 2011). However, the mesocortical dopaminergic pathway has also been implicated, with elevated LMA correlated to increased striatal dopamine release, akin to the correlation observed between striatal dopamine release and psychosis risk in ultra-high risk groups (Abi-Dargham et al., 2000, Flagstad et al., 2004).

1.10.2: Assessing negative symptoms

1.10.2.1: Social withdrawal

Originally used as a test for anxiety-like behaviours, the social interaction test was adapted by Sams-Dodd (1995, 1997) to be used as an index of social withdrawal in patients with schizophrenia, although the lack of clinically effective treatments means there is no 'gold standard' with which to evaluate the predictive validity of the paradigm. Despite this, clozapine, which alleviates social deficits in some patients (Murphy et al., 2006), produces some improvements in rodent social interaction tasks where complete physical contact between subjects is allowed, and where factors such as duration of interaction and ano-genital sniffing are measured (Presti-Torres et al., 2012). However, less promising results have been obtained when the subjects are placed in separate chambers, and nose-poking is used to indicate the potential desire to interact (Gururajan et al., 2012).

The mechanisms underlying social interaction in rats are thought to primarily involve the VTA-NAc neuronal circuit and be mediated in part by activation of dopamine D_1 receptors. Novel optical tools have used fibre photometry to try and establish which projections may play a causal role in driving or inhibiting social behaviours. Studies using these techniques have shown increased VTA cell body fluorescence during social interaction, and infusion of D_1 receptor antagonist SCH23390 attenuated the pro-social effects elicited by VTA-dopamine neuron stimulation (Gunaydin et al., 2014). Cannabinoid-1 (CB_1) receptors are also thought to be crucial for social interation. For example, PCP-

induced social withdrawal (5mg/kg i.p. sub-chronic PCP, twice daily for 7d) was attenuated following administration of cannabinoid receptor (CB_1 and CB_2) agonist CP55,940 (0.01mg/kg, i.p.) and following administration of fatty acid amide hydrolase (FAAH) inhibitor URB597 (0.1 and 0.3 mg/kg i.p.), which inhibits endocannabinoid degradation (Seillier et al., 2013). More work is needed to determine fully how neuronal circuits are affected in developmental models however, neonatal-PCP isolation-rearing has been shown to induce robust social deficits, as measured by social interaction (McIntosh, 2014).

1.10.2.2: Anhedonia

Anhedonia is typically assessed through sucrose preference tests and through progressive ratio schedules to determine an animal's breaking point (motivational state and willingness to receive a rewarding stimulus; (Simpson et al., 2012)). When given a choice between water and sucrose, rodents have a stronger preference for a sucrose-containing solution (Willner, 2005) and animal models for schizophrenia characteristically show a lower preference for sucrose in this paradigm (Strekalova et al., 2004, Neill et al., 2014). A fact which has been greatly exploited to determine reinforcement behaviour. For example, acute, sub-chronic and chronic exposure to PCP at various doses decreases sucrose consumption (Turgeon and Hoge, 2003, Baird et al., 2008, Neill et al., 2010).

There is considerable debate as to whether sucrose consumption is truly a marker of a hedonic response and whether it can be used as a measure of

negative symptoms in animal models. For example the anticipation of reward is believed to be affected in schizophrenia, not necessarily the immediate response following reinforcement (Gard et al., 2007, Barnes et al., 2014), and so the consummatory response to sucrose as a marker may not be the most efficient way of assessing the emotional disturbances present in animal models (Neill et al., 2014). In light of this, effort-related choice tasks are currently considered to have high translational value to assess motivation and goal-directed behaviour in rodents (Der-Avakian et al., 2016). Developed by Salamone's lab group, rats are trained to choose between high effort and low effort choices which yield high or low rewards respectively (Markou et al., 2013, Salamone et al., 2016a, Salamone et al., 2016b). In this paradigm, $GlyT_1$ inhibitor Bitopertin (10mg/kg p.o.) reversed the haloperidol-induced suppression on lever pressing and haloperidol-induced attenuation of high-effort over low-effort barrier choice for rewards in Sprague-Dawley rats (Yohn et al., 2017), suggesting the effort-related choice task to be a suitable paradigm with which to assess motivational dysfunction.

1.10.2.3: Communication (via USVs)

The emission of ultrasonic vocalisations (USVs) by rats is considered to be an important form of communication influencing their social behaviour. The acoustic features of these vocalisations can vary throughout the rat's development and three distinct types have thus far been categorised according to their average peak frequency. These are namely 22 kHz, 50 kHz and 40 kHz calls. Both 22 kHz and 50 kHz calls are emitted by juvenile and adult rats

whereas 40 kHz calls are primarily emitted by rat pups, usually following isolation from either the dam and/or litter (Portfors, 2007, Burgdorf et al., 2013).

The higher frequency (50 kHz) USVs can occur in a variety of situations including rough-and-tumble play, mating, food consumption, social isolation, or placement in a fresh housing cage (Brudzynski, 2015). Generally, these calls are categorised into three main groups of flat (constant frequency) and frequency modulated (step and trill) based on their pattern analysis. Recently, these call subtypes have been broken down even further into up to fourteen different groups (Wright et al., 2010), highlighting the acoustic diversity of 50 kHz USVs emitted by rats.

It is generally assumed that these higher frequency 50 kHz calls are reflective of positive emotional states. This is based on studies focusing on the effects of drugs of abuse on the number of USV emissions. For example, amphetamine (2mg/kg), morphine (1mg/kg) and MDMA (10-15mg/kg) showed significant increases in trill type call emissions compared to vehicle controls (Simola, 2015). In addition, amphetamine (0.25-2mg/kg) has been shown to dose-dependently increase the total number of 50 kHz USV emissions, particularly the trill subtype (Wright et al., 2010), suggesting that trill calls are associated with reward. By comparison, flat type calls have been suggested to re-establish social contact. For example, flat call emissions were significantly higher in singly-tested as opposed to pair-tested rats (Wohr et al., 2008). Furthermore, adult male rats have higher call rates during transient isolation from their cage

mates, which decreases across the isolation period (Wohr et al., 2008). By contrast, lower frequency (22 kHz) USVs are classed as 'alarm' calls, emitted often in the presence of aversive stimuli such as foot shocks, bright light and predatory odours (Portfors, 2007, Brudzynski, 2015) and are therefore thought to reflect negative affective states. Consequently, it appears that USV emissions by adult rats can be influenced by the surrounding environment and are not solely specific to reward-associated situations.

Communication is often impaired in neuropsychiatric disorders including schizophrenia and Autism Spectrum Disorders (ASD), and very few effective models exist specifically to determine effects on communication. Some studies have bred rat strains for high and low USV emissions, typically using Long Evans (Burgdorf et al., 2013) or Wistar rats (Wohr and Schwarting, 2007, Wohr et al., 2008) however the majority of studies focus on the changes to USV emissions in approach behaviour or vocal responses following 50 kHz or 22 kHz playback (Wohr and Schwarting, 2007, Burgdorf et al., 2013), and less so in non-aggressive social environments such as during social interaction with a novel conspecific. Therefore understanding the complexity behind these calls would be beneficial to improving the validity of rodent models, as well as improving the translational relevance of using rodents to predict the clinical efficacy of novel therapeutics, particularly for negative symptoms.

1.11: Concluding remarks

Schizophrenia is a neurodevelopmental disorder influenced by genetic and environmental factors. In particular, negative symptoms of the disease remain poorly treated although $GlyT_1$ inhibitors remain a key area of interest, with improvements to negative symptoms seen in pre-clinical studies and in clinical trials. Additionally, adjunctive therapies with compounds such as oxytocin or anti-oxidants may have some therapeutic potential based on additional physiological and neurobiological changes observed in schizophrenia patients. Although much more work is required to fully elucidate how such compounds can improve negative symptoms, improving animal models and improving the way in which we assess negative symptoms in rodents can help determine which therapies are more likely to have an effect in the human population.

1.12: Thesis aims

The overall aims of this thesis are to investigate the ability of $GlyT_1$ inhibitor RO4993850, analogue to Bitopertin, and neuropeptide oxytocin to improve social behaviours in Lister-hooded rats. Chapter 2 will focus on producing a functional map of the $GlyT_1$ inhibitor RO4993850 to establish some of the key brain loci involved in mediating its effects and provide insight into its precise mechanism of action. Chapter 3 will utilise the same $GlyT_1$ inhibitor to determine its ability to reverse deficits in social behaviour, also investigating neurochemical changes induced in the 'dual-hit' neonatal-PCP isolation-rearing model to further characterise its face validity. Finally, chapter 4 will investigate the effects of oxytocin on a battery of behaviour tests and determine a possible mechanism of action for how the neuropeptide mediates its effects.

Chapter 2: Assessing neuronal activation induced by $GlyT_1$ inhibitor RO4993850 in the rat brain

2.1: Introduction

For years it has proved difficult to fully determine the precise neural mechanisms by which various drugs work within the brain. Administration of pharmacological compounds can activate or inhibit signalling pathways both directly and indirectly following binding to their primary target. Therefore, understanding the mechanism of action for drugs is of vital importance when determining their viability for psychiatric diseases. One of the most commonly used methods to determine the specific brain loci activated and subsequent neuronal metabolic activity following drug administration, is the use of immunohistochemistry with metabolic neuronal markers (Dragunow and Faull, 1989).

2.1.1: C-fos as a neuronal marker

There are a number of different proteins that can be used as markers of neuronal activity, however one of the most commonly used as a functional mapping tool is c-fos. The c-fos protein, an IEG originally identified as a proto-oncogene in a chicken with a nephroblastoma (Nishizawa et al., 1987), consists of 380 amino acids and is part of the Fos protein family. It contains a leucine zipper motif which allows dimerization with other proteins derived from oncogenes, particularly c-Jun; a member of the Jun protein family. The synergistic binding of these dimers at the AP-1 transcriptional activating factor

complex binding site is required to mediate long-term responses to signals that regulate growth and development (Franza et al., 1988). C-fos can be visualised as staining of the cell nucleus by immunohistochemistry using antibodies developed specifically for the c-fos protein (Hoffman et al., 1993). Members of the Fos and Jun protein families are classified as IEGs and therefore do not require *de novo* synthesis prior to transcription. Thus, they are the first genes to be activated by external stimuli, and induction is both rapid and transient (Greenberg et al., 1986).

Since the first experiments conducted on differentiated PC12 cells, showing effective c-fos induction with depolarising agents (Morgan and Curran, 1986), studies in the rat have revealed increased c-fos expression following exposure to external stimuli, including seizures (Morgan et al., 1987), brain injury (Dragunow et al., 1990b), exposure to stress (Kovacs, 1998, Turgeon et al., 2007), and during learning and memory tasks such as NOD (Zhu et al., 1995, Zhu et al., 1997, Jenkins et al., 2004, Albasser et al., 2010). In effect, the administration or exposure to various growth factors, neurotransmitters, drugs, and physiological manipulations are able to increase expression of the c-fos gene within the central nervous system (CNS; (Herrera and Robertson, 1996)).

C-fos expression occurs in both neurons and glial cells, particularly following cortical injury (Dragunow and Robertson, 1988, Dragunow et al., 1990b, Bennett and Schwartz, 1994, Herrera and Robertson, 1996), but increase in expression is largely thought to be a consequence of synaptic activation

(Herrera and Robertson, 1996, Kovacs, 1998). The IEGs can induce or inhibit the transcription of target genes, depending on which combination of proto-oncogene dimerization occurs, contributing to long term changes within the brain (MacGibbon et al., 1994). It is generally believed that dimerization of c-fos to c-Jun induces gene transcription, whilst dimerization to Jun-B inhibits gene transcription (Herrera and Robertson, 1996). Studies by Dragunow and Robertson (1988) and Hughes et al. (1992) also suggest that the various IEGs are differentially regulated in terms of their basal expression, and whilst levels of c-Jun were found to be quite high in the dentate gyrus (DG) of adult male Sprague-Dawley rats, baseline levels of c-fos were found to be very low within the same region.

Therefore, the expression of c-fos or other IEGs by individual neurons makes it a useful way to determine activation within specific brain sub-regions and produce a functional anatomical map for neuronal populations affected by various stimuli, including the administration of pharmacological agents. For example, dopamine D_2 receptor antagonist haloperidol dose-dependently induces c-fos immunoreactivity in striatal neurons, an effect which is reversed following administration of a dopamine D_2 receptor agonist (Miller, 1990). This reversal was also seen following administration of NMDA receptor antagonist MK-801 (Dragunow and Faull, 1990, Dragunow et al., 1990c) suggesting the increase in c-fos expression within the striatum to be mediated in part via NMDA receptors. The intracellular signalling pathways involved in c-fos production following activation of dopamine D_1 (direct pathway) and NMDA receptors are shown in Figure 2.1. Further studies have suggested that the

subsequent release of glutamate in the corticostriatal pathway is the primary response leading to increased c-fos expression, with the activation of NMDA receptors playing a key role in c-fos accumulation (Dragunow et al., 1990a, Aronin et al., 1991, Fu and Beckstead, 1992). Thus, the low levels of c-fos transcription under basal conditions and its rapid induction on downstream target genes and signalling pathways means the protein continues to remain a useful mapping tool to determine the mechanism of action for various compounds.

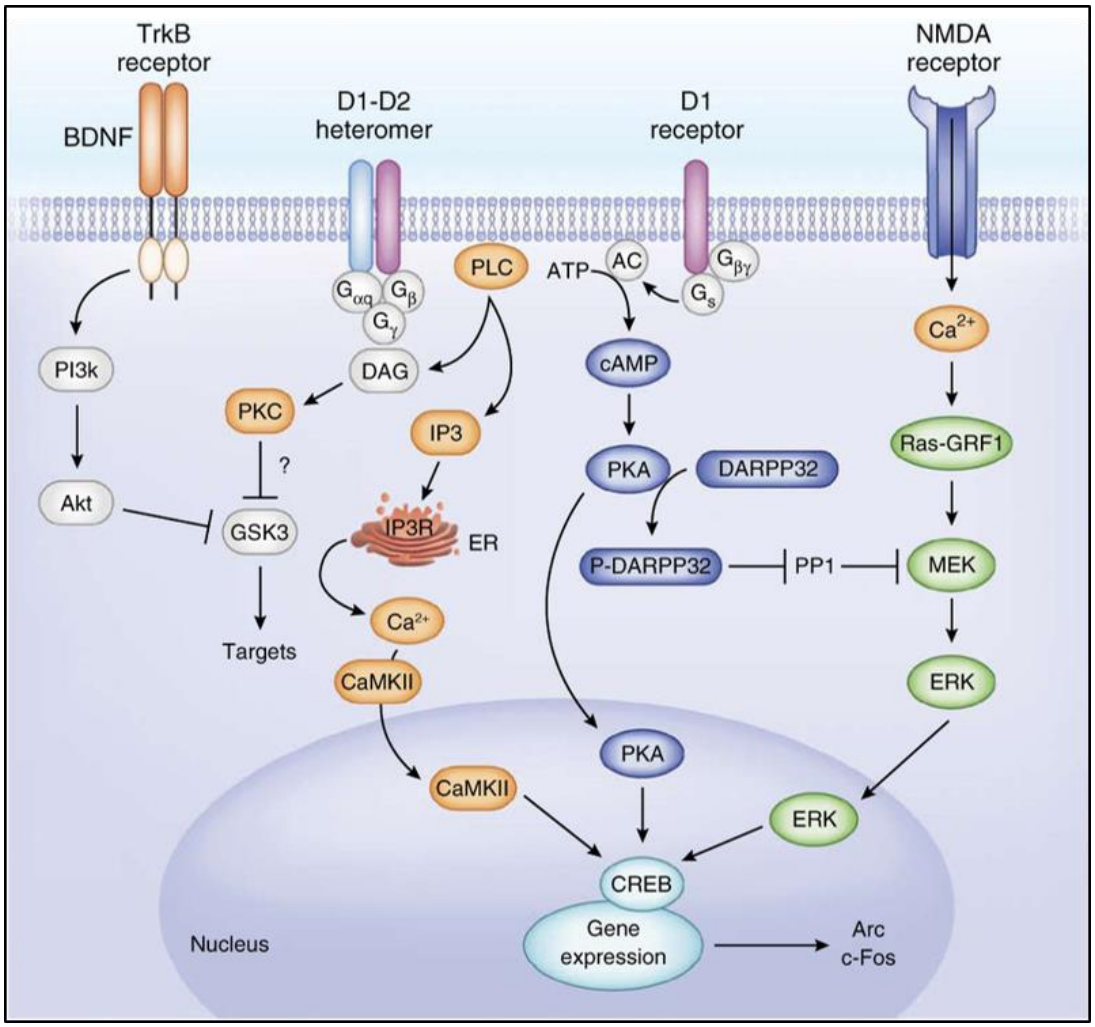


Figure 2.1: Intracellular signalling pathways that lead to c-fos production. Following activation of specific receptors, intracellular signalling pathways can lead to downstream activation of several genes. Receptors include NMDA receptors, the TrkB receptor, dopamine-induced D_1-D_2 heteromers, and dopamine D_1-like receptors which can induce two different signalling pathways (including direct modulation of the NMDA receptor via $G_{\beta}\gamma$ proteins and via activation of protein kinase A (PKA), which can translocate to the nucleus and act directly on CREB). Figure taken from (Calabresi et al., 2014). AC, adenylyl cyclase; Arc, activity-regulated cytoskeleton-associated protein; DAG, diacylglycerol; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinases; IP3, inositol 1,4,5-trisphosphate; MEK, mitogen-activated protein kinase; PKC, protein kinase C; P-DARPP-32, phosphorylated dopamine- and cAMP-regulated phosphoprotein 32 kDa; PP1, protein phosphatase 1; PI3k, phosphatidylinositide 3-kinases; Ras-GRF1, Ras-guanine nucleotide-releasing factor 1; CaMKII, calcium/calmodulin-dependent protein kinase type II; PLC, phospholipase C; CREB, cAMP responsive element binding protein; GSK3, glycogen synthase kinase-3.

2.1.2: Glycine Transporter Inhibitors

Glycine Transporter ($GlyT_1$) inhibitors, as discussed in Chapter 1: section 1.8.4.2, represent a novel treatment approach for disorders involving NMDA receptor hypofunction. The subsequent binding of both glycine and glutamate to the $GluN_1$ (Hirai et al., 1996) and $GluN_2$ (Laube et al., 1997) subunits of the NMDA receptor respectively, is necessary for the activation of these glutamatergic receptors (Johnson and Ascher, 1987, Kleckner and Dingledine, 1988). The localisation of $GlyT_1$ transporters and NMDA receptors have been found to overlapwithin forebrain areas (Cubelos et al., 2005), with $GlyT_1$ transporters tightly regulating synaptic glycine concentrations and therefore modulating NMDA receptor function. Normal levels of glycine within the CSF fall in the range of 7-10µM (Ferraro and Hare, 1985) although affinity for glycine at its NMDA receptor binding site is much lower, ranging between 0.1-3µM (Danysz and Parsons, 1998), suggesting that synaptic levels of glycine are kept at non-saturating levels, most likely via astrocytes (Shibasaki et al., 2017). The exact concentrations have been difficult to ascertain although several lines of evidence suggest glycine levels are maintained between nanomolar and low micromolar concentrations, close to the EC50 for the synaptic NMDA receptor glycine sites (Semba and Patsalos, 1993, Bergeron et al., 1998).

The use of $GlyT_1$ inhibitors as a viable treatment approach for schizophrenia is based on evidence showing improvements to negative, positive and cognitive symptom scores in patients following administration of exogenous glycine, and agonists for the glycine binding site such as D-serine (Javitt et al., 1994,

Heresco-Levy et al., 1996, Leiderman et al., 1996, Heresco-Levy et al., 2005). The low brain penetration of D-serine, the non-optimal gastrointestinal tolerability of the very high doses of glycine needing to be administered, together with the difficulties in identifying small molecule agonists at the glycine-site of NMDA receptors have led to the search for $GlyT_1$ inhibitors as an indirect way to increase synaptic glycine levels and subsequently induce NMDA receptor activation. The viability of this approach is supported *in vivo* (Black et al., 2009, Boulay et al., 2010, Burket et al., 2015, Chaki et al., 2015, Komatsu et al., 2015). For example, 0.1mg/kg TASP0315003, a novel $GlyT_1$ inhibitor currently in development, was shown to improve PCP-induced deficits in social interaction in mice and MK-801-induced cognitive dysfunction in NOD and social recognition paradigms in rats following oral administration 4h prior to each behavioural test (Chaki et al., 2015).

Clinical studies have also shown some promise, summarised in (Singer et al., 2015), although the $GlyT_1$ inhibitor Bitopertin (RG1678) developed by F. Hoffmann-La Roche remains the only compound to date to reach phase III in clinical trials. Bitopertin inhibits glycine reuptake in the brain and increases glycine levels to the same extent as those seen in preclinical models (Hofmann et al., 2016). Furthermore, when adjunctively administered to antipsychotics for 8 weeks at a dose of 10, 30 or 60mg, it significantly reduced negative symptom scores at the 10mg and 30mg doses in a randomized, double-blind phase II clinical trial (Umbricht et al., 2014). Another glycine reuptake inhibitor, AMG-747, has shown similar improvements in negative symptoms in patients with schizophrenia when added to current antipsychotic treatment in a double-

blind phase II clinical trial (Dunayevich et al., 2017). Unfortunately, results of phase III trials in the same populations with Bitopertin have been disappointing (Bugarski-Kirola et al., 2017).

Although Bitopertin improved total PANSS symptom scores at the 30mg/kg dose when taken as a monotherapy in patients with acute schizophrenia, it failed, the same as olanzapine, to reach statistical significance from placebo in a randomised, double-blind phase II/III trial (Bugarski-Kirola et al., 2014). Nonetheless, in one of the three phase III clinical studies in patients with sub-optimally controlled positive symptoms, Bitopertin, at the dose of 10mg, induced a statistically significant improvement in the Positive Symptoms Factor Scores scale (Bugarski-Kirola et al., 2014, Bugarski-Kirola et al., 2017), suggesting further therapeutic potential of $GlyT_1$ inhibitors beyond the treatment of negative symptoms alone**.** Therefore, despite disheartening results in recent trials, these compounds warrant further investigation.

2.2: Aims

The $GlyT_1$ inhibitor, RO4993850, is an analogue of Bitopertin, and like its sister compound, has been shown to restore social deficits in a neurodevelopmental rat model for schizophrenia (McIntosh, 2014). However, the precise brain loci mediating these effects following systemic administration remain unknown.

This study therefore aims to illustrate some key brain loci involved in mediating the effects of $GlyT_1$ inhibitors, in particular, the impact of RO4993850 on c-fos expression in selected brain regions including the prefrontal and orbitofrontal (OFC) cortices, NAc and amygdala following systemic administration in the rat. Information obtained from this study will be useful in outlining a functional map of $GlyT_1$ inhibitors within the CNS and provide insight into their mechanism of action.

2.3: Methods

2.3.1: $GlyT_1$ inhibitor induced c-fos activation

2.3.1.1: Animals

Twenty-four drug-naïve adult male Lister-Hooded rats weighing 150-200g on arrival (Charles River UK) were housed in groups of four in individually ventilated cages, under a controlled temperature (21 ± 2°C) and humidity (45 ± 15%) with a 12h light/dark cycle (lights on 06:00-18:00h). All experiments were performed in the light phase, with food (Beekay Standard diet) and water available *ad libitum*. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986 and the ARRIVE guidelines (Kilkenny et al., 2010) with approval of the University of Nottingham Local Ethical Committee (AWERB).

2.3.1.2: Experiment Design

All animals received a (0.9%w/v) sterile saline intraperitoneal (i.p.) injection 24h before drug injection to habituate them to the procedure. The animals were divided in a pseudorandom manner into three treatment groups (n=8 per treatment group) to receive an i.p. injection of either vehicle (Gelatine 7.5%/NaCl 0.62%; 2ml/kg) or $GlyT_1$ inhibitor RO4993850 (Gelatine 7.5%/NaCl 0.62%/active-RO4993850; 1mg/kg or 3mg/kg). Doses were selected based on previous studies which had found RO4993850 to attenuate deficits in a NOD paradigm in neonatal-PCP isolation-reared rats (McIntosh, 2014). All drugs

were delivered ready-formulated by F. Hoffmann-La Roche Ltd and administered using a blind protocol.

2.3.1.3: Transcardial Perfusions

Post-injection, the animals were left undisturbed in the home cage for 2h before being deeply anaesthetised with isoflurane and transcardially perfused with 180ml phosphate-buffered saline (PBS) and 180ml 4% v/v paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4 (fixative solution). The 2h interval was selected based on in-house studies at Roche to allow for a 30min drug pre-treatment time and maximal c-fos production since previous reports have shown peak Fos protein production to occur 90-120min post-drug administration (Zangenehpour and Chaudhuri, 2002), although it is worth noting that some studies have also used a shorter interval time of 60min (Ebling et al., 1991, Cullinan et al., 1995). Brains were removed from the skull and post-fixed in 4% PFA solution at 4°C for 24h before being transferred into a cryo-preservative solution containing 20% sucrose in 0.1M PBS, pH 7.4.

2.3.1.4: C-fos immunohistochemistry

Each brain was sequentially cut in the coronal plane into 60µm thick sections on a freezing microtome (Anglia Scientific) and submersed in PBS. Endogenous peroxidase activity was blocked by incubating the sections in 0.5% v/v hydrogen peroxide in PBS for 10min. Sections were then washed twice with PBS and incubated with PBST (0.01M PBS, 1% BSA, 0.3% Triton-X) and blocked in 0.02%

v/v goat serum for 1h at room temperature before incubation in PBST containing c-Fos rabbit polyclonal antibody (1:1000 dilution; ab7963; Abcam) for 24h at 4°C.

After 24h sections were washed in a second PBST solution (0.01M PBS, 0.3% BSA and 0.1% Triton-X) before incubation in PBST containing biotinylated goat anti-rabbit secondary antibody (1:200 dilution; Vector Laboratories) for 2h. The sections were subsequently washed before processing with avidin-biotin-horseradish peroxidase complex in PBST (Vectastain Elite Kit, Vector Laboratories) for 2h. The reaction was developed using 0.05% 3, 3'-diaminobenzidine (DAB; Substrate Kit, Vector Laboratories) in the presence of 0.01% v/v hydrogen peroxide and stopped by washing 3 times in PBS. The sections were mounted on gelatin-coated microscope slides, left to dry and then dehydrated using a graded series of alcohols (70%, 95% and 100%), cleared in xylene and coverslipped with DPX mounting media.

2.3.1.5: Cell Quantification

The brain structures of interest were outlined according to Paxinos and Watson's stereotaxic atlas (Paxinos and Watson, 1986) and the areas analysed included the PFC, OFC, amygdala, NAc and dorsal and ventral striatum. Regions were selected because of the known distribution of $GlyT_1$ and the likelihood of involvement in social behaviour. Sections at specific Bregma points containing one or more of the desired brain regions (see figure 2.2; A) were imaged as .TIFF files at a magnification of 50x using a Zeiss Axioplan microscope system with

QCapture Pro 7 software, cropped to isolate the specific brain region in question, and measured, with at least 6 sections used for each region of interest. Fos-positive nuclei were visualised by the dark reaction product confined to the nucleus and quantified using the cell counter functions in ImageJ software (NIH). Only cell-positive neurons reaching 70% optical density were included and the experimenter was blind to treatment groups when quantifying images. Changes in c-fos expression between treatment groups were analysed using one-way ANOVA. The differences in means were further analysed by Sidak post-hoc tests, with significance set at $p<0.05$.

2.3.2: PCP induced c-fos activation

Due to the initial difficulties in optimising c-fos immunoreactivity, 6 group-housed drug-naïve adult male Lister-hooded rats (150-200g on arrival, Charles River UK) were used as a control; 3 received sterile saline (0.9%; 2ml/kg i.p.) and 3 received PCP (10mg/kg i.p.). This dose of PCP was selected based on studies showing acute administration of 10mg/kg to induce sufficient c-fos expression in frontal and striatal brain regions in both rats and mice (Santana et al., 2011, Castane et al., 2015). The same experiment design was used, with rats returned to the home cage post-injection and left undisturbed for 2h before transcardial perfusion with 4% PFA. The same reagents, antibody concentrations and optimised immunohistochemical procedure were also used. Changes to c-fos expression was determined in the same brain regions using the same AP coordinates from Bregma. Differences in c-fos

immunoreactivity between treatment groups were analysed using unpaired Student's *t*-tests with Holm-Sidak correction. Significance was set at $p<0.05$.

2.4: Results

2.4.1: $GlyT_1$ inhibitor induced c-fos activation

Compared to vehicle, RO4993850 caused a significant increase in the number of c-fos immunoreactive cells in both the rostral dorso-lateral ($F_{(2,22)}$=13.710, p<0.001), lateral ($F_{(2,23)}$=7.647, p=0.03) and ventral ($F_{(2,23)}$=6.877, p=0.05) OFC, and caudal ventral ($F_{(2,20)}$=13.536, p<0.001) and lateral ($F_{(2,20)}$=21.750, p<0.001) OFC. Further analysis revealed this increased pattern of c-fos activation to be dose-dependent, with significance achieved between both low and high doses in the rostral dorsolateral (p<0.001), and caudal lateral (p=0.006) OFC, Sidak post-hoc.

RO4993850 also increased c-fos expression in the rostral ($F_{(2,23)}$=2.784, p=0.085) and caudal ($F_{(2,20)}$=3.349, p=0.058) prelimbic (PRL), infralimbic (IL; $F_{(2,20)}$=7.231, p=0.005) and limbic cortices (CG1; $F_{(2,20)}$=4.785, p=0.022). However, RO4993850 failed to produce any concomitant substantial elevation in c-fos expression in the NAc or basomedial amygdala (BMA), although a small but significant increase at the low dose occurred in the basolateral amygdala (BLA; $F_{(2,21)}$=4.696, p=0.022; Figure 2.3).

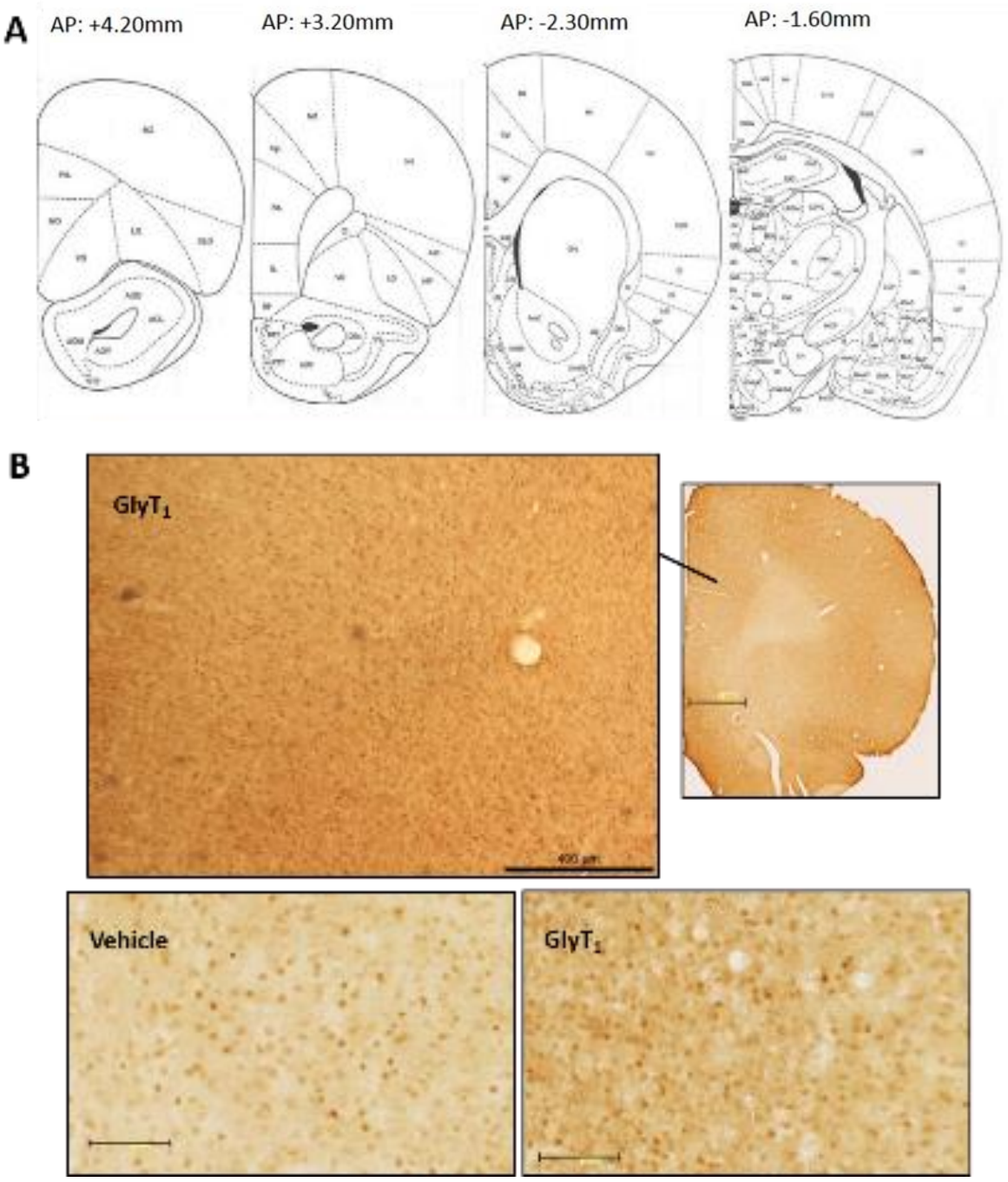
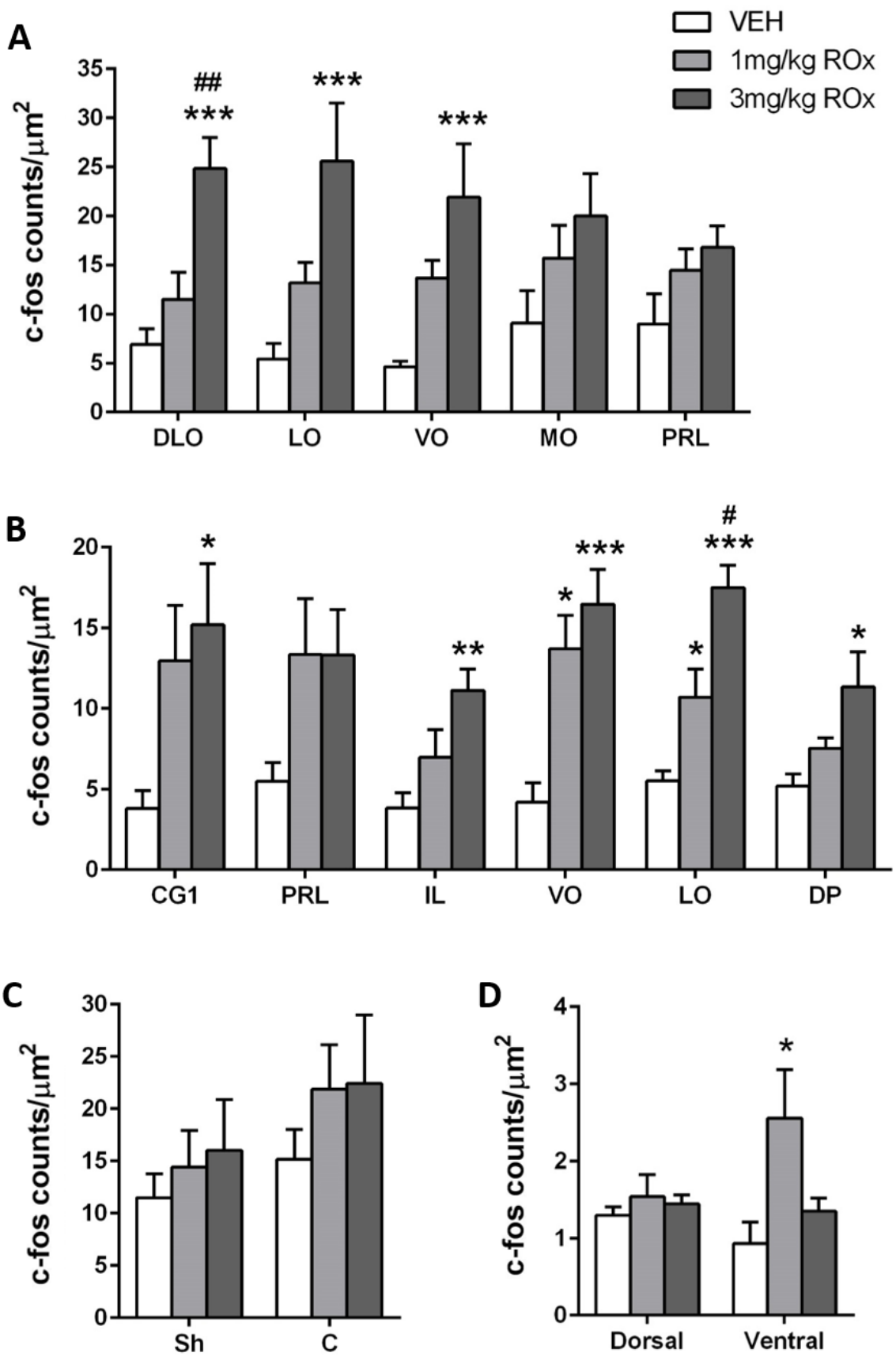


Figure 2.2. Diagrammatic representation of rat brain areas selected for c-fos immunohistochemistry: A; Coronal sections taken at AP locations measured from Bregma as indicated from Paxinos and Watson (1986) for the measurement of c-fos immunoreactivity, sequentially depicted left-right as the rostral and caudal prefrontal cortex, striatum, nucleus accumbens and amygdala. B; 50x microscopic image (scale bar 400µm) and zoomed in 20x microscopic images (scale bar 100µm) of c-fos positive nuclei (defined by dark brown dots) in the rostral prelimbic cortex of rats treated with vehicle or acute $GlyT_1$ inhibitor RO4993850 3mg/kg i.p.


A
VEH
1mg/kg ROx
3mg/kg ROx
c-fos counts/μm²
DLO
LO
VO
MO
PRL
B
CG1
PRL
IL
VO
LO
DP
C
Sh
C
D
Dorsal
Ventral

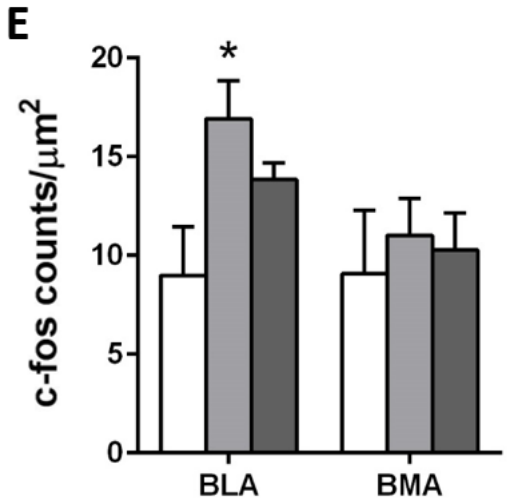


Figure 2.3: Density of c-fos counts (counts/µm^2) in various brain sub-regions: A; Rostral prefrontal cortex (PFC), B; Caudal PFC, C; Nucleus Accumbens, D; Striatum and E; Amygdala. Columns show the mean ± SEM c-fos counts for rats treated with an acute dose of $GlyT_1$ inhibitor RO4993850 (ROx; 1 or 3mg/kg) or Vehicle (VEH; 2ml/kg) i.p. before returning undisturbed to the home cage for 2h.

One-way ANOVA for each brain sub-region revealed a significant effect of treatment in the rostral dorsolateral ($F_{(2,22)}$=13.710, p<0.001), lateral ($F_{(2,23)}$=7.647, p=0.03), and ventral ($F_{(2,23)}$=6.877, p=0.05) OFC sub-regions, as well as in the caudal VO ($F_{(2,20)}$=13.536, p<0.001) and LO ($F_{(2,20)}$=21.750, p<0.001) OFC. A significant treatment effect was also observed in the infralimbic ($F_{(2,20)}$=7.231, p=0.005), and limbic cortices ($F_{(2,20)}$=4.785, p=0.022), ventral striatum ($F_{(2,23)}$=4.238, p=0.028) and basolateral amygdala ($F_{(2,21)}$=4.696, p=0.022). *p<0.05, **p<0.01, ***p<0.001 VEH vs 3mg/kg ROx; $^{\#}$p<0.05, $^{\#\#}$p<0.01 1mg/kg vs 3mg/kg ROx; Sidak post-hoc; n=8 per treatment group. OFC; Orbitofrontal cortex, PFC; Prefrontal Cortex; DLO; Dorsolateral OFC, LO; Lateral OFC, VO; ventral OFC, MO; medial OFC, PRL; prelimbic cortex, CG1; Limbic cortex 1, IL; infralimbic cortex, DP; dentate putamen, C; NAc Core, Sh; NAc Shell, BLA; Basolateral amygdala; BMA; Basomedial amygdala).

2.4.2: PCP induced c-fos activation

Compared to saline-treated animals, PCP-treated rats showed substantially increased c-fos immunoreactivity in several sub-regions, reaching significance in the rostral medial OFC (M=14.2 vs M=67.4; $t_{(76)}$=3.879, p=0.0002) and caudal PRL (M=14.4 vs. M=72.4; $t_{(76)}$=4.228, p<0.0001) and IL cortices (M=16.2 vs M=76.8; $t_{(76)}$=4.118, p<0.0001). Strong trends of elevated c-fos expression were also noted in the rostral ventral OFC (M=25.3 vs M=64.5; $t_{(76)}$=2.864, p=0.005), rostral PRL (M=25.5 vs M=61.4; $t_{(76)}$=2.621, p=0.0106) and caudal limbic cortex (M=9.7 vs M=47.6; $t_{(76)}$=2.762, p=0.007; Figure 2.5). Additionally, elevated expression was observed in the core of the NAc, dorsal striatum and amygdala although significance was not reached in these regions. The observations with PCP served to confirm the validity of the c-fos approach used herein with a compound whose pattern of activation is well documented.

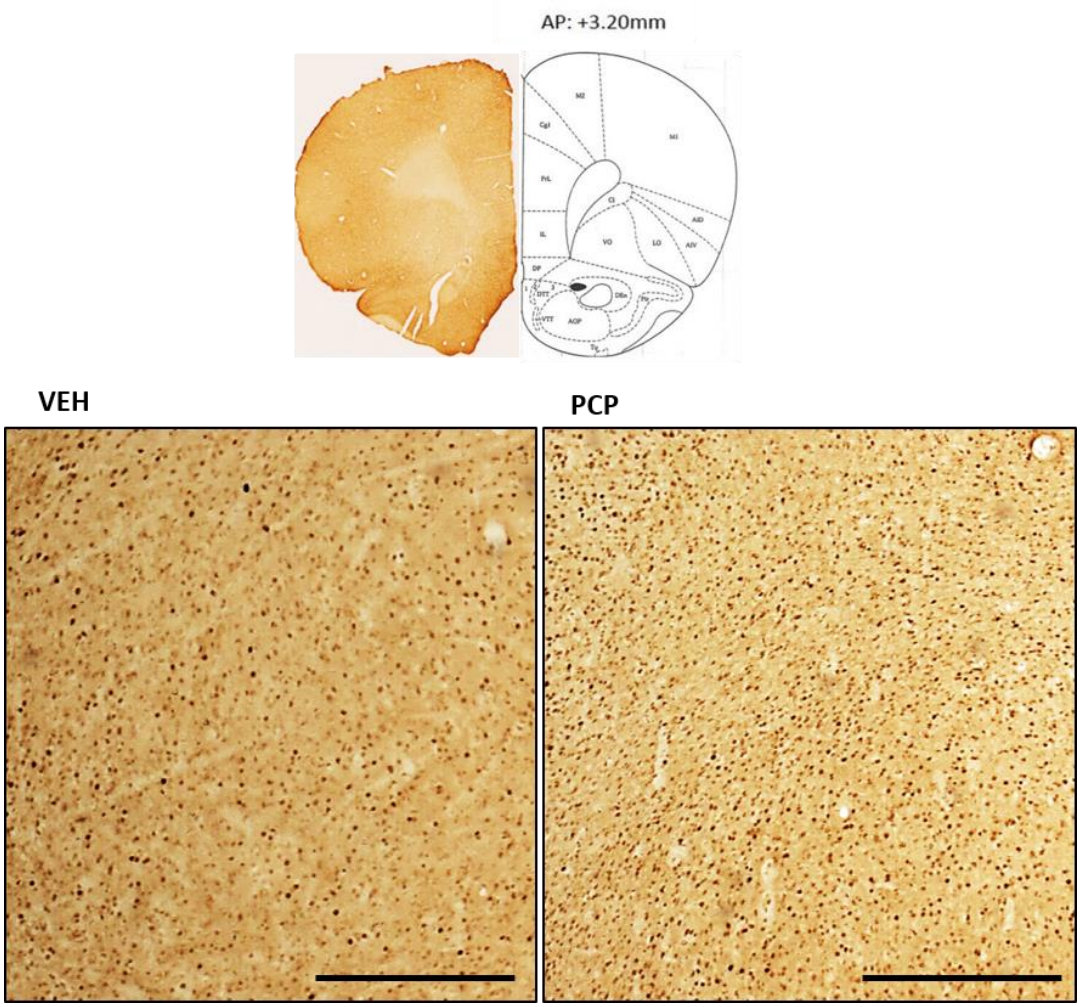
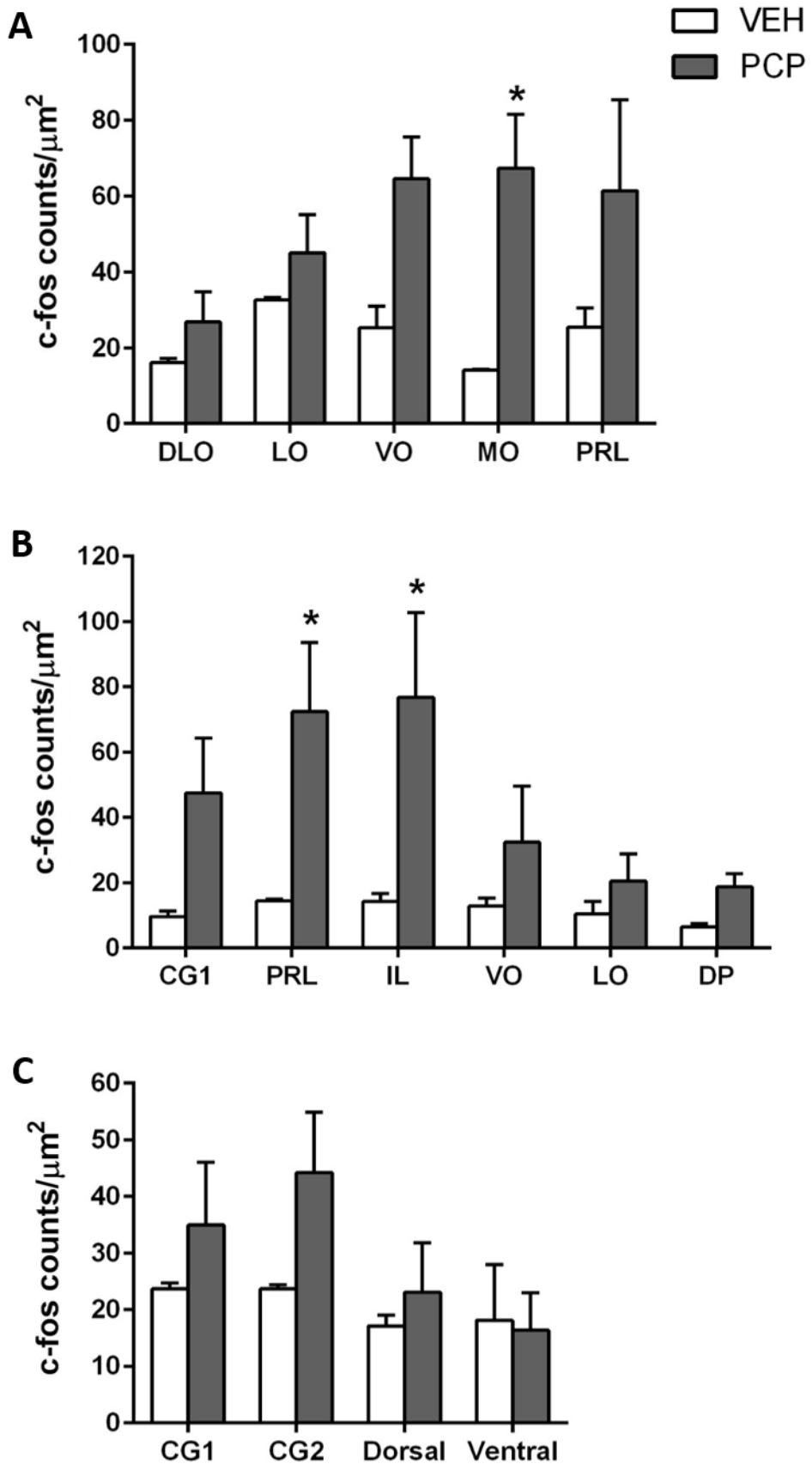


Figure 2.4. Example images taken from the ventral OFC. 10x microscopic image (scale bar 400µm) of c-fos positive nuclei (defined by dark brown reaction product) in the ventral orbital frontal cortex of rats treated with vehicle (VEH; 2ml/kg) or acute phencyclidine (PCP; 10mg/kg) i.p.


A
VEH
PCP
c-fos counts/μm²
100
80
60
40
20
0
*
DLO
LO
VO
MO
PRL
B
c-fos counts/μm²
120
100
80
60
40
20
0
*
*
CG1
PRL
IL
VO
LO
DP
C
c-fos counts/μm²
60
50
40
30
20
10
0
CG1
CG2
Dorsal
Ventral

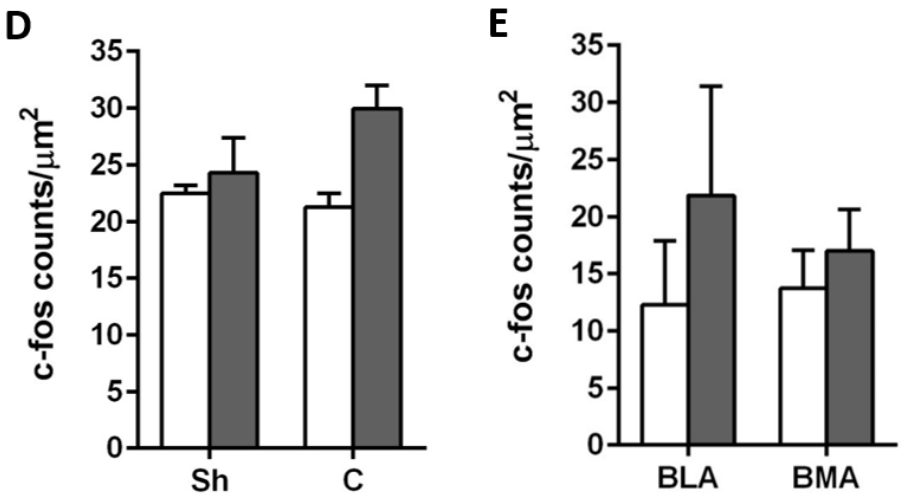


Figure 2.5. Density of c-fos counts (counts/µm^2) in various brain sub-regions: A; Rostral prefrontal cortex (PFC), B; Caudal PFC, C; Nucleus Accumbens, D; Striatum and E; Amygdala. Columns show the mean ± SEM c-fos counts for rats treated with an acute dose of Phencyclidine (PCP; 10mg/kg) or Vehicle (VEH; 0.9% NaCl; 2ml/kg) i.p. before returning undisturbed to the home cage for 2h.

There was a significant effect of PCP treatment in the rostral medial OFC ($p=0.0002$), caudal prelimbic ($p<0.0001$) and infralimbic cortices ($p<0.0001$); unpaired Student's t-tests with Holm-Sidak correction; n=3 per treatment group. *$p<0.05$ compared to VEH. OFC; Orbitofrontal cortex, PFC; Prefrontal cortex; DLO; Dorsolateral OFC, LO; Lateral OFC, VO; ventral OFC, MO; medial OFC, DP; Dentate Putamen, PRL; prelimbic cortex, CG1; limbic cortex 1, IL; infralimbic cortex, C; NAc Core, Sh; NAc Shell, BLA; Basolateral amygdala; BMA; Basomedial amygdala)

2.5: Discussion

2.5.1: $GlyT_1$ inhibitor induced c-fos activation

Acute systemic administration of the $GlyT_1$ inhibitor RO4993850 selectively activated neurons in the rostral and caudal PFC. However, no substantial c-fos elevation was observed in the NAc or amygdala. Additionally, within the striatum, very few c-fos positive nuclei were counted across all three treatment groups. This pattern of c-fos activation induced by the compound suggests it causes the preferential activation of neurons in the OFC and cingulate cortices.

Currently, only one other $GlyT_1$ inhibitor has been investigated by determining changes to c-fos expression levels. The potent and selective $GlyT_1$ inhibitor NFPS selectively induced a significant increase in c-fos immunoreactivity in the PFC and NAc, but less so in the dorsolateral striatum of adult male Sprague-Dawley rats (Kinney et al., 2003). This supports our findings regarding potential downstream dopaminergic pathway activation following administration of $GlyT_1$ inhibitors although our study showed no substantial c-fos elevation in the NAc. This could possibly be attributed to the different chemical structures between the two $GlyT_1$ inhibitors. For example, NFPS, differently to RO4993850, can act as a pseudo-irreversible inhibitor (Aubrey and Vandenberg, 2001) and induce side effects in rodents including dose-related respiratory distress, motor dysfunction and seizures (Harsing et al., 2006, Perry et al., 2008).

2.5.2: Antipsychotic-induced c-fos activation

Although few studies have examined the changes to c-fos expression induced by $GlyT_1$ inhibitors, some antipsychotics have been investigated by such methods. For example, clozapine dose-dependently induced c-fos activation in the PFC whereas raclopride and haloperidol induced no substantial elevation in c-fos levels. All three antipsychotics however, were shown to increase c-fos expression in the NAc albeit with differing patterns of expression i.e. dorsomedial-ventromedial and anterior regions of the NAc respectively. Contrastingly, haloperidol and raclopride induced striatal c-fos immunoreactivity although clozapine showed no substantial changes in expression (Robertson and Fibiger, 1992). Additionally, Wistar rats pre-treated with clozapine 5mg/kg i.p. were shown to have attenuated PCP-induced c-fos mRNA expression in glutamatergic cells in cortical and thalamic brain areas, whilst increased expression was noted in GABAergic interneurons when clozapine was administered in combination with PCP (Kargieman et al., 2007, Santana et al., 2011).

It is worth noting that some of the differences observed in c-fos expression *in vivo* could be due to underlying neurobiological differences which have not been fully documented. For example, different rat strains have been used in the aforementioned studies i.e. Wistar and Lister-hooded, and it is thought that Lister-hooded rats have a different circadian rhythm of activity in the home cage which may contribute to some of the differences in c-fos expression seen compared to other rat strains (Chiasson et al., 1996, Herrera and Robertson,

1996, Clemens et al., 2014). Despite this, haloperidol, risperidone and clozapine appear to induce both overlapping and unique patterns of c-fos immunoreactivity although it is not yet fully understood how this translates into their antipsychotic activity. Original hypotheses suggested that the unique pattern of c-fos immunoreactivity (particularly within the NAc) correlates to specific neuronal activation involving antagonism of dopaminergic D_3 receptors. Pre-treatment of 7-hydroxy-N, N-di-n-propyl-2-aminotetralin (7-OH-DPAT; a D_3 receptor agonist) and quinpirole (a selective D_2 and D_3 receptor agonist) 15min prior to clozapine (20mg/kg) significantly decreased expression in the NAc and in the medial PFC respectively (Guo et al., 1995).

Recent work has highlighted a link between psychosis, dopamine overflow and the associative striatum in schizophrenia (Kegeles et al., 2010a, Simpson et al., 2010, Brunelin et al., 2013). In particular, first-episode-psychosis patients were found to have increased D_2 receptor availability in the associative striatum and increased glutamate levels, the latter of which was normalised following four week antipsychotic treatment with risperidone (de la Fuente-Sandoval et al., 2013). Therefore it is possible that the altering expressions in c-fos immunoreactivity indicates potential activity on negative or cognitive symptoms although this needs to be further validated.

2.5.3: The role of the OFC in schizophrenia

The selective activation of the OFC by the $GlyT_1$ inhibitor RO4993850 is of particular interest as accumulating evidence suggests the regions involvement

in emotion, reward and decision making beyond some forms of cognition, such as reversal learning, due to its critical role in sensory integration and feedback processing in both animals and humans (Rolls, 1996, Bechara et al., 2000). For example, patients with schizophrenia showed a poorer performance in the Iowa gambling task; a psychological task thought to stimulate decision-making involving the OFC (Bechara et al., 1994), trail-making task; a psychological test of visual attention and task-switching (Bowie and Harvey, 2006) and Wisconsin card-sorting task; a psychological test of attention set-shifting (Berg, 1948) compared to healthy controls (Nakamura et al., 2008).

The ability to integrate information to determine potential reward (anticipatory anhedonia) is thought to involve the OFC, although there is as yet no direct evidence of impaired OFC function in determining the computational value of specific stimuli (Cools et al., 2002, Barch and Dowd, 2010, Gradin et al., 2011). Nonetheless, impairments within this region are thought to relate to the motivational construct of negative symptoms observed in schizophrenia, particularly contributing to the avolition and asociality observed (Dowd and Barch, 2010, Kring and Barch, 2014, Strauss et al., 2014, Barnes et al., 2015). This is supported from imaging studies where abnormalities have been observed within ventral striatum-NAc and dorsal striatum-PFC circuitry (Croxson et al., 2009, Dowd and Barch, 2012, Millan et al., 2014). Additionally, the links between these regions and the amygdala, a region known to play an important role in social cognition, emotional processing, mood and reward (Croxson et al., 2009, Dowd and Barch, 2010, Millan et al., 2014) further

highlights the complex neurobiology underlying negative symptoms. Therefore, the specific neuronal activation elicited in brain regions herein suggests potential interactions between the OFC and NAc and could highlight one way in which the $GlyT_1$ inhibitor may improve social behaviours.

2.5.4: Impaired signalling within the OFC

Electrophysiology studies in adult male Sprague-Dawley rats have shown that the antipsychotics haloperidol (0.1mg/kg, i.p.) and clozapine (10mg/kg i.p.), and $mGlu_{2/3}$ receptor agonist LY354740 (10mg/kg, i.p.) are able to reverse MK-801-induced disruption (0.1mg/kg, i.p. 20min later) of NMDA receptor function within the OFC, despite each of the compounds having a different a mechanism of action (Homayoun and Moghaddam, 2008). It is worth noting however that recently tested $mGlu_{2/3}$ receptor agonists failed against positive and negative symptoms in clinical trials (Stauffer et al., 2013, Downing et al., 2014), questioning the use of acute MK-801 as a model to induce cognitive dysfunction, further highlighting the need for improved animal models that better reflect schizophrenia symptomatology, as previously discussed in Chapter 1, section 1.9.

Despite this, previous work showing the ability of $GlyT_1$ inhibitor RO4993850 to improve social and cognitive deficits in a 'two-hit' (neonatal-PCP combined with isolation-rearing) neurodevelopmental rat model of schizophrenia (McIntosh, 2014) combined with the apparent specific targeting of OFC neurons shown herein, suggests the OFC could be a necessary and common

target required for a compound to have therapeutic potential as a treatment for negative symptoms (Homayoun and Moghaddam, 2008). For example, a particular subset of schizophrenia patients with severe negative symptoms (classed as deficit syndrome) show hypofrontality, predominantly in the medial and lateral prefrontal cortices, along with reduced cerebral blood flow in the OFC compared to non-deficit syndrome patients (Kanahara et al., 2013), further supporting the importance of targeting this brain region.

Although $GlyT_1$ are expressed on glutamatergic synapses, there is also the possibility of a secondary and downstream impact on dopaminergic signalling following activation of the OFC, which could provide an alternate way $GlyT_1$ inhibitors are able to mediate their effects although this would need to be further validated. For example, a study by Lodge (2011) showed that the medial PFC and OFC differentially regulate dopaminergic activity in the VTA. Burst-like activation of the medial PFC induced an increase in dopamine neuron firing whereas OFC activation inhibited dopamine activity. Therefore the pattern of cortical activation is also critical for determining the impact on dopaminergic output.

2.5.5: Morphological changes in the OFC

The importance of the OFC in schizophrenia is suggested in MRI studies showing morphological changes to this region in patients suffering from the disorder, as discussed in Chapter 1, section 1.7. For example, grey matter volume deficits in the OFC have been observed in chronically-treated

schizophrenia sufferers, although no correlation was shown between OFC volume and performance in the Iowa gambling task (Nakamura et al., 2008).

Despite this, it has not yet been possible to determine whether antipsychotic treatment or pathophysiology of disease progression is responsible for these morphological changes. Furthermore, MRI studies between first-episode drug-naïve patients and healthy controls have produced some interesting but often inconsistent results. For example, drug-naïve schizophrenia patients have been shown to have differential cortical surface sizes in the OFC, but no change to grey matter volume compared to controls (Crespo-Facorro et al., 2000). In another study however, patients had no overall change to cortical surface size, but cortical thickness was lower in the medial PFC and higher in the OFC compared to healthy individuals. Interestingly, the decrease in cortical thickness in the prefrontal region was also correlated to positive symptom severity (Xiao et al., 2015). Negative symptoms severity by contrast, has been correlated to increased OFC volume (Lacerda et al., 2007). These results were replicated in a study by (Yue et al., 2016) who observed that increases in grey matter volume in the thalamus and PFC were correlated to improvements in positive and negative symptoms respectively.

Whilst the specific neuropathophysiological mechanisms underlying the morphological abnormalities of the OFC remain unclear, it is likely to be caused by disease progression rather than an effect of antipsychotic therapy as these structural changes are present prior to the onset of treatment. Determining whether antipsychotics are able to alter brain morphology could however be

of interest in determining their therapeutic potential. For example, schizophrenia patients were shown to have increased grey matter volume in the PFC and thalamus following 8 weeks treatment with atypical antipsychotics clozapine, quetiapine, aripiprazole and olanzapine at doses equivalent to 285mg/day chlorpromazine (Yue et al., 2016). As the $GlyT_1$ inhibitor RO4993850 also selectively targets the OFC, it would also be of interest to determine whether treatment with the compound is able to induce cortical changes in the same way as some antipsychotics.

2.5.6: PCP-induced c-fos activation

Within the study, 10mkg/kg PCP selectively activated key brain regions, particularly the PFC and OFC. The general increase in c-fos immunoreactivity observed in the rostral and caudal PFC and OFC, core NAc, amygdala and striatum is in agreement with existing literature (Santana et al., 2011, Castane et al., 2015). For example, both 10mg/kg and 15mg/kg PCP was shown to elevate c-fos mRNA expression and c-fos positive neurons in several cortical, sub-cortical and thalamic brain areas (Kargieman et al., 2007, Turgeon et al., 2007, Santana et al., 2011).

Although PCP in the current study did not cause significant elevations in c-fos expression in several areas, this is most likely due to the small samples sizes used in the study which was not designed to evaluate the effect of PCP but merely to validate the technique. Despite this, the differences in c-fos immunoreactivity are sufficient in providing validity for our

immunohistochemistry protocol as the exact same was used for both parts of the study. Additionally, it supports the conclusion that the $GlyT_1$ inhibitor RO4993850 contributed to the majority of c-fos activation observed, and less so from the handling and injection process, as one flaw with using c-fos as a marker of neuronal activation is that peak c-fos production can occur from 60-120min, and can appear within 30min following an external stimulus (Hoffman et al., 1993, Zangenehpour and Chaudhuri, 2002), and so it cannot often be linked to a single event or time point. One common method often used therefore is to compare c-fos protein production in specific brain regions with the drug of interest in the presence of specific receptor inhibitors to determine which downstream signalling pathways might also be involved in eliciting the mechanism of action. For example clozapine is thought to be linked to the epidermal growth factor receptor (EGFR) and ERK signalling pathway (Pozzi et al., 2003, Pereira et al., 2012, Pereira et al., 2013).

2.5.7: Summary

Observing changes in basal c-fos immunoreactivity following drug administration has been used routinely to identify specific brain region activation and to map functional networks within the CNS. In particular, induction of c-fos immunoreactivity was examined to predict which antipsychotics were likely to produce extrapyramidal side-effects (Robertson and Fibiger, 1992, Robertson et al., 1994). Results from this study have suggested that acute systemic administration of RO4993850 primarily targets

the PFC (of the regions examined), potentially leading to NMDA receptor activation and may, as a secondary consequence, lead to dopaminergic pathway activation, particularly within the OFC sub-region (Gibert-Rahola and Villena-Rodriguez, 2014).

It would be of interest to determine whether the compound is also able to reverse or alter some of the observed neuropathological changes observed in animal models of schizophrenia following chronic administration, potentially providing a biomarker to predict drug efficacy in the clinic. The use of c-fos as a neuronal marker therefore acts as a positive starting point to determine whether key brain areas are being targeted by the compound, and to further elucidate the exact mechanisms that contribute to the neuropsychopharmacological effects of $GlyT_1$ inhibitors. As a consequence, the next chapter of this thesis investigates how specific brain region activation potentially translates to changes in social behaviour, neuropathology and dopamine overflow following treatment with the same $GlyT_1$ inhibitor compound.

Chapter 3: Effects of chronic $GlyT_1$ inhibitor treatment on social behaviours in a neurodevelopmental rat model

3.1: Introduction

3.1.1: Neonatal-PCP isolation-rearing

Two-hit animal models are becoming increasingly used as a way to overcome the incomplete phenotypic and neurochemical syndrome produced by current rodent models which use a single adverse early-life manipulation. The rationale behind the neonatal-PCP isolation-rearing model is that individually, both neonatal-PCP administration and isolation-rearing can induce behavioural, neurochemical and molecular changes in adult rats (Anastasio and Johnson, 2008a, Fone and Porkess, 2008a, Boctor and Ferguson, 2010, Jones et al., 2011a).

Therefore the combination of these two manipulations; repeated 10mg/kg s.c. PCP administration on PND 7, 9 and 11 in neonatal rat pups, combined with social isolation post-weaning (PND 21-25) could produce more robust behavioural changes in adult male Lister-hooded rats. Furthermore, these changes may better resemble the core symptoms of schizophrenia, such as locomotor hyperactivity (shown to have predictive validity for positive symptoms), deficits in social interaction (thought to correlate to negative symptoms), impaired PPI (a measure of sensorimotor gating), together with cognitive deficits including impaired contextual-fear learning (to assess associative learning and memory) and impaired performance in a NOD

paradigm (Gaskin et al., 2014). The isolation procedure of repeated neonatal-PCP administration specifically on PND 7, 9 and 11, and the minimum length of time required raising rats in isolation (at least five weeks) prior to behavioural testing has been determined in previous studies (Jones et al., 2011c). Lister-hooded rats show a more pronounced effect of isolation-induced hyper-locomotor activity compared to other rat strains such as Sprague-Dawley (Geyer et al., 1993), and a weaning age of PND 21-25 corresponds to a critical stage of cortical and limbic brain development. Therefore, external influences at this age, such as social isolation can disrupt normal neuronal development (Einon and Morgan, 1977).

3.1.2: $GlyT_1$ inhibitor RO4993850

$GlyT_1$ inhibitors, as discussed in previous chapters, remain potential therapeutics and compounds of interest for the treatment of negative symptoms in schizophrenia. Chapter 2 illustrated a functional map of neuronal activation following administration of $GlyT_1$ inhibitor RO4993850, with a dose-dependent induction of c-fos within the PFC similar to that achieved by clozapine following acute systemic administration (10-30mg/kg s.c.) in adult male Wistar rats perfused 2h post-injection (Robertson and Fibiger, 1992). However, it is also important to determine whether activation of specific brain areas causally translates to a behavioural effect in both animal models and patients with schizophrenia.

Previous studies have used this same $GlyT_1$ inhibitor in the two-hit neonatal-PCP isolation-rearing neurodevelopmental rat model for schizophrenia, and acute administration of 1 or 3mg/kg RO4993850 attenuated novel-arena induced hyperactivity, ameliorated social deficits in a social interaction task, and reversed cognitive deficits in a NOD task (McIntosh, 2014). However, one weakness with the majority of preclinical studies is that acute systemic administration of compounds is often used to determine therapeutic efficacy, whilst in the clinic chronic treatment is required for a number of weeks before any therapeutic benefit is seen. Therefore, this study aimed to determine the effects of chronic $GlyT_1$ inhibitor administration on adult rats.

3.1.3: Aims

This study was split into two sections. The aim of study one was to determine the effects of chronic $GlyT_1$ inhibitor RO4993850 administration on social behaviours, including deficits in social interaction and changes to USV emissions (which act as a communication index), using neonatal-PCP isolation-reared rats (part 1). In addition, changes to c-fos and parvalbumin immunoreactivity were determined in key brain areas; c-fos to identify any regional-specific activation, and calcium binding protein parvalbumin to determine any changes in expression of a sub-group of GABAergic interneurons known to be affected in both animal models and schizophrenia, either by the 'dual-hit', or following chronic administration with $GlyT_1$ inhibitor RO4993850 (part 2).

The second study tested the hypothesis that $GlyT_1$ inhibitors may influence dopaminergic activity to mediate their effects, as determined by the functional map developed in Chapter 2. Therefore, dopamine overflow was measured by microdialysis in the PFC and striatum in freely-moving neonatal-PCP isolation-reared rats following an acute systemic dose of the $GlyT_1$ inhibitor RO4993850.

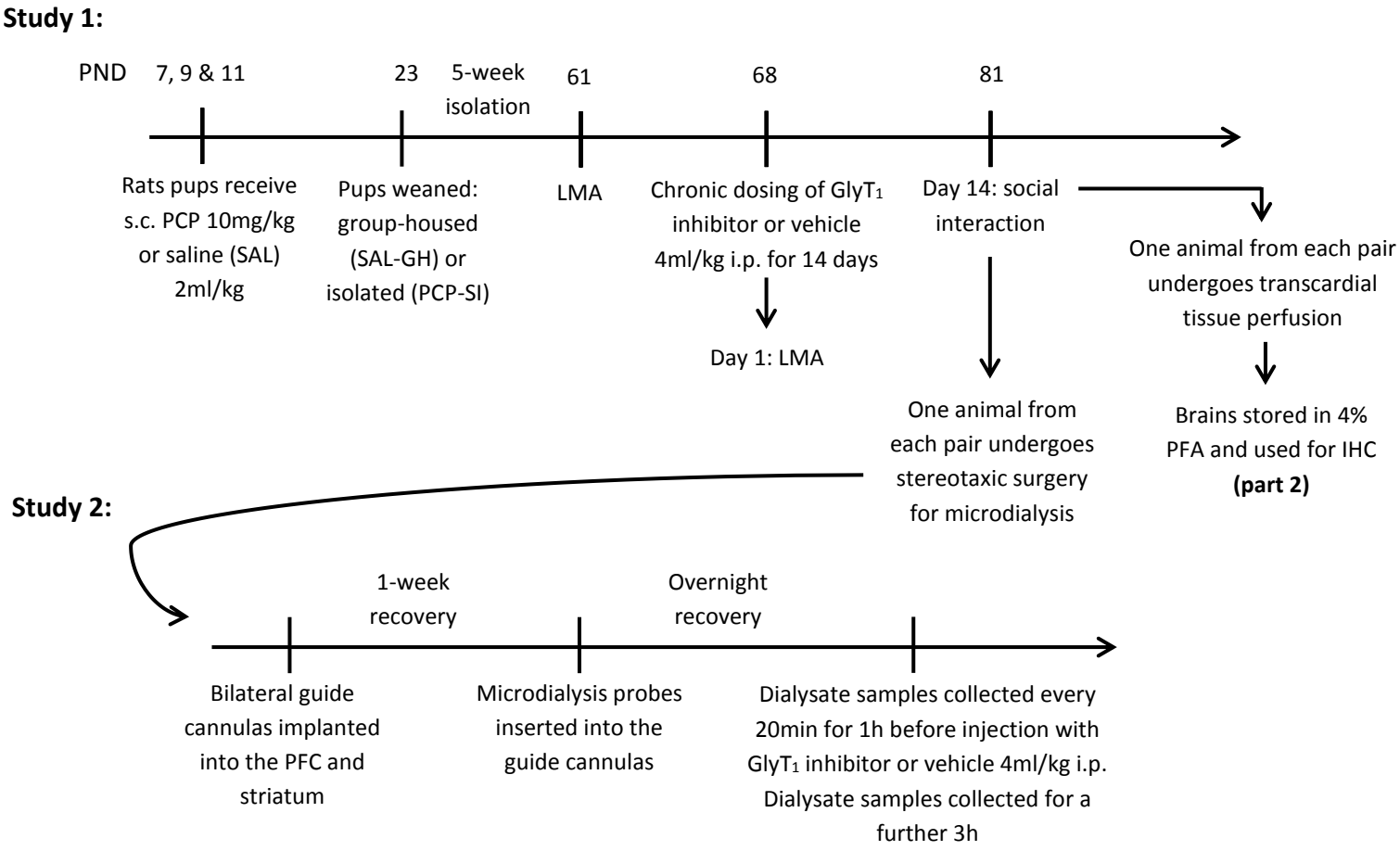
3.2: Methods

3.2.1: Neonatal-PCP Isolation-Rearing

Sixty-eight male Lister-hooded rat pups were housed with their dams and on PND 7, 9 and 11 received subcutaneous (s.c.) injections of either sterile saline (SAL; 0.9% w/v NaCl; 2ml/kg) or phencyclidine hydrochloride (PCP; 10mg/kg; Sigma Aldrich UK) dissolved in 0.154M saline. Injections took place between 10:00 and 12:00h on each day. Following injection, rat pups were returned to the home cage and observed for 2h to monitor adverse effects induced from PCP administration. Rat pups continued to be housed with their dams until weaning (PND 23) at which point they were either group-housed (GH) or put into individual cages (social isolation, SI) for 5 weeks, dependent on whether they received neonatal saline or PCP respectively. Rats were weaned PND 23 based on previous studies indicating a robust onset of 'behavioural syndrome' when Lister-hooded rats were weaned on this day (Schubert et al., 2009).

Rats were housed under a controlled temperature (21 ± 2^{o}C) and humidity (45 ± 15%), with a 12h light/dark cycle (lights on 06:00-18:00h). All experiments were performed in the light phase, with food (Beekay standard diet) and water available *ad libitum*. Bedding and cages were changed at the same time once a week and rats received minimal handling throughout the study period to avoid disruption of the 'isolation syndrome'. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act, 1986 and the ARRIVE guidelines (Kilkenny et al., 2010) with approval of AWERB.

3.2.2: Experimental Design



3.3: Study 1:

Part 1: Effects of neonatal-PCP isolation-rearing on behaviour

3.3.1: Locomotor Activity (LMA)

Drug-free locomotor activity (LMA) was monitored following the 5-week isolation (PND 61) to confirm development of the 'isolation syndrome' as novel-arena induced hyperactivity is one of the most consistent findings reported in the isolation-rearing model. The following week (PND 68) LMA was reassessed following treatment with $GlyT_1$ inhibitor RO4993850.

Rats were placed in Plexiglas chambers (39cm × 23.5cm × 24.5cm) with an exchangeable floor and removable metal grid roof. The chambers were located within frames containing two layers of infrared photocell beams: lower layer (8 × 4, length × width) and upper layer 8 beams (width only), and consecutive breaks of two adjacent beams produced a count of horizontal ambulatory LMA whilst a single upper tier beam break was recorded as vertical rears (Photobeam Activity System, San Diego Instruments, USA; (Watson et al., 2012b). Cumulative beam breaks were recorded in 5min epochs for 60min, after which animals were returned to their home cage. Activity and rearing were calculated as mean ± SEM for every consecutive 5min epoch during the experiment time course and analysed using two-way repeated measures ANOVA, followed with Sidak post-hoc analysis where appropriate. Statistical significance was set to $p<0.05$.

3.3.2: Chronic Injections

The week following drug-free LMA, animals were divided into four treatment groups to receive chronic administration of either $GlyT_1$ inhibitor RO4993850 (Gelatine 7.5%/NaCl 0.62%/active-RO4993850; 3mg/kg;) or associated vehicle (Gelatine 7.5%/NaCl 0.62%; 4ml/kg) i.p. for 14 consecutive days (n=16 SAL-GH Vehicle; n=16 SAL-GH RO4993850 3mg/kg; n=16 PCP-SI Vehicle; n=16 PCP-SI RO4993850 3mg/kg). The 3mg/kg dose was chosen based on its ability to reverse social deficits in neonatal-PCP isolates (McIntosh, 2014). Drugs were formulated by F. Hoffmann-La Roche Ltd. On day one of injections (PND 68), LMA was recorded 30min post-injection to determine acute effects of the compound, using the same method as defined above. On the final day of injections (PND 81), rats were matched for weight, PCP/housing and treatment for a social interaction paradigm 30min post-injection.

3.3.3: Social Interaction

Social interaction was used as an index to measure the negative symptoms of schizophrenia. Rats from two different litters were matched for age, weight (<30g difference to prevent dominance) and developmental treatment (i.e. SAL-GH or PCP-SI). As behaviour between each rat pair is influenced by the behaviour of each individual rat, both the animals had the same treatment within the trial to establish the effect of the $GlyT_1$ inhibitor on social interaction. It would be possible to also compare changes to social behaviour between rats with different treatments i.e. Vehicle vs RO4993850 however an increased

number of animals would be needed within the study and it would likely not prove to be more informative than pairing rats for each treatment alone. Each pair of rats were sprayed on the back with either pink or blue hair dye (Superdrug instant colour hairspray, Superdrug Stores PLC, UK) to permit discrimination on video-tracking software following injection before placing them in an unfamiliar circular open field (diameter 75cm) under controlled conditions (21°C ± 2°C), illuminated with a low light intensity of 40LUX for 10min. A colour camera was located directly above the open field and connected to a PC for digital recordings of the experiment using Ethovision software (Noldus). Behaviours, including body sniffing, ano-genital sniffing, following, lying side-by-side, crawling over-and-under, pinning and biting and boxing, were manually scored post-trial from video recordings. In order to ensure consistency, videos were scored twice and compared to ensure similar scores were obtained. After the trial, rats were removed from the open field and returned to their home cage.

3.3.4: Ultrasonic Vocalisations (USVs)

During the social interaction test, USVs were also recorded using an electret microphone (Emkay, Avisoft Bioacoustics, Germany) connected to an ultrasound detection unit (Ultrasound Gate, customised model 112, Avisoft Bioacoustics, Berlin). The resulting signals were digitalised and saved as .wav files. The temporal and frequency characteristics of calls were extracted using associated Avisoft analysis software (SAS-Lab Pro, v. 4.38, Avisoft Bioacoustic,

Berlin). Spectrographs were produced using the following parameters; 488 Hz frequency resolution, 512 FFT length, 75% frame size and 93.75% time window overlap. A lower cut-off frequency of 22 kHz and a higher cut-off frequency of 85 kHz was used to reduce the level of background noise.

Various parameters were detected automatically including peak frequency, amplitude and min-max bandwidth. As temporal parameters, latency to the first call and mean call duration were determined. The total number of USVs emitted were measured and all USVs were grouped into pro-social 50 kHz and aversive 22 kHz subtypes based on spectrograph and bandwidth parameters. Additionally, the 50 kHz calls were grouped into three main categories of flat, step and trill based on their specific pattern analysis (Brenes et al., 2016), as outlined below:

- **Flat calls:** peak frequency changes were equal to or lower than 5 kHz although the difference between start and end frequencies could be greater than 5 kHz if the USV had an upward or downward direction.

- **Step calls**: a flat USV that had at least one short element at least 5 kHz higher or lower than the fundamental call

- **Trill calls**: at least one frequency modulation that had a longer element than those present in step calls and/or a common zig-zag shape which often appeared as a series of inverted-u shapes.

Calls which did not fit into any of the three categories were excluded from the analysis comparing different 50 kHz call-subtypes. Example spectrographs highlighting the different call categories are shown in Figure 3.8. The total

number of calls for each category, as well as call parameters including changes in call bandwidth and duration were log_{10} transformed prior to two-way ANOVA analysis for each call subtype.

3.4: Study 1:

Part 2: Cortical effects in neonatal-PCP isolation-reared rats

3.4.1: C-fos immunoreactivity following social interaction

For each pair of animals that underwent a social interaction trial, one was transcardially perfused with 4% PFA using the same protocol as outlined in chapter 2, section 2.3.1.3-5, to determine changes to c-fos expression following a behavioural task between different treatment groups (n=7-8 per treatment group; 31 rats in total). Differences in cell counts were analysed by two-way ANOVA for each brain sub-region of interest.

3.4.2: Parvalbumin immunoreactivity following social interaction

Free-floating hippocampal and prefrontal sections (prepared the same way as above) were washed in PBS four times before incubation in block, 0.02% v/v goat serum in PBST (0.01M PBS, 1% BSA, 0.3% Triton-X) for 1h at room temperature, followed by incubation in PBST containing parvalbumin rabbit polyclonal antibody (1:500 dilution; ab11427 (Abcam) for 24h at 4°C. After 24h, sections were washed three times in a second PBST solution (0.01M PBS, 0.3% BSA and 0.1% Triton-X) before incubation in PBST containing the secondary antibody; goat anti-rabbit (Alexa Fluor 568; 1:500 dilution; ab175471 (Abcam)) for 1h at room temperature. The sections were then washed twice in PBST and

twice in PBS before mounting on gelatin-coated microscope slides and left to dry in darkness. DAPI was applied at a 1:1000 dilution for 30s in darkness before rinsing with distilled water. Slides were coverslipped with DABCO mounting medium. Once coverslipped, slides were kept in darkness and stored at 4°C until analysis completed. NB immunofluorescence was used to stain for parvalbumin due to readily available antibody within the laboratory and for the purpose of easier visualisation for dual-staining with other antibodies of interest. These included GAD_{67}, reduced in prefrontal GABAergic interneurons in the PFC in schizophrenia (Lazarus et al., 2015) and $Kv_{3.1}$, a potassium voltage-gated channel important in generating high frequency firing of fast-spiking GABAergic interneurons (Boddum et al., 2017). Unfortunately dual-stains were not able to be visualised with currently available antibodies on our tissue.

Parvalbumin positive interneurons were manually counted using appropriate filters on a fluorescent microscope (Nikon EFD-3 fluorescent microscope; Optiphot 2, Spot Insight QE camera). Differences in parvalbumin immunoreactivity between treatment groups (n=7-8 per treatment group; 31 rats in total) were determined by two-way ANOVA for each brain sub-region of interest. Key areas for investigation were hippocampal sections divided into the cornu ammonis (CA1 and CA2/3) and DG and the rostral PFC, as outlined by Paxinos and Watson's stereotaxic atlas (1986). At least 5-6 coronal sections from each treatment group were used for analysis and compared under the microscope to ensure similar Bregma locations for each rat. Sub-region boundaries in the hippocampus were also cross-checked using an online rat

atlas image viewer available at this website http://cmbn-navigator.uio.no/rat_hippocampus_atlas/.

3.5: Study 2:

Effects on dopamine overflow following an acute dose of RO4993850

3.5.1: Stereotaxic surgery

The remaining animal from each rat pair during social interaction was kept for stereotaxic surgery and bilateral implantation of guide cannulas into the PFC and striatum (n=8 per treatment group; 32 rats in total). Rats were anaesthetised with isoflurane; 5% isoflurane in 0.3L/min O_2 and 0.6L/min N_2O, and mounted in a stereotaxic frame. CMA 12 guide cannulas (Linton Instrumentation, Diss, UK) were implanted relative to Bregma into the medial PFC (AP: +3.2, L: -0.7, V: -2.3) and striatum (AP: +0.48, L: +2.5, V: -4.0) according to the rat brain atlas of Paxinos and Watson (1986). The cannulas, a copper-wire tether and three skull-embedded stainless steel screws were cemented with dental acrylic. Carprofen (rimadyl) 1mg/kg was administered s.c. pre-surgery, with lignocaine administered topically around the sutures post-surgery. No further analgesia was administered for the duration of the study.

3.5.2: Collection of Dialysate Samples

Following one-week recovery, rats were briefly anaesthetised (for 5-10min with isoflurane; 5% isoflurane in 0.3L/min O_2 and 0.6L/min N_2O, for insertion of CMA 12 microdialysis probes (0.5mm x 4.0mm membrane length with

molecular cut-off 20,000 Da; Linton Instrumentation) into the guide cannulas. Once implanted, the microdialysis probes were perfused continuously with aCSF (made up of 125mM NaCl, 1.25mM KCl, 0.5mM $MgCl_2$, 13.5mM $NaHCO_3$, 0.2mM NaH_2PO_4, 0.90mM Na_2HPO_4, 0.30mM Na_2SO_4 and 0.5mM $CaCl_2$) at 1.0µl/min using dual-syringe pumps (Harvard Apparatus). Rats were placed into an open-field arena (which they could freely explore) with the microdialysis FEP tubing attached to a dual channel stainless steel swivel (Linton Instrumentation) and tether connected to a swivel mount (Linton Instrumentation) on the edge of the arena.

The following day, three baseline samples were collected at 20min intervals before injection with either $GlyT_1$ inhibitor RO4993850 3mg/kg i.p. or associated vehicle dependent on which chronic treatment was received prior to social interaction. Samples were collected in 20min intervals for a further 3h into vials containing 5µl 0.1M perchloric acid and 0.03% sodium metabisulphite (Sigma Aldrich, UK) before immediate placement on dry ice until stored into a -80^{o}C freezer. At the end of the study, rats were euthanised with 1ml sodium pentobarbital (Euthatal) i.p. and brains dissected and stored in 4% PFA at 4^{o}C for histological verification of correct probe placement.

3.5.3: Confirmation of Probe Placement

After at least one-week in 4% PFA, brains were washed in PBS and cut into sections using an acrylic rat brain 1mm Sagittal matrix (Braintree Scientific) and photographic images taken; Figure 3.15.

3.5.4: Analysis of Monoamines

Microdialysis samples were analysed using High Performance Liquid Chromatography (HPLC) with electrochemical detection (ECD). Frozen dialysate samples (stored at -80°C) were defrosted at room temperature before injection of 15µl into a Targa C18 3µM column (100 x 1.0mm; Higgins Analytical) using a Perkin Elmer Series 200 autosampler (Antec Leyden, The Netherlands). To prevent pressure build-up, a c18 opti-guard 1mm guard column (Sigma Aldrich, UK) was attached. Monoamines, including dopamine and 5-hydroxytryptamine (5-HT), were measured along with their major metabolites; 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA). However, the 5-HT and 5-HIAA peaks often co-eluted preventing quantification and so were subsequently excluded from the overall analysis.

The mobile phase, made up of 20mM potassium dihydrogen phosphate, 20mM sodium acetate, 0.1mM ethylenediaminetetraacetic acid (EDTA), 0.15mM octanesulfonic acid and 10% methanol (pH 3.9) was circulated through the system at a flow rate of 0.4ml/min via a Dionex P680 pump. Analytes were detected using a SenCell with Ag/AgCl reference electrode set at 0.75V and connected to a DECADE II SDC Detector 1 (Antec Leyton). Quantification was determined using Clarity software (Data Apex) against standards. Changes in neurotransmitter levels from baseline were determined by repeated measures ANOVA followed by Tukey post-hoc analysis, with statistical significance set at $p<0.05$.

3.6: Results:

Study 1:

Part 1: Effects of neonatal-PCP isolation-rearing on behaviour

3.6.1: Novel-arena induced drug-free LMA

3.6.1.1: Total ambulatory count

In order to assess any impact of rearing on neophobia, rat behaviour was examined in a novel computerised arena. Drug-free novel-arena induced LMA was measured for the first time on PND 61, 5 weeks post-weaning. Two-way repeated measures ANOVA of total LMA counts revealed a significant main effect of time ($F_{(11, 726)}$=151.8, p<0.001), indicating that rats habituated to the arena. There was also a significant main effect of PCP/housing ($F_{(11,66)}$=9.397, p=0.0031) showing PCP-SI rats were more hyperactive than SAL-GH rats which was significant at the 10min time-point (p<0.001; Sidak post-hoc). There was also a significant PCP/housing x time interaction ($F_{(11, 726)}$=2.260, p=0.0105; Figure 3.1; A) indicating activity levels between both cohorts to similize over time, reinforced following a comparison of the total ambulatory activity across the experiment where PCP-SI rats had significantly greater activity (Mean=2165 ± 60) compared to SAL-GH controls (Mean=1879 ± 72; $t_{(66)}$=3.065, p=0.0031; unpaired Student's *t*-test; Figure 3.1; B).

3.6.1.2: Total rearing activity

Two-way repeated measures ANOVA of the time-course of rearing activity between PCP-SI and SAL-GH rats revealed a significant main effect of PCP/housing ($F_{(1, 67)}$=8.945, p=0.0039), time ($F_{(11, 737)}$=82.28, p<0.001), and a PCP/housing x time interaction ($F_{(11, 737)}$=1.893, p=0.0371) indicating that over the 1h experiment, rearing decreased as animals habituated to the arena. Overall, PCP-SI rats exhibited more rears than SAL-GH rats, particularly at the 35 (p<0.01), 45 (p<0.05), 50 (p<0.01) and 55 (p<0.05) min time-points; Sidak post-hoc (Figure 3.1; C). This profile was confirmed by analysis of total rears such that PCP-SI rats reared significantly more (Mean=228.2 ± 19.4) than controls (Mean=162.4 ± 10.0); p=0.0031; unpaired Student's *t*-test; Figure 3.1; D). These results suggest that neonatal-PCP isolation rearing had induced the expected behavioural 'isolation' syndrome.

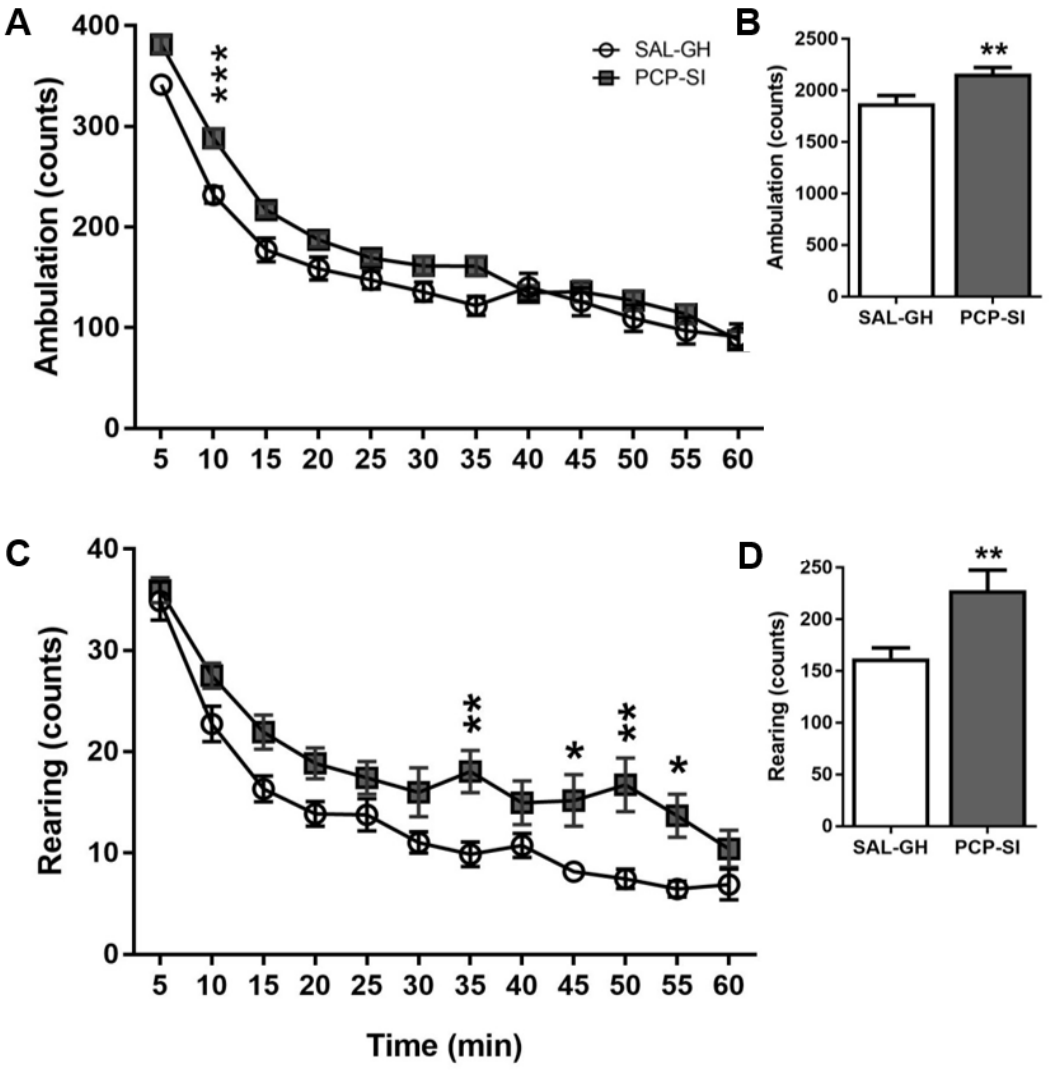


Figure 3.1: Novel-arena drug-free locomotor activity (LMA). Comparative differences (mean ± S.E.M.) in A; time-course of ambulatory count (cumulative counts/5min epoch), B; total ambulatory count, C; time-course of rearing (cumulative counts/5min epoch), and D; total number of rears over 60min drug-free LMA on PND 61 in adult male Lister-hooded rats administered with either saline (SAL; 0.9% w/v NaCl; 2ml/kg) or phencyclidine (PCP; 10mg/kg) s.c. PND 7, 9 and 11, followed by subsequent isolation-(PCP-SI) or group-housing (SAL-GH) PND 23. A; Two-way repeated measures ANOVA revealed a significant main effect of time ($F_{(11,\ 726)}$=151.8, p<0.001), PCP/housing ($F_{(11,66)}$=9.397, p=0.0031) and a time x PCP/housing interaction ($F_{(11,\ 726)}$=2.260, p=0.0105). B; **p=0.01, unpaired Student's *t*-test. C; Two-way repeated measures ANOVA revealed a significant main effect of time ($F_{(11,\ 737)}$=82.28, p<0.001), PCP/housing ($F_{(1,\ 67)}$=8.945, p=0.0039) and a time x PCP/housing interaction ($F_{(11,\ 737)}$=1.893, p=0.0371). D; **p=0.01, unpaired Student's t-test. *p<0.05, **p<0.01, ***p<0.001; Sidak post-hoc compared to SAL-GH; n=34.

3.6.2: LMA following acute RO4993850

3.6.2.1: Total ambulatory count

LMA was reassessed the following week (PND 68), this time following acute systemic treatment with the $GlyT_1$ inhibitor RO4993850 (3mg/kg or vehicle 4ml/kg i.p. 30min prior to rat placement in the arena). Two-way ANOVA of total cumulative activity revealed no significant effect of PCP/housing ($F_{(1,64)}$=0.1444, p=0.7052) or treatment ($F_{(1,64)}$=0.2212, p=0.6397). There was also no significant PCP/housing x treatment interaction ($F_{(1,64)}$=0.05937, p=0.808; Figure 3.2; A), indicating similar levels of LMA between SAL-GH and PCP-SI rats in both treatment groups.

3.6.2.2: Total rearing activity

Comparison of rearing activity however, showed that PCP-SI rats exhibited significantly more rears over the 1h experiment compared to SAL-GH controls (Figure 3.2; B). Two-way ANOVA showed a significant main effect of PCP/housing ($F_{(1,64)}$=13.82, p=0.0004) but no significant main effect of treatment with the $GlyT_1$ inhibitor RO4993850 ($F_{(1,64)}$=0.5067, p=0.4792). There was also no PCP/housing x treatment interaction ($F_{(1,64)}$=1.392, p=0.2425). Therefore, acclimatisation to the novel arena was unaltered by $GlyT_1$ inhibition although post-hoc analysis showed RO3994850 SAL-GH rats to exhibit significantly ($p<0.01$) fewer rears than RO3994850 PCP-SI rats, suggesting it might have differential effects in the two-hit neurodevelopmental groups.

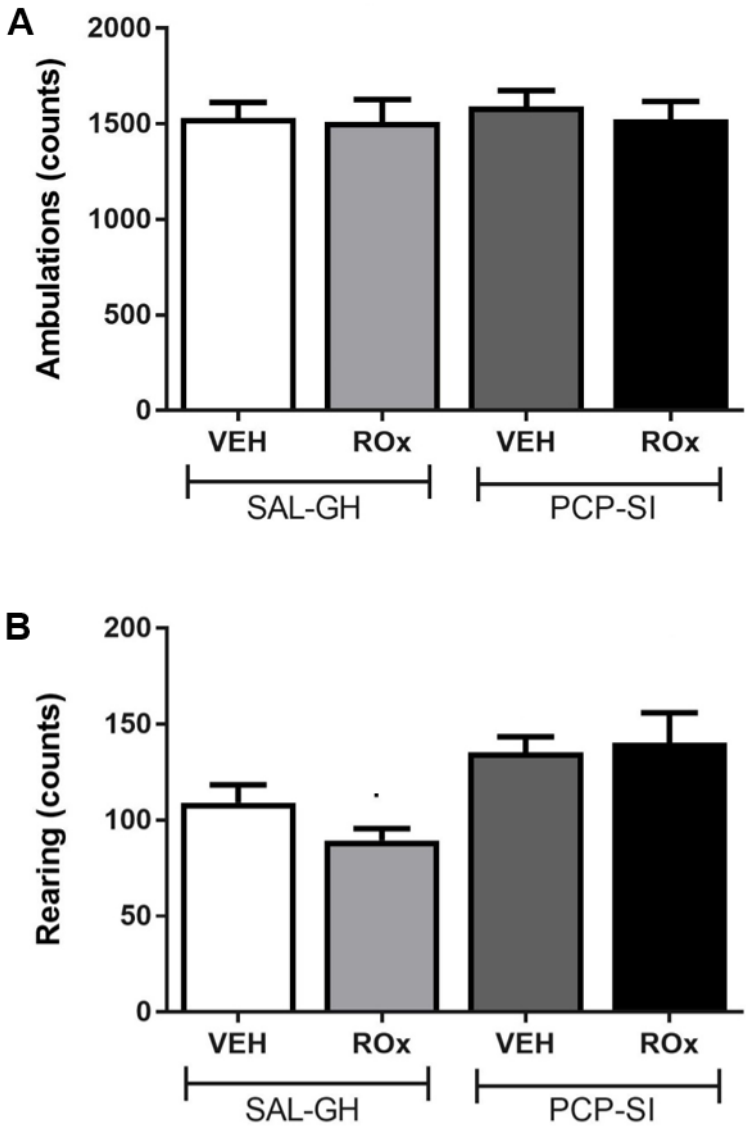


Figure 3.2: Locomotor activity (LMA) following treatment with a GlyT$_1$ inhibitor. Comparative differences (mean ± S.E.M.) in A; total ambulatory count and B; total number of rears over 60min on PND 68 in adult male Lister-hooded rats administered with either saline (SAL; 0.9% NaCl; 2ml/kg) or phencyclidine (PCP; 10mg/kg) s.c. PND 7, 9 and 11, followed by subsequent isolation (PCP-SI) or group-housing (SAL-GH) PND 23, and acute vehicle (VEH; 4ml/kg) or RO4993850 (ROx; 3mg/kg) i.p. 30min prior to placement in an activity box. A; Two-way ANOVA of activity revealed no significant effect of PCP/housing or treatment ($p>0.05$). B; Two-way ANOVA of total rears revealed a significant main effect of PCP/housing ($F_{(1, 64)}=13.82$, $p=0.0004$), but no effect of treatment or PCP/housing x treatment interaction ($p>0.05$); n=16 SAL-GH: VEH, n=18 SAL-GH: ROx; n=18 PCP-SI: VEH; n=16 PCP-SI: ROx.

3.6.3: Social interaction following chronic treatment with $GlyT_1$ inhibitor RO4993850

3.6.3.1: Total social interaction

In order to assess any impact on social behaviours, effects of the $GlyT_1$ inhibitor were examined on social interaction in a novel arena. Neonatal-PCP combined with social isolation from weaning decreased the total time spent in pro-social interactive behaviours. Pro-social behaviours included ano-genital sniffing, body sniffing, crawling over-and-under, following and lying side-by-side. The social interaction deficit in PCP-SI rats was attenuated following chronic treatment with $GlyT_1$ inhibitor RO4993850 for 14 days. Two-way ANOVA of total pro-social behaviour revealed no significant effect of PCP/housing ($F_{(1,29)}$=2.144, p=0.1539) or treatment ($F_{(1,29)}$=2.652, p=0.1142) but did show a PCP/housing x treatment interaction ($F_{(1,29)}$=13.46, p=0.001). Further analysis with Tukey post-hoc tests showed significant differences between SAL-GH and PCP-SI vehicle-treated rats ($p<0.01$) indicating neonatal-PCP and isolation-rearing to attenuate social interaction. The reduced interaction in PCP-SI vehicle rats was restored by RO399485O ($p<0.01$). Thus, chronic treatment with the $GlyT_1$ inhibitor RO4993850 increased the overall time engaged in pro-social behaviours in PCP-SI rats but induced little change in control (SAL-GH) rats (Figure 3.3).

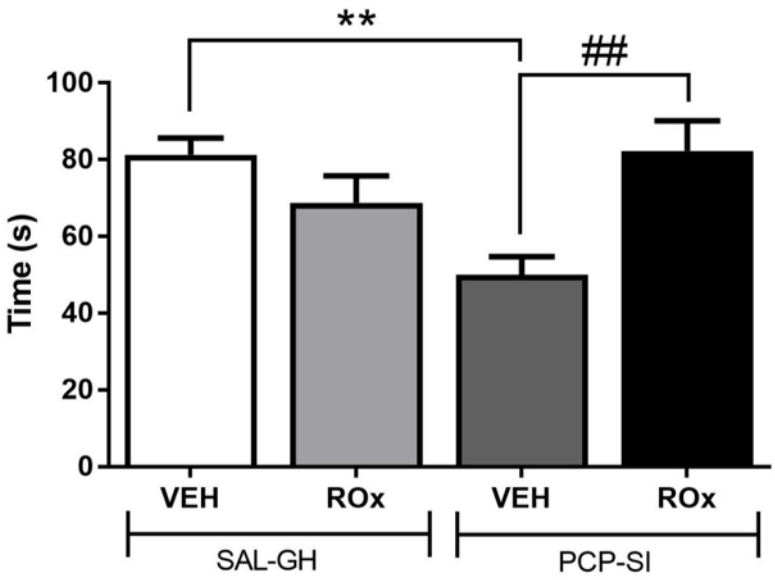


Figure 3.3: Total time spent in social interaction. Comparative differences (mean ± S.E.M.) in the total time spent in pro-social behaviours (excl. boxing/biting behaviour) on PND 81 in adult male Lister-hooded rats. Rats were reared with either neonatal saline (SAL; 2ml/kg) and group-housed (SAL-GH) or phencyclidine (PCP; 10mg/kg) s.c. and isolated (PCP-SI) PND 23 for 5 weeks before chronic treatment with vehicle (VEH; 4ml/kg) or GlyT$_1$ inhibitor RO4993850 (ROx; 3mg/kg) i.p. for 14 days PND 68-81. Drug treatment was administered 30min prior to the 10min social interaction trial. Two-way ANOVA of total time interacting revealed no significant effect of PCP/housing or treatment ($p>0.05$), but there was a significant PCP/housing x treatment interaction ($F_{(1,\ 29)}=13.46$, $p<0.001$) **$p<0.01$ compared to SAL-GH: VEH, $^{\#\#}p<0.01$ compared to PCP-SI: VEH; Tukey post-hoc; n=16 SAL-GH: VEH, n=18 SAL-GH: ROx; n=18 PCP-SI: VEH; n=16 PCP-SI: ROx.

3.6.3.2: Total time in individual behaviours

Analysis of time spent in individual component behaviours was performed to gain a more thorough account of the social changes observed. ANOVA revealed a significant main effect of PCP/housing on ano-genital sniffing ($F_{(1,29)}$=99.66, p<0.0001), body sniffing ($F_{(1,29)}$=26.64, p<0.0001) and boxing/biting ($F_{(1,29)}$=4.527, p=0.042) behaviours. In contrast, there was no significant main effect of treatment with the $GlyT_1$ inhibitor RO4993850 on any of the individual behaviours, although the change in body sniffing was close to significance ($F_{(1,29)}$=3.839, p=0.0598). Tukey post-hoc analysis showed that SAL-GH rats engaged in significantly more ano-genital sniffing behaviour than PCP-SI rats regardless of drug treatment (p<0.001). Additionally, body sniffing did not differ in either SAL-GH group but PCP-SI rats treated with RO4993850 engaged in significantly more body sniffing compared to PCP-SI rats given vehicle (p<0.05). No other scored behaviours reached post-hoc significance (Figure 3.4). NB: None of the rats engaged in pinning or passive lying-side-by-side during any of the social interaction trials.

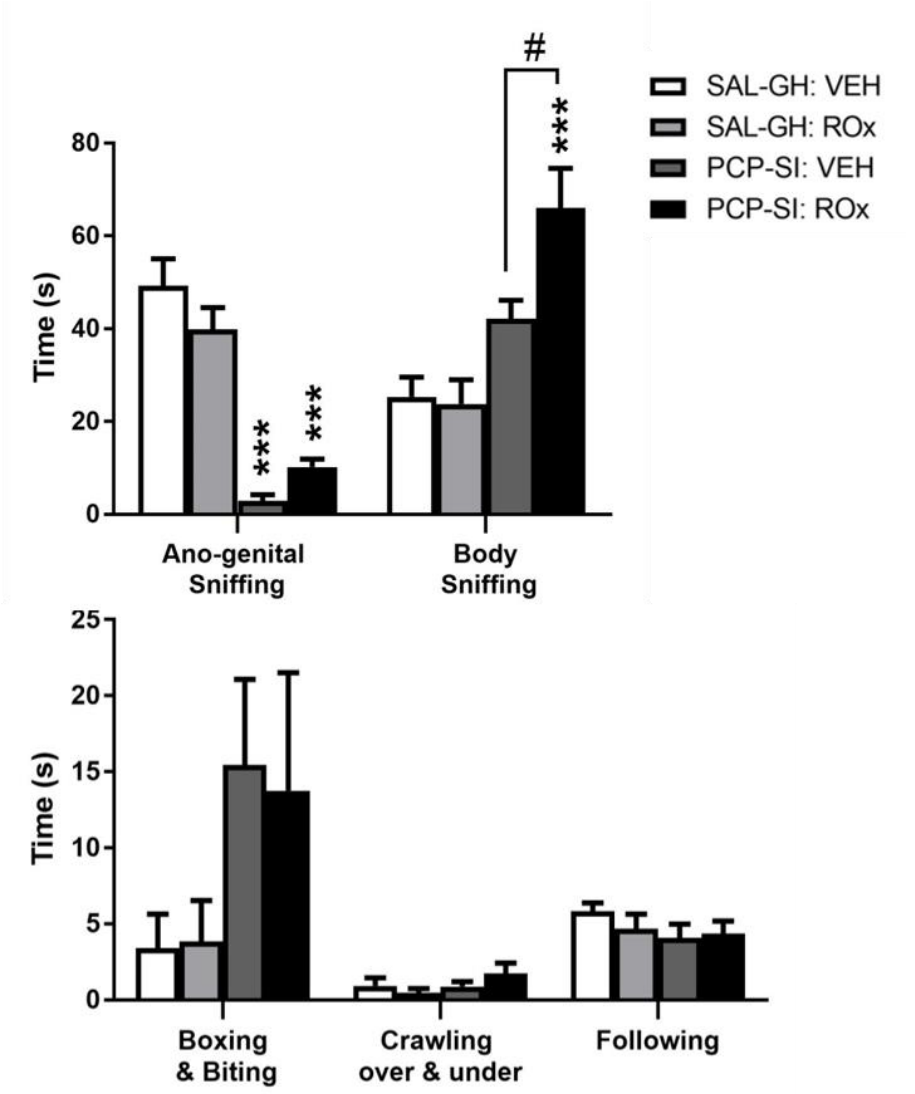


Figure 3.4: Time spent in individual interactive behaviours during social interaction. Comparative differences (mean ± S.E.M.) in total time spent engaging in individual behaviours during the 10min social interaction trial on PND 81 in adult male Lister-hooded rats reared and treated as indicated in the methods during social interaction. Two-way ANOVA of each individual behaviour revealed a significant main effect of PCP/housing on ano-genital sniffing ($F_{(1, 29)}$=99.66, p<0.0001), body sniffing ($F_{(1, 29)}$=26.64, p<0.0001) and boxing/biting ($F_{(1, 29)}$=4.527, p=0.042), but no significant effect of treatment on any behaviour (p>0.05). There was however, a significant PCP/housing x treatment interaction for ano-genital sniffing ($F_{(1, 29)}$=4.791, p=0.0368) and body sniffing ($F_{1, 29)}$=4.938, p=0.0342). ***p<0.001 compared to SAL-GH: VEH, $^{\#}$p<0.05 compared to PCP-SI: VEH; Tukey post-hoc; n=16 SAL-GH: VEH, n=18 SAL-GH: ROx; n=18 PCP-SI: VEH; n=16 PCP-SI: ROx. NB. A different time scale is used to show ano-genital and body sniffing from other, less frequent behaviours. Additionally, lying side-by-side and pinning are not shown as none occurred during the social interaction trials.

3.6.3.3: Latency to first behaviour

In order to further investigate the change in behavioural profile induced by isolation rearing, the latency to each behavioural component was investigated. Two-way ANOVA of time taken to the first behaviour observed between each rat pair revealed a significant main effect of PCP/housing ($F_{(1,29)}$=5.532, p=0.0257), as well as significant main effect of treatment ($F_{(1,29)}$=5.252, p=0.0294). There was however no PCP/housing x treatment interaction ($F_{(1,29)}$=1.609, p=0.2147) indicating that both housing condition (neonatal-PCP combined with isolation-rearing) and treatment with the $GlyT_1$ inhibitor RO4993850 influenced the time taken to engage in a social behaviour with a novel conspecific. Tukey post-hoc analysis revealed a significant difference in time taken between SAL-GH vehicle and PCP-SI RO3994850 treated rats ($p<0.05$) and a trend towards significance between PCP-SI vehicle and PCP-SI RO3994850 treated rats (p=0.0723), indicating that treatment with the $GlyT_1$ inhibitor reduced the amount of time taken for PCP-SI rats to engage in social behaviour, but had less of an impact in SAL-GH animals (Figure 3.5; A).

Typically, SAL-GH rats engaged in ano-genital sniffing first, regardless of treatment group. By contrast, PCP-SI rats initially engaged in body sniffing, regardless of treatment, reflecting the individual behaviours predominantly seen by these groups described previously (Figure 3.4).

3.6.3.4: Latency to ano-genital sniffing

Overall, PCP-SI rats took longer to engage in ano-genital sniffing than SAL-GH rats, such that there was a significant main effect of PCP/housing ($F_{(1,29)}$=9.612, p=0.0043; two-way ANOVA). The overall time taken was significantly reduced following chronic treatment with $GlyT_1$ inhibitor RO4993850 ($F_{(1,29)}$=5.532, p=0.0257), such that both SAL-GH and PCP-SI rats showed reduced latency to engage in ano-genital sniffing. Two-way ANOVA also revealed a significant PCP/housing x treatment interaction ($F_{(1,29)}$=4.857, p=0.0356). Post-hoc significance (Tukey post-hoc) was achieved between SAL-GH vehicle and PCP-SI vehicle ($p<0.01$) rats, as well as PCP-SI vehicle and RO4993850 ($p<0.05$) rat treatment groups (Figure 3.5; B).

3.6.3.5: Latency to body sniffing

Analysis of body sniffing revealed no significant main effect of PCP/housing ($F_{(1,29)}$=1.525, p=0.2268) or treatment ($F_{(1,29)}$=0.01105, p=0.9170) and no PCP/housing x treatment interaction ($F_{(1,29)}$=0.4848, p=0.4918), indicating that the initiation of body sniffing was unaffected by housing conditions or treatment in the social interaction test (Figure 3.5; C).

3.6.3.6: Latency to boxing and biting

For some trials, no boxing/biting behaviour was observed between rat pairs. In this instance a time of 600s was used (to account for the 10min duration of each social interaction trial). Two-way ANOVA of time to the first boxing/biting episode between each rat pair revealed a main effect of PCP/housing just reaching significance ($F_{(1,29)}$=4.063, p=0.05). There was however, no significant main effect of treatment ($F_{(1,29)}$=0.8422, p=0.3662) or PCP/housing x treatment interaction ($F_{(1,29)}$=0.9737, p=0.3319), indicating that PCP-SI rats took slightly less time to engage in their first boxing/biting behaviour compared to SAL-GH controls; (Figure 3.5; D).

3.6.3.7: Distance moved during social interaction

Analysis of total distance moved during the 10min social interaction test, often an index of total ambulatory activity, showed that overall PCP-SI rats had marginally more overall movement and therefore marginally more overall activity during the trial than SAL-GH rats; two-way ANOVA revealed a main effect of PCP/housing ($F_{(1,29)}$=3.627, p=0.0668) that was close to significance. However, this activity was not influenced by treatment ($F_{(1,29)}$=0.015, p=0.9023). There was also no PCP/housing x treatment interaction ($F_{(1,29)}$=0.9160, p=0.3464; Figure 3.6).

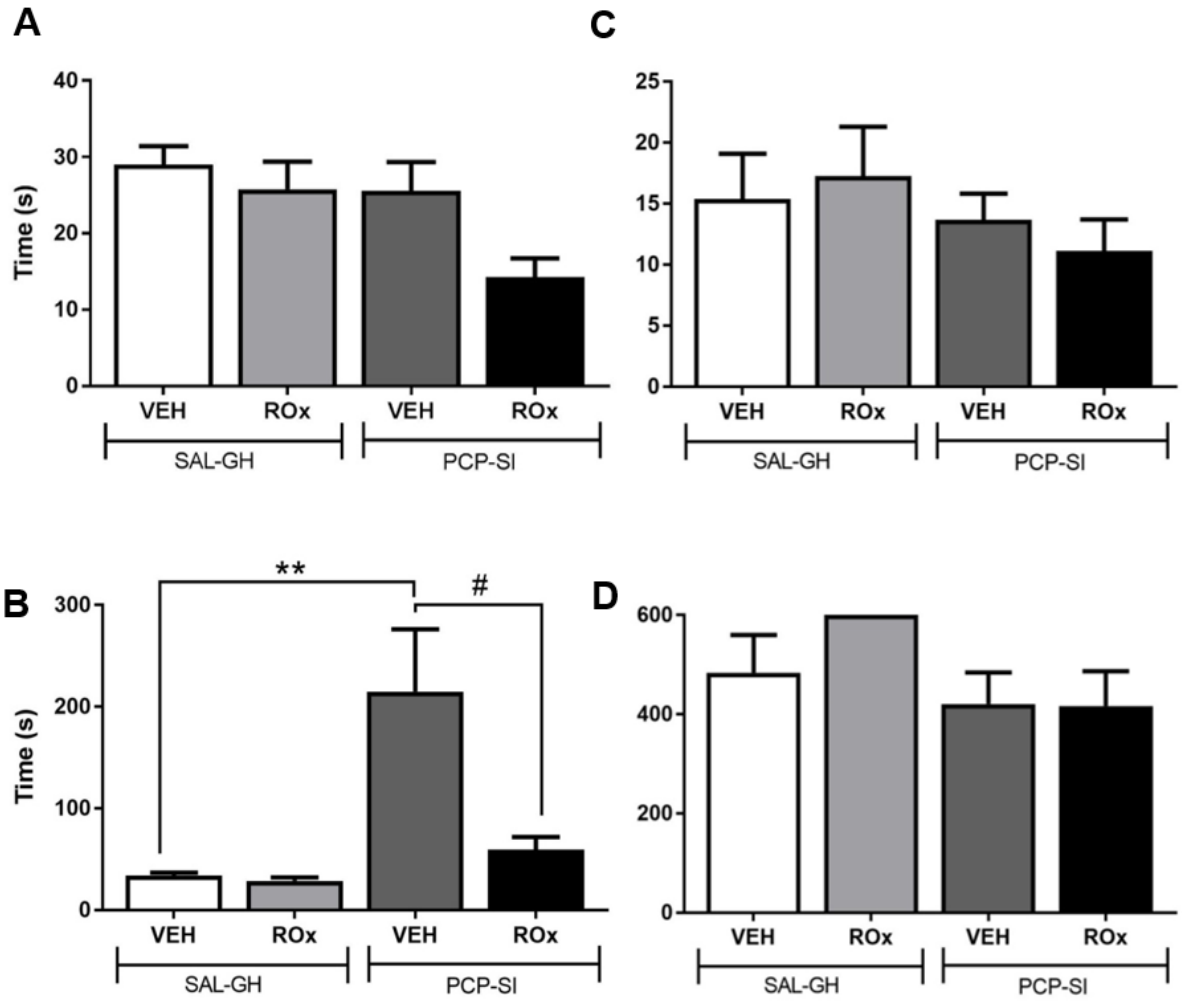


Figure 3.5: Latency to engage in interactive behaviours. Comparative differences (mean ± S.E.M.) in the latency to engage in A; any social interaction, B; ano-genital sniffing, C; body sniffing and D; boxing/biting behaviour between each rat pair during the 10min social interaction trial PND 81 in adult male Lister-hooded rats. A; Two-way ANOVA of time to first scored behaviour revealed a significant main effect of PCP/housing ($F_{(1, 29)}$=5.532, p=0.0257) and treatment $F_{(1, 29)}$=5.252, p=0.0294) but no PCP/housing x treatment interaction (p>0.05). B; Two-way ANOVA of time to first ano-genital sniffing revealed a significant main effect of PCP/housing ($F_{(1,29)}$=9.612, p=0.0043), treatment ($F_{(1,29)}$=5.532, p=0.0257) and a PCP/housing x treatment interaction ($F_{(1,29)}$=4.857, p=0.0356). C; Two-way ANOVA of time to first body sniffing revealed no significant main effect of PCP/housing or treatment (p>0.05). D; Two-way ANOVA of time to first boxing/biting behaviour revealed no significant main effect of PCP/housing or treatment (p>0.05). **p<0.01 compared to SAL-GH: VEH, $^{\#}$p<0.05 compared to PCP-SI: VEH; Tukey post-hoc. n=16 SAL-GH: VEH, n=18 SAL-GH: ROx; n=18 PCP-SI: VEH; n=16 PCP-SI: ROx.

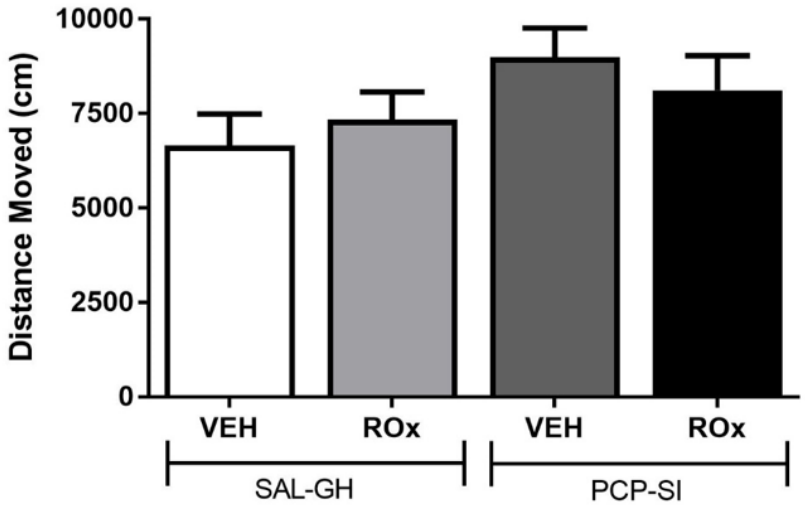


Figure 3.6: Total distance moved during social interaction. Differences in in the total distance moved (cm, mean ± S.E.M.) during the 10min social interaction trial recorded on PND 81 in adult male Lister-hooded rats. Two-way ANOVA of total distance moved revealed no significant effect of PCP/housing or treatment ($p>0.05$). n=16 SAL-GH: VEH, n=18 SAL-GH: ROx; n=18 PCP-SI: VEH; n=16 PCP-SI: ROx.

3.6.4: USVs during social interaction

USVs were recorded during the social interaction task and categorised into social (50 kHz) and aversive (22 kHz) calls following Fast Fourier transformation and pattern analysis. Differences in the total number of emissions, total duration of calls and changes in call bandwidth were subsequently determined between the 50 kHz and 22 kHz calls emitted by all four groups following a $\log_{10}$ transformation to normalise the data before two-way ANOVA analysis.

3.6.4.1: Changes in the number of USVs emitted

50 kHz calls

Interestingly, PCP-SI rats emitted a greater number of 50 kHz calls overall compared to SAL-GH rats; a two-way ANOVA of total calls revealed a significant main effect of PCP/housing ($F_{(1,29)}$=6.821, p=0.0141) which reached post hoc significance (p<0.05 SAL-GH: VEH vs PCP-SI: VEH; Tukey post-hoc). The number of calls emitted following chronic treatment with $GlyT_1$ inhibitor RO499380 was substantially reduced (~95%) in PCP-SI rats but failed to reach significance ($F_{(1,29)}$=1.726, p=0.1992) due to variation. There was also no PCP/housing x treatment interaction ($F_{(1,29)}$=1.960 p=0.1722; data not shown).

Following categorisation of the pro-social 50 kHz calls into three subtypes; flat, step and trill, PCP-SI rats were shown to emit the highest number of calls in each category, reaching significance for flat ($F_{(1, 29)}$=5.422, p=0.0271), step ($F_{(1, 27)}$=4.166, p=0.05) and trill ($F_{1, 25)}$=5.383, p=0.0288). For trill-type calls, post-hoc

significance was also achieved between both vehicle treated groups ($p<0.05$; Sidak post-hoc).

However, chronic treatment with RO4993850 failed to significantly influence the number of flat ($F_{(1, 29)}=0.1356$, $p=0.7153$), step ($F_{(1, 27)}=0.0084$, $p=0.9278$) and trill ($F_{(1, 25)}=2.061$, $p=0.1635$) USVs emitted. There was also no significant PCP/housing x treatment interaction ($p>0.05$) for any of the three call categories (Figure 3.7; A-C).

22 kHz calls

The number of 22 kHz calls emitted showed a similar profile. PCP-SI rats emitted a greater number of aversive calls and ANOVA revealed a main effect of PCP/housing ($F_{(1, 6)}=4.161$, $p=0.0875$) that was close to significance. Treatment with RO4993850 reduced the number of calls but this failed to reach significance ($F_{(1, 6)}=2.792$, $p=0.1458$), although a PCP/housing x treatment interaction ($F_{(1, 6)}=6.889$, $p=0.0393$) was revealed. Therefore treatment with the $GlyT_1$ inhibitor reduced the number of USVs emitted in socially impaired rats (PCP-SI) but not in SAL-GH control rats, which emitted a similar number of USVs. Post-hoc significance was achieved in the number of 22 kHz USV emissions between SAL-GH and PCP-SI vehicle treated rats, and between PCP-SI vehicle and PCP-SI RO4993850 treated rats ($p<0.05$; Sidak post-hoc). It must be noted however, that the majority (86 out of 90) of aversive calls recorded from PCP-SI vehicle treated animals were from one rat pair and associated with boxing and biting behaviour (discussed later; Figure 3.7; D).

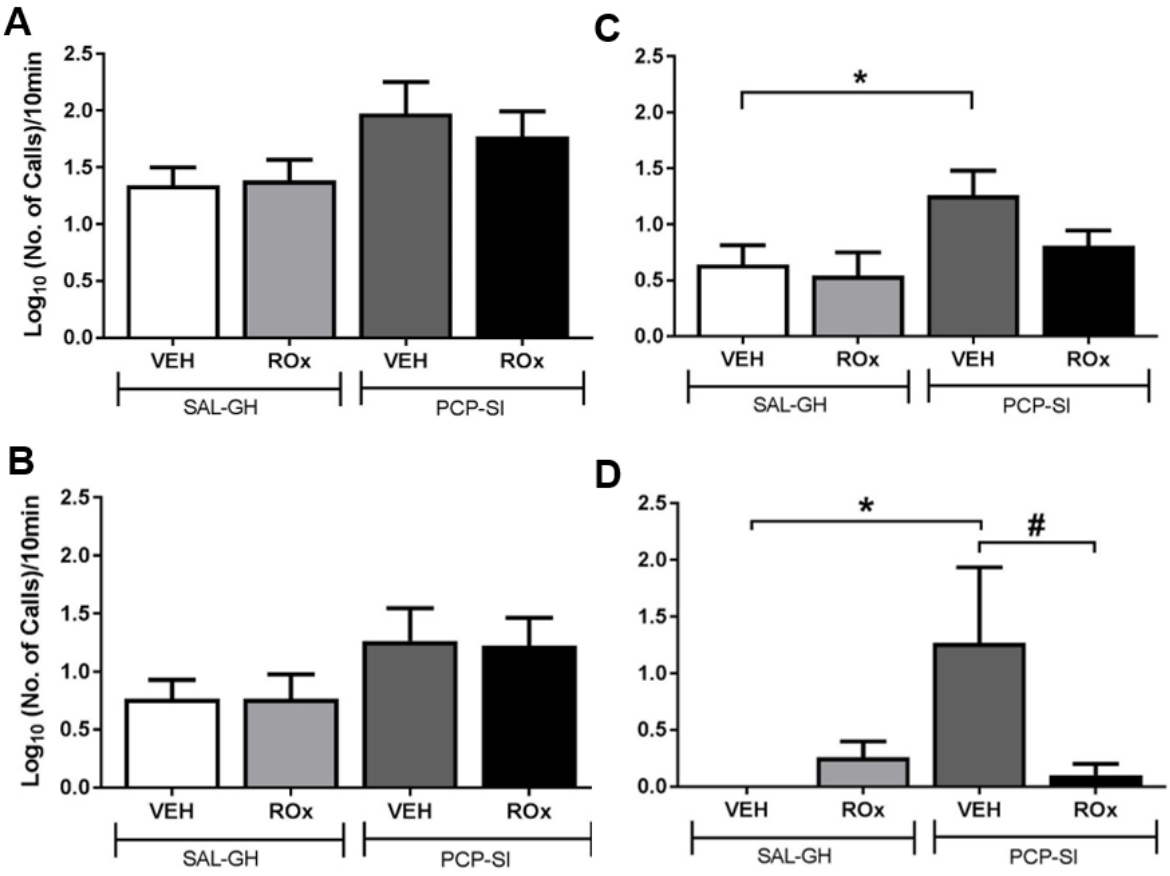


Figure 3.7: Number of ultrasonic vocalisations emitted between each rat pair during social interaction. Comparative differences (mean ± S.E.M.) in number of pro-social 50 kHz A; flat, B; step, C; trill and 22 kHz D; aversive ultrasonic vocalisations (USVs) emitted by both rats in each pair during the 10min social interaction trial PND 81 in adult male Lister-hooded rats reared and treated as described in the methods. 50 kHz USVs characterised following Fast Fourier transformation and pattern analysis, with the total number of emissions and subsequent analysis determined following a $\log_{10}$ transformation to normalise the data. A; Two-way ANOVA of the total flat-type calls emitted revealed a significant main effect of PCP/housing ($F_{(1, 29)}$=5.422, p=0.0271), but no significant effect of treatment or PCP/housing x treatment interaction (p>0.05). B; Two-way ANOVA of total step-type calls emitted revealed no significant main effect of PCP/housing or treatment (p>0.05). C; Two-way ANOVA of total trill-type calls emitted revealed a significant main effect of PCP/housing ($F_{1, 25)}$=5.383, p=0.0288), but no significant main effect of treatment or PCP/housing x treatment interaction (p>0.05). D; Two-way ANOVA of aversive calls emitted revealed a significant main effect of PCP/housing ($F_{1, 6)}$=4.161, p=0.0875) but no significant main effect of treatment (p>0.05). There was however a PCP/housing x treatment interaction ($F_{1, 6)}$=6.889, p=0.0393). *p<0.05 compared to SAL-GH: VEH, $^{\#}$p<0.05 compared to PCP-SI: VEH; Sidak post-hoc. n=16 SAL-GH: VEH, n=18 SAL-GH: ROx; n=18 PCP-SI: VEH; n=16 PCP-SI: ROx.

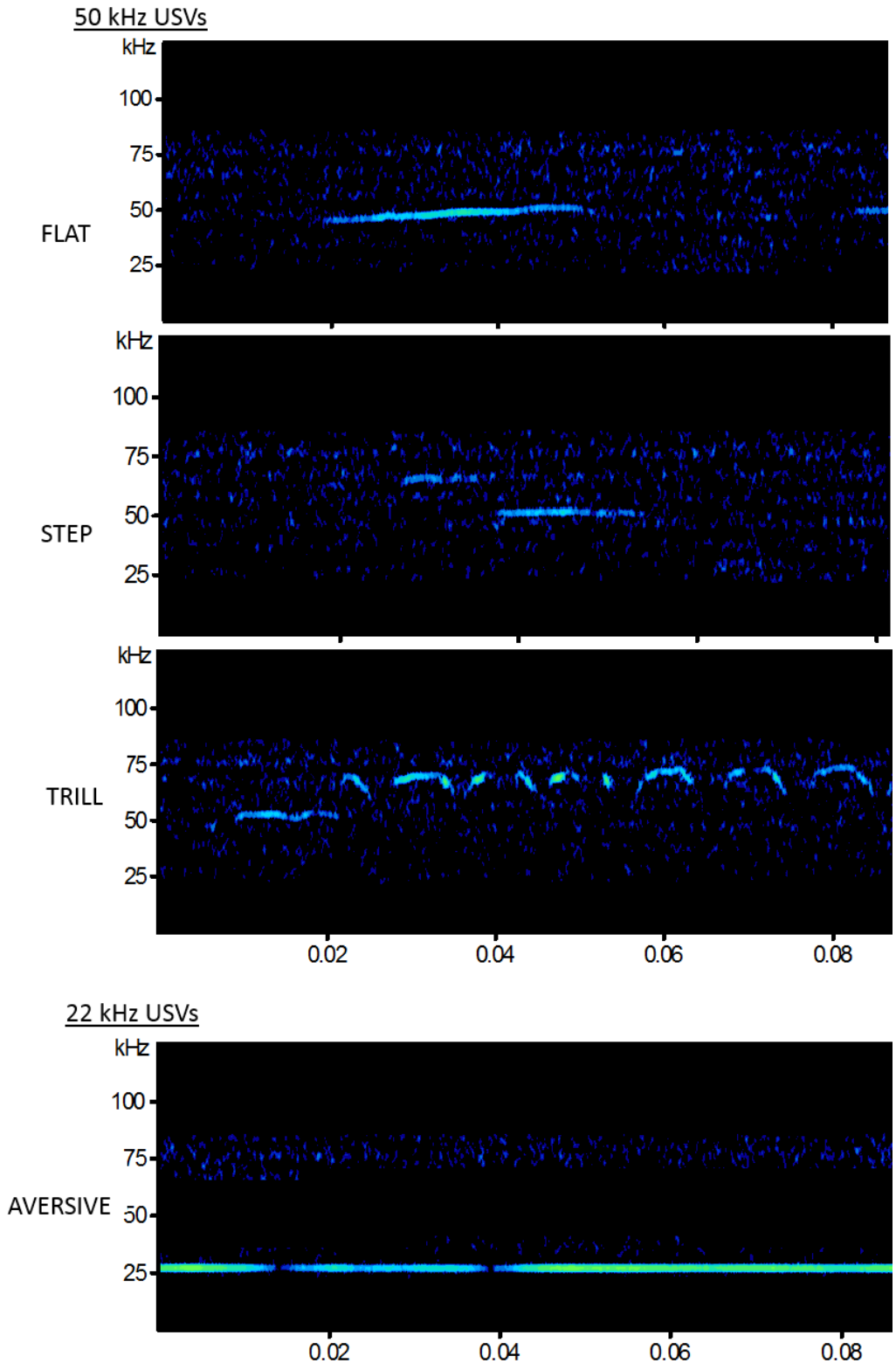


Figure 3.8: Example spectrograph images of ultrasonic vocalisations. Spectrographs show pro-social 50 kHz calls (time scales in s) defined into three subtypes of flat, step and trill according to pattern analysis, as well as 'aversive' 22 kHz calls.

3.6.4.2: Changes in call duration

50 kHz calls

For each 50 kHz call subtype (flat, step and trill) SAL-GH rats emitted calls that were significantly shorter in duration compared to PCP-SI rats; ANOVA showed a significant main effect of PCP/housing for flat ($F_{(1,29)}$=156.634, p=0.0001), step ($F_{(1,27)}$=57.646, p=0.0001) and trill ($F_{(1,25)}$=32.824, p=0.0001) USVs. The difference in call duration between SAL-GH vehicle and both PCP-SI groups (vehicle and RO4993850 treated rats) also reached post-hoc significance (p<0.001; Sidak post-hoc) for each call subtype.

Treatment with the $GlyT_1$ inhibitor RO4993850 for 14 days showed little influence on step ($F_{(1, 27)}$=0.460, p=0.498) and trill ($F_{(1, 25)}$=0.0000, p=1.000) call duration. However, there was a trend towards significance in flat call duration ($F_{(1, 29)}$=3.635, p=0.057). There was also a significant PCP/housing x treatment interaction for flat calls ($F_{(1, 29)}$=11.683, p=0.001), but only a close to significant effect for step calls ($F_{(1, 27)}$=3.344, p=0.068) and no significant PCP/housing x treatment interaction for the trill call subtype ($F_{(1, 25)}$=1.840, p=0.176). Further post-hoc analysis revealed a significant difference in call length for flat type calls between PCP-SI vehicle and RO4993850 treated rats (p<0.001; Sidak post-hoc), but no difference in SAL-GH rats. This suggests that the $GlyT_1$ inhibitor RO4993850 decreases flat call duration specifically in socially impaired rats (PCP-SI) with less of an effect in SAL-GH controls (Figure 3.9; A-C).

22 kHz calls

In contrast there was no significant main effect of PCP/housing ($F_{(1, 6)}$=1.673, p=0.199), treatment ($F_{(1, 6)}$=2.178, p=0.143) or PCP/housing x treatment interaction ($F_{(1, 6)}$=0.134, p=0.716) on the length of 22 kHz USV emissions suggesting that neither housing conditions nor treatment had any effect. However, this is not surprising considering the low number of USV emissions at this bandwidth (Figure 3.9; D).

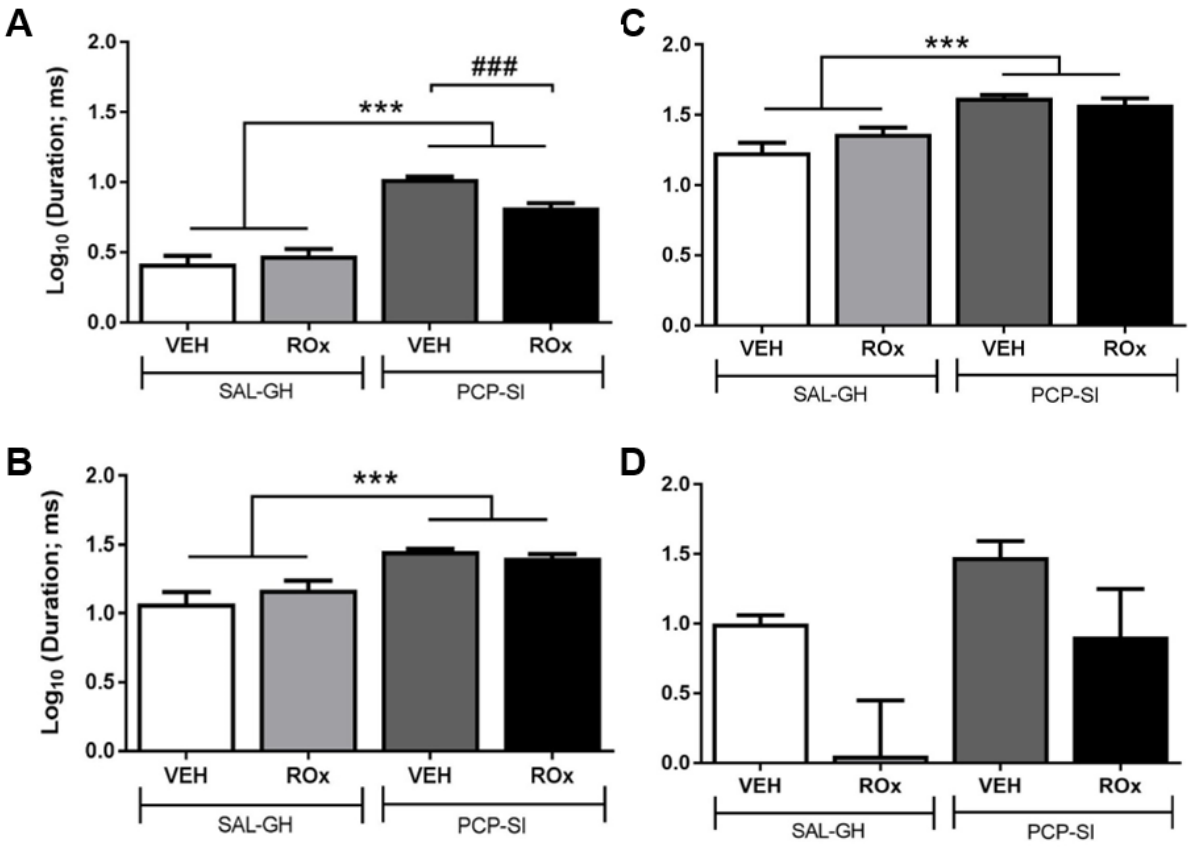


Figure 3.9: Duration of ultrasonic vocalisations emitted between each rat pair during social interaction. Comparative differences (mean ± S.E.M.) in the total duration (ms) of pro-social 50 kHz A; flat, B; step, C; trill and 22 kHz D; aversive ultrasonic vocalisations (USVs) emitted by both rats in each pair during the 10min social interaction trial PND 81 in adult male Lister-hooded rats. 50 kHz USVs characterised following Fast Fourier transformation and pattern analysis, with the total USV duration and subsequent analysis determined following a log_{10} transformation. A; Two-way ANOVA of the total flat-type call duration revealed a significant main effect of PCP/housing ($F_{(1, 29)}$=156.634, p=0.0001), but only a close to significant effect of treatment ($F_{(1, 29)}$=3.635, p=0.057). There was however, a significant PCP/housing x treatment interaction ($F_{(1, 29)}$=11.683, p=0.001). B; Two-way ANOVA of total step-type call duration revealed a significant main effect of PCP/housing ($F_{(1, 27)}$=57.646, p=0.0001), but no significant main effect of treatment or PCP/housing x treatment interaction (p>0.05). C; Two-way ANOVA of total trill-type call duration revealed a significant main effect of PCP/housing ($F_{1, 25)}$=32.842, p=0.0001), but no significant main effect of treatment or PCP/housing x treatment interaction (p>0.05). D; Two-way ANOVA of aversive call duration revealed no significant main effect of PCP/housing or treatment (p>0.05). ***p<0.001 compared to SAL-GH: VEH, $^{###}$p<0.001 compared to PCP-SI: VEH; Sidak post-hoc. n=16 SAL-GH: VEH, n=18 SAL-GH: ROx; n=18 PCP-SI: VEH; n=16 PCP-SI: ROx.

3.6.4.3: Changes in call bandwidth

50 kHz calls

Differences in call bandwidth (change in minimum and maximum peak frequencies) for each of the 50 kHz call subtypes were also analysed. Overall, PCP-SI rats showed greater differences in call bandwidth compared to SAL-GH rats; a two-way ANOVA of change in call bandwidth revealed a significant main effect of PCP/housing for flat ($F_{(1,29)}$=141.962, p=0.0001), step ($F_{(1, 27)}$=59.967, p=0.0001) and trill ($F_{(1, 25)}$=44.655, p=0.0001) call subtypes. The difference in call bandwidth between SAL-GH vehicle and both PCP-SI rat groups (vehicle and RO4993850) also reached post-hoc significance (p<0.001; Sidak post-hoc) for each call subtype.

This difference in call bandwidth between both PCP-SI rat groups was reduced following chronic treatment with $GlyT_1$ inhibitor RO4993850; two-way ANOVA of call bandwidth revealed a significant main effect of treatment for flat ($F_{(1, 29)}$=8.393, p=0.004) and trill ($F_{(1, 25)}$=7.520, p=0.006) but not step ($F_{(1, 27)}$=1.712, p=0.191) call subtypes. There was also a PCP/housing x treatment interaction for the change in bandwidth of flat-type calls ($F_{(1, 29)}$=7.668, p=0.006) but not step and trill (p>0.05) call subtypes. Post-hoc significance (Sidak post-hoc) was also achieved between PCP-SI vehicle and RO4993850 treated rats for flat (p<0.001), step (p<0.05) and trill (p<0.05) calls (Figure 3.10; A-C).

22 kHz calls

Two-way ANOVA of aversive 22 kHz call bandwidth did not reveal significant effects of PCP/housing ($F_{(1,6)}$=0.703, p=0.404) or treatment ($F_{(1,6)}$=1.019, p=0.315). Therefore the aversive calls produced in the frequency range of 20-32 kHz did not have a substantial change in individual call bandwidth between SAL-GH or PCP-SI rats, or those treated with the $GlyT_1$ inhibitor, characteristic for calls of this nature (Figure 3.10; D).

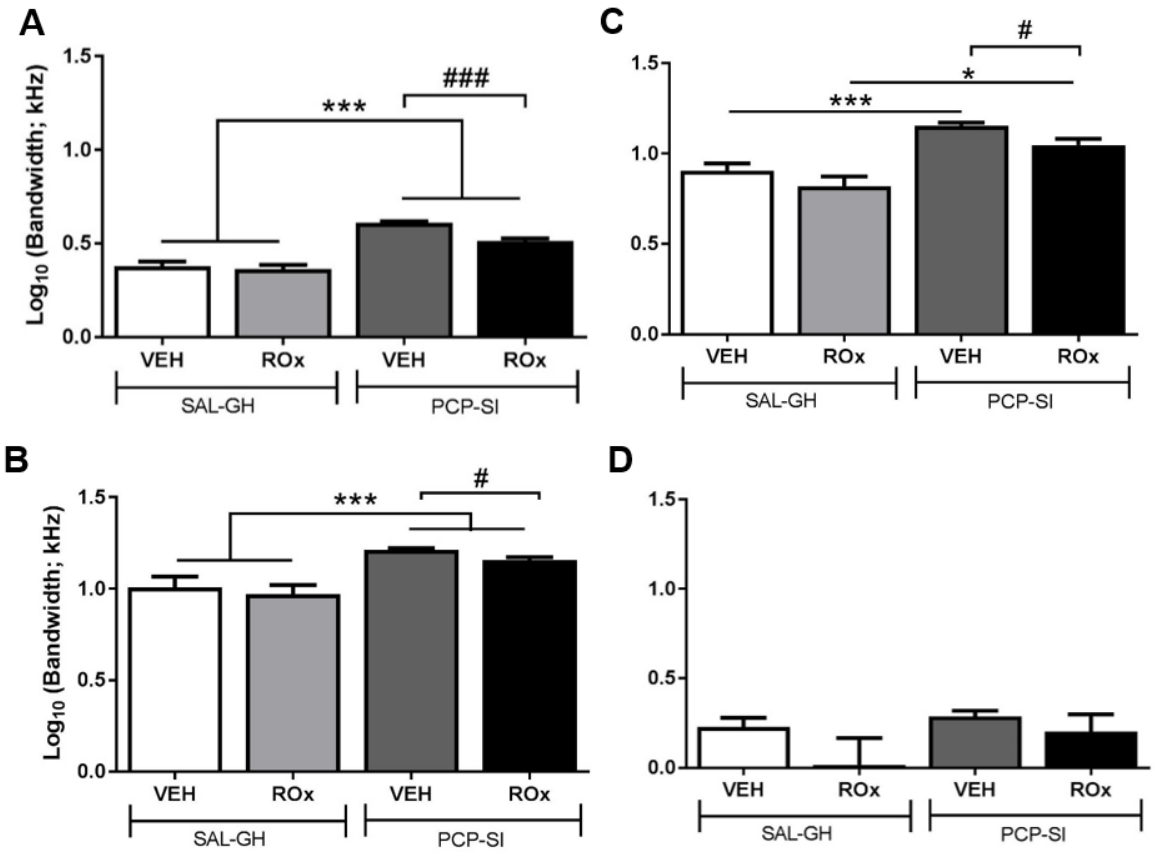


Figure 3.10: Changes in call bandwidth for each call sub-type emitted between each rat pair during social interaction. Comparative differences (mean ± S.E.M.) in the change in call bandwidth (difference between minimum and maximum peak frequencies; kHz) of pro-social 50 kHz A; flat, B; step, C; trill and 22 kHz D; aversive ultrasonic vocalisations (USVs) emitted by both rats in each pair during the 10min social interaction trial PND 81. 50 kHz USVs characterised following Fast Fourier transformation and pattern analysis, with the total change in call bandwidth and subsequent analysis determined following a $\log_{10}$ transformation. A; Two-way ANOVA of the total flat-type call change in bandwidth revealed a significant main effect of PCP/housing ($F_{(1, 29)}$=141.962, p=0.0001), treatment ($F_{(1, 29)}$=8.393, p=0.004) and a PCP/housing x treatment interaction ($F_{(1, 29)}$=7.668, p=0.006). B; Two-way ANOVA of total step-type call change in bandwidth revealed a significant main effect of PCP/housing ($F_{(1, 27)}$=59.967, p=0.0001), but no significant main effect of treatment or PCP/housing x treatment interaction (p>0.05). C; Two-way ANOVA of total trill-type call change in bandwidth revealed a significant main effect of PCP/housing ($F_{1, 25)}$=44.655, p=0.0001) and treatment ($F_{(1, 25)}$=7.520, p=0.006), but no PCP/housing x treatment interaction (p>0.05). D; Two-way ANOVA of aversive call change in bandwidth revealed no significant main effect of PCP/housing or treatment (p>0.05). *p<0.05, ***p<0.001 compared to SAL-GH: VEH, $^{\#}$p<0.05, $^{\#\#\#}$p<0.001 compared to PCP-SI: VEH; Sidak post-hoc. n=16 SAL-GH: VEH, n=18 SAL-GH: ROx; n=18 PCP-SI: VEH; n=16 PCP-SI: ROx.

3.6.5: Comparing behaviour to USV emissions

3.6.5.1: Behaviour during the first USV

A comparison of which socially-interactive behaviours rats were engaging in (any of the manually scored behaviours including ano-genital sniffing, body sniffing, crawling over-and-under, lying side-by-side, pinning, following and boxing/biting behaviour) at the time of the first detectable USV emission is shown in Table 3.1. In general, SAL-GH rats had a tendency to be engaged in ano-genital sniffing behaviour, whereas PCP-SI rats were predominantly engaged in body sniffing behaviour, reflecting the individual behaviours predominantly seen by the rat during the social interaction trial (Figure 3.4).

Table 3.1: Behaviour observed between rat pairs during first USV call

	SAL-GH VEH	SAL-GH ROx	PCP-SI VEH	PCP-SI ROx
Ano-genital Sniffing	4	5		
Body Sniffing	2	2	8	8
Following	1			
No detectable behaviour	1	1	1	

Despite differing first behaviours observed between SAL-GH and PCP-SI rats, there was no difference in the length of time taken until emission of the first USV. Two-way ANOVA of time taken revealed no significant main effect of PCP/housing ($F_{(1,29)}$=0.4944, p=0.4876) or treatment ($F_{(1,29)}$=0.2112, p=0.6492). There was also no significant PCP/housing x treatment interaction

($F_{(1,29)}$=0.01747, p=0.8958), indicating the time taken to emit a USV to be independent of housing or treatment conditions (data not shown).

3.6.5.2: Behaviour during 22 kHz USVs

All the various behavioural components (ano-genital sniffing, body sniffing, crawling over-and-under, following and boxing/biting behaviour) recorded during the social interaction trial were matched to concomitant aversive calls. The majority of aversive 22 kHz calls detected in PCP-SI vehicle treated rats (86 out of 90) were from one trial and associated with boxing and biting behaviour. However, the remaining calls were associated with body sniffing or rearing.

3.6.5.3: Correlation of behaviour with the number of USVs

In general terms, the extent of social behaviour in the rat during social interaction, as well at the number of USV emissions, appear to be influenced by housing conditions (neonatal-PCP combined with isolation-rearing). Despite this however, no correlation occurred between the number of calls (analysed following a $\log_{10}$ transformation) and the total time spent in pro-social interaction behaviours; r=0.08363, p=0.6436.

3.6.6: Study 1:

Part 2: Cortical effects in neonatal-PCP isolation-reared rats

3.6.6.1: C-fos immunoreactivity following chronic RO3994850

In order to determine which key cortical and limbic brain regions might be activated in association with social isolation and/or altered by the $GlyT_1$ inhibitor, changes to c-fos immunoreactivity in various brain sub-regions were determined following the social interaction test using one rat from each pair, which underwent perfusion fixation at 90min post-trial. Two-way ANOVA for each brain region revealed no significant main effect of PCP/housing on total c-fos immunoreactivity, although a trend towards significance was seen in the rostral ventral OFC ($F_{(1, 28)}$=3.000, p=0.096), dorsal striatum ($F_{(1, 28)}$=3.789, p=0.063) and NAc Shell ($F_{(1, 28)}$=3.941, p=0.058), suggesting neonatal-PCP isolation-rearing to exhibit little influence on the extent of c-fos activity and thereby neuronal activation within these sub-regions.

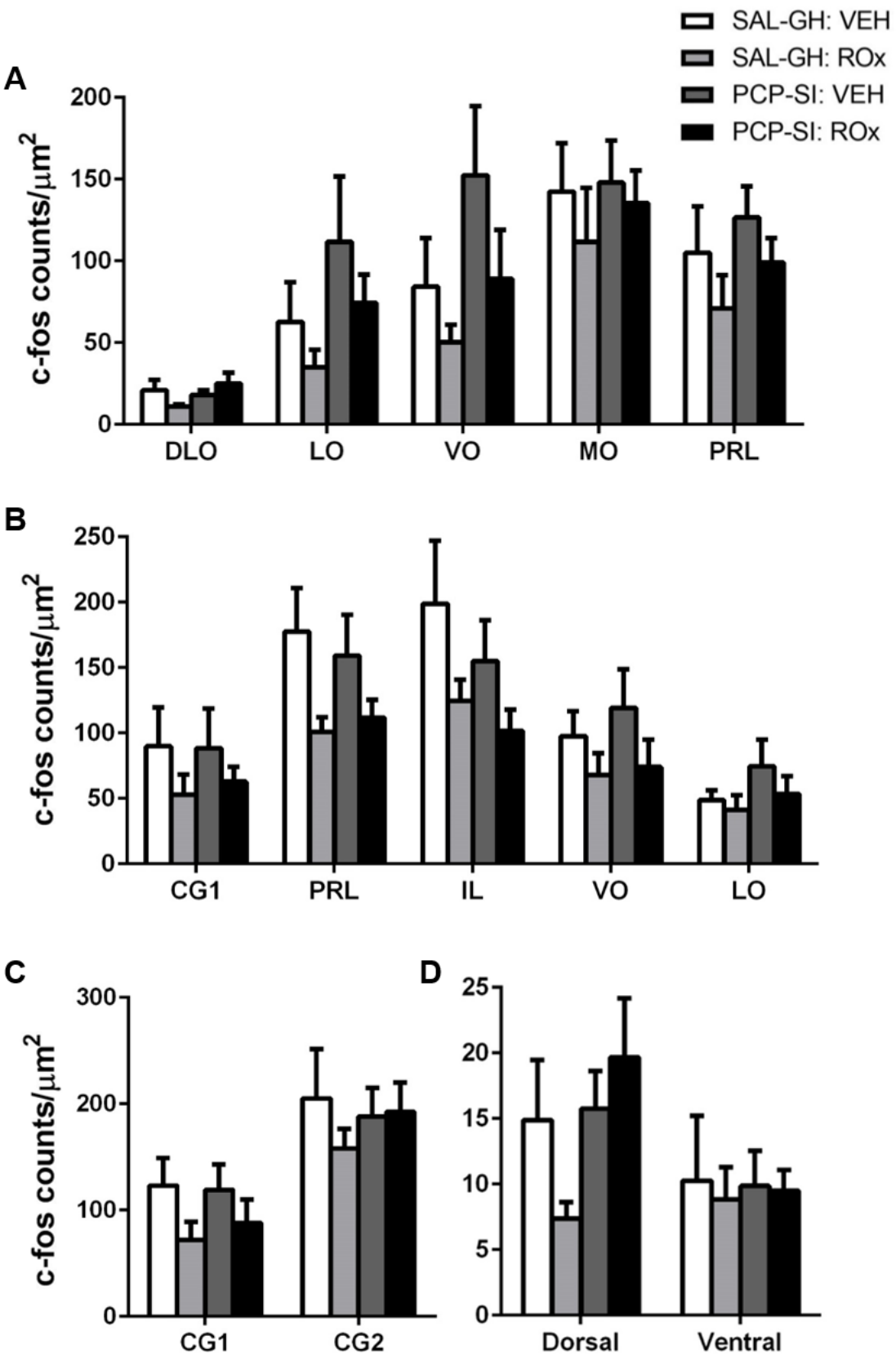
The $GlyT_1$ inhibitor RO4993850 decreased total c-fos immunoreactivity within the PFC, revealing a significant main treatment effect in the caudal PRL ($F_{(1,27)}$=6.336, p=0.019) and IL ($F_{(1,28)}$=4.769, p=0.039) cortices but no other brain regions. A significant PCP/housing x treatment interaction was seen in the BMA ($F_{(1, 27)}$=9.839, p=0.004) but only a trend towards significance was observed in the rostral dorsolateral OFC ($F_{(1, 28)}$=3.469, p=0.074) and BLA ($F_{(1, 27)}$=3.834, p=0.062). Further post-hoc analysis revealed no significant effects between groups except for the BMA where the number of calls in SAL-ROx was

significantly less than SAL-GH (p=0.046; Tukey post-hoc; all ANOVA results outlined in Table 3.2 and Figure 3.11).

Table 3.2: C-fos immunoreactivity in selective brain regions

Brain Region	PCP/housing			Treatment			PCP/housing x treatment Interaction		
	df	F	p-value	Df	F	p-value	df	F	p-value
Rostral PFC									
DLO	(1, 28)	1.520	0.229	(1, 28)	0.106	0.747	**(1, 28)**	**3.469**	**0.074**
LO	(1, 28)	2.776	0.108	(1, 28)	1.509	0.231	(1, 28)	0.034	0.855
VO	**(1, 28)**	**3**	**0.096**	(1, 28)	2.487	0.127	(1, 28)	0.226	0.639
MO	(1, 26)	0.289	0.596	(1, 26)	0.636	0.433	(1, 26)	0.115	0.738
PRL	(1, 28)	1.412	0.246	(1, 28)	2.192	0.151	(1, 28)	0.024	0.878
Caudal PFC									
CG1	(1, 27)	0.032	0.860	(1, 27)	1.765	0.196	(1, 27)	0.058	0.811
PRL	(1, 27)	0.024	0.878	**(1, 27)**	**6.336**	**0.019**[a]	(1, 27)	0.343	0.563
IL	(1, 28)	1.3	0.265	**(1, 28)**	**4.769**	**0.039**[a]	(1, 28)	0.131	0.720
LO	(1, 28)	1.539	0.226	(1, 28)	0.911	0.349	(1, 28)	0.209	0.651
VO	(1, 28)	0.381	0.543	(1, 28)	2.719	0.112	(1, 28)	0.113	0.739
Cingulate Cortex									
CG1	(1, 28)	0.07	0.794	**(1, 28)**	**3.403**	**0.077**[a]	(1, 28)	0.202	0.657
CG2	(1, 28)	0.09	0.767	(1, 28)	0.523	0.476	(1, 28)	0.766	0.390
Striatum									
Dorsal	**(1, 28)**	**3.789**	**0.063**	(1, 28)	0.281	0.6	(1, 28)	2.866	0.103
Ventral	(1, 28)	0.003	0.958	(1, 28)	0.093	0.763	(1, 28)	0.034	0.854
Nucleus Accumbens									
Core	(1, 28)	2.254	0.146	(1, 28)	1.360	0.255	(1, 28)	0.000	0.985
Shell	**(1, 28)**	**3.941**	**0.058**	(1, 28)	0.023	0.882	(1, 28)	1.995	0.170
Amygdala									
BLA	(1, 27)	0.809	0.377	(1, 27)	0.280	0.602	**(1, 27)**	**3.834**	**0.062**
BMA	(1, 27)	0.021	0.885	(1, 27)	0.646	0.429	**(1, 27)**	**9.839**	**0.004**[a]

[a] $p<0.05$


SAL-GH: VEH
SAL-GH: ROx
PCP-SI: VEH
PCP-SI: ROx
A
c-fos counts/μm²
200
150
100
50
0
DLO
LO
VO
MO
PRL
B
c-fos counts/μm²
250
200
150
100
50
0
CG1
PRL
IL
VO
LO
C
c-fos counts/μm²
300
200
100
0
CG1
CG2
D
25
20
15
10
5
0
Dorsal
Ventral

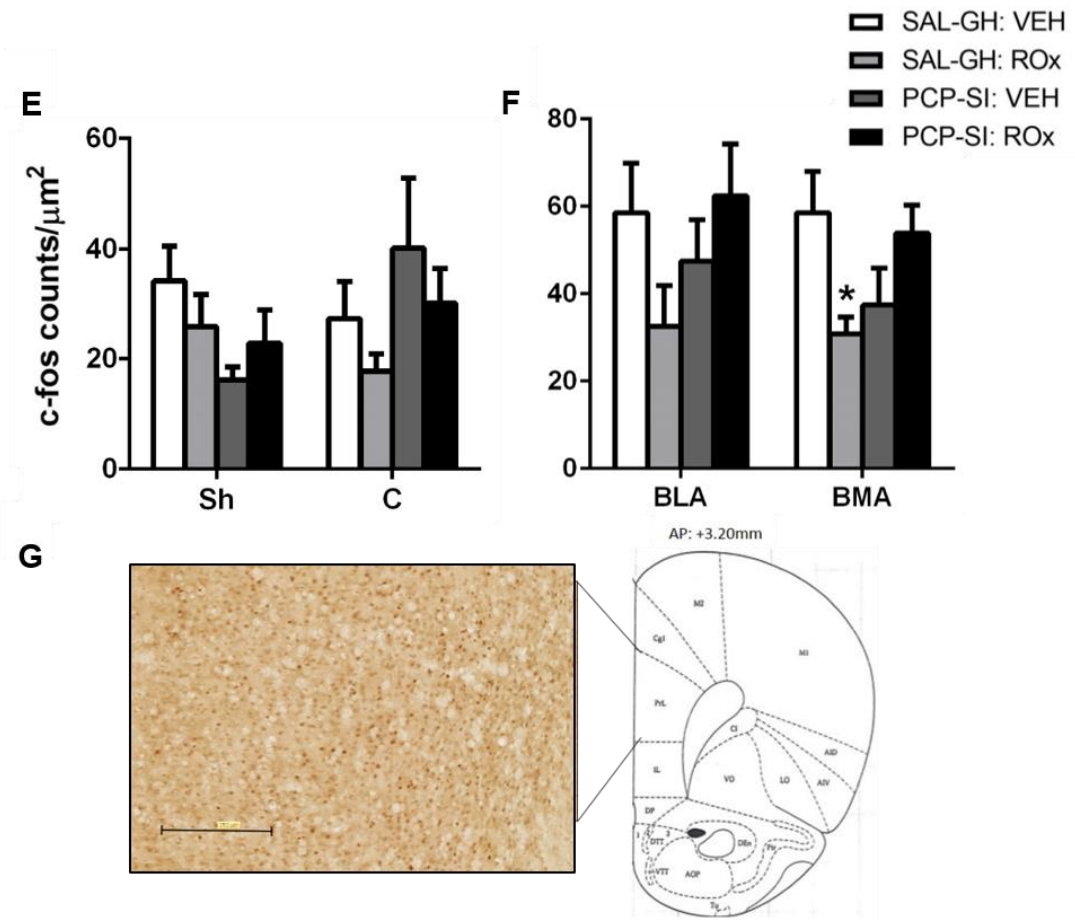


Figure 3.11: Density of c-fos counts (counts/µm^2) in various brain sub-regions. Columns shown comparative differences (mean ± S.E.M.) of c-fos counts in the A; rostral PFC, B; caudal PFC, C; cingulate cortex, D; striatum, E; NAc and F; amygdala following a social interaction trial PND 81 in adult male Lister-hooded rats. Tissue collected for immunohistochemistry 90min post-trial and perfusion-fixed with 4% PFA. G; example image of a PRL 60µm coronal section used for quantification of c-fos immunoreactivity, scale bar 250µm. Two-way ANOVA for each brain region revealed no significant main effect of PCP/housing although a trend towards significance was seen in the rostral VO ($F_{(1,\ 28)}$=3.000, p=0.0096), dorsal striatum ($F_{(1,\ 28)}$=3.789, p=0.063) and NAc Sh ($F_{(1,\ 28)}$=3.941, p=0.058). There was however a significant main effect of treatment in the caudal PRL ($F_{(1,27)}$=6.336, p=0.019) and IL ($F_{(1,28)}$=4.769, p=0.039) cortices. A significant PCP/housing x treatment interaction was seen in the BMA ($F_{(1,\ 27)}$=9.839, p=0.004) but only a trend towards significance was seen in the rostral DLO ($F_{(1,\ 28)}$=3.469, p=0.074) and BLA ($F_{(1,\ 27)}$=3.834, p=0.062). *p<0.05 compared to SAL-GH: VEH; Tukey post-hoc. n=8 SAL-GH: VEH, n=8 SAL-GH: ROx; n=9 PCP-SI: VEH; n=8 PCP-SI: ROx. OFC; Orbitofrontal cortex, PFC; Prefrontal Cortex; DLO; Dorsolateral OFC, LO; Lateral OFC, VO; ventral OFC, MO; medial OFC, PRL; prelimbic cortex, CG1; Limbic cortex 1, IL; infralimbic cortex, NAc; Nucleus accumbens, C; NAc Core, Sh; NAc Shell, BLA; Basolateral amygdala; BMA; Basomedial amygdala.

3.6.6.2: Parvalbumin immuno-reactivity following chronic RO4993850

In order to ascertain any impact of PCP/housing and/or treatment on the number of parvalbumin positive GABA interneurons shown to be sensitive to a number of neurodevelopmental manipulations, as shown in Chapter 1, Table 1.1, parvalbumin immunoreactivity was determined in the rostral PFC and hippocampus (CA1, CA2/3 and DG) following the social interaction test using one rat from each pair, perfused 90min post-trial.

Within the hippocampus, PCP-SI rats had significantly fewer parvalbumin positive cells compared to SAL-GH rats. ANOVA revealed a significant main effect of PCP/housing in the CA1 ($F_{(1,27)}$=23.86, p=0.0001), CA2/3 ($F_{(1,27)}$=13.84, p=0.0009) and DG ($F_{(1,27)}$=6.213, p=0.0191), also reaching Tukey post-hoc significance (p<0.05) in all three regions (Figure 3.12; A). Conversely however, there was no substantial change in parvalbumin expression within the rostral PFC (Figure 3.12; B). No specific sub-region (dorsolateral, ventral, lateral and medial OFC or PRL) revealed a significant main effect of PCP/housing (p>0.05) however there was a trend towards significance in the dorsolateral OFC ($F_{(1,24)}$=3.724, p=0.0655). Chronic treatment with RO4993850 failed to alter the number of parvalbumin positive cells in any hippocampal (CA1, CA2/3 and DG) or rostral prefrontal region (p>0.05), indicating the compound to have little influence following two weeks treatment.

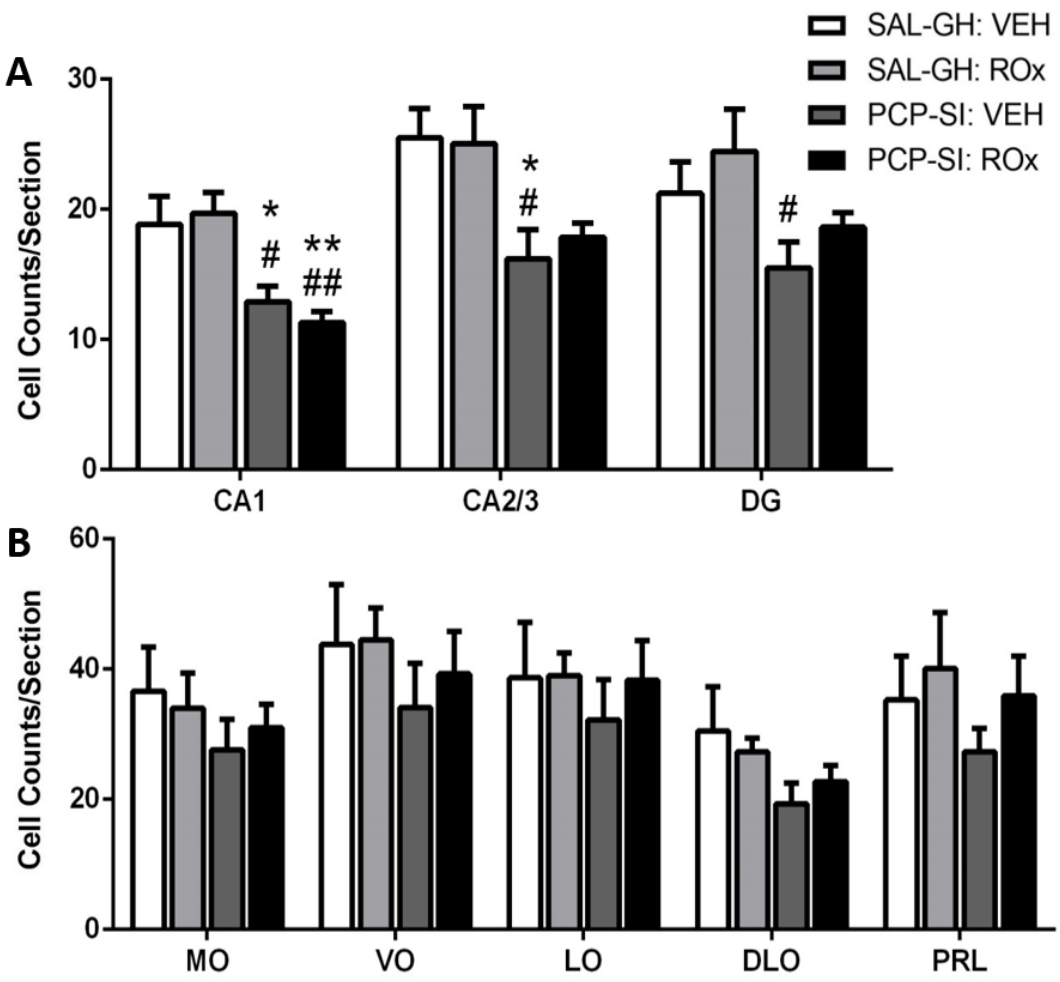


Figure 3.12: Mean cell counts for parvalbumin immunoreactivity in various brain sub-regions. Columns shown comparative differences (mean ± S.E.M.) of parvalbumin counts in the A; hippocampus and B; rostral PFC following a social interaction trial PND 81 in adult male Lister-hooded rats administered with either saline (SAL; 0.9% NaCl; 2ml/kg) or phencyclidine (PCP; 10mg/kg) s.c. PND 7, 9 and 11, followed by subsequent isolation (PCP-SI) or group-housing (SAL-GH) PND 23, before chronic treatment with vehicle (VEH; 4ml/kg) or GlyT$_1$ inhibitor RO4993850 (ROx; 3mg/kg) i.p. for 14 days PND 68-81. Treatment administered 30min prior to the trial and tissue collected for immunohistochemistry collected 90min post-injection. A; Two-way ANOVA of parvalbumin expression revealed a significant main effect of PCP/housing for all three hippocampal sub-regions; CA1 ($F_{(1,27)}$=23.86, p=0.0001), CA2/3 ($F_{(1,27)}$=13.84, p=0.0009) and Dentate Gyrus (DG; $F_{(1,27)}$=6.213, p=0.0191). However, there was no significant effect of treatment or PCP/housing x treatment interaction (p>0.05) for all hippocampal regions. B; Two-way ANOVA of parvalbumin expression revealed no significant effect of PCP/housing or treatment in any sub-region. *p<0.05, **p<0.01, compared to SAL-GH: VEH; $^{\#}$p<0.05, $^{\#\#}$p<0.01 compared to SAL-GH: ROx; Tukey post hoc. n=8 SAL-GH: VEH, n=8 SAL-GH: ROx; n=9 PCP-SI: VEH; n=8 PCP-SI: ROx. OFC; Orbitofrontal cortex, DLO; Dorsolateral OFC, LO; Lateral OFC, VO; ventral OFC, MO; medial OFC, PRL; prelimbic cortex.

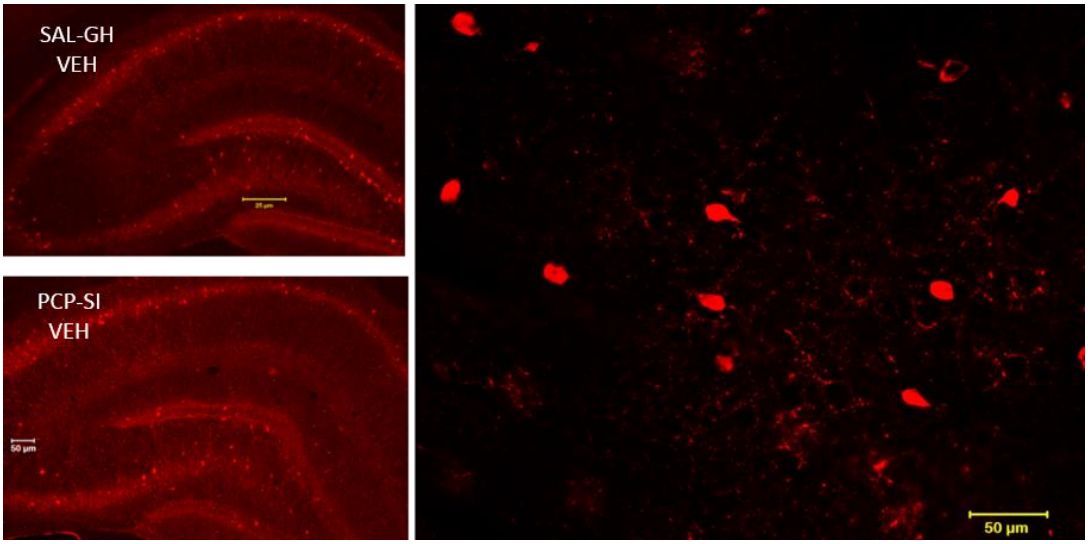


Figure 3.13: Parvalbumin staining of hippocampal sections. Example images of parvalbumin immunoreactivity (defined by cytoplasmic red stain) in the dorsal hippocampus. Whole hippocampal sections (left); scale bar: 25µm. Sample image (right): scale bar: 50µm, of zoom used to manually quantify parvalbumin count in the hippocampus and rostral PFC of SAL-GH and PCP-SI rats treated with saline vehicle or GlyT$_1$ inhibitor RO4993850 for 14 days.

3.6.7: Study 2:

Effects on dopamine overflow following an acute dose of $GlyT_1$ inhibitor RO4993850

In an attempt to establish a potential neuronal mechanism for the observed changes in social behaviour produced by RO4993850, microdialysis was used to measure changes in dopamine release in the PFC and striatum. This was done using the same rats as in Study 1, administering an acute systemic dose of $GlyT_1$ inhibitor RO4993850. Changes to serotonin levels (5-HT) could not be determined due to very high pmol/ml levels of the major metabolite 5-HIAA which merged with 5-HT to produce co-eluting peaks. Probe placement was determined post-microdialysis collection and example pictures of probe placement are seen in Figure 3.15. In some cases dopamine levels were below the detection limit, resulting in small sample sizes for some treatment groups. Changes to dopamine, DOPAC and HVA levels were calculated as a percentage change from basal and analysed by a two-way repeated measures ANOVA. Basal levels for dopamine and its metabolites are shown in Table 3.3.

3.6.7.1: PFC

There was no effect of PCP/housing ($F_{1, 14)}$=0.052, p=0.823) or $GlyT_1$ inhibitor treatment ($F_{(1, 14)}$=0.209, p=0.654) on dopamine overflow within the PFC. There was also no PCP/housing x treatment interaction ($F_{(1, 14)}$=0.029, p=0.868; Figure 3.14; A). Analysis of dopamine metabolites DOPAC and HVA also revealed no significant main effect of PCP/housing or treatment ($p > 0.05$) at any time point

throughout the study. There was also no PCP/housing x treatment interaction (data not shown). Therefore, it appears unlikely that acute systemic administration of the $GlyT_1$ inhibitor increases basal dopamine in the PFC within 3h in adult Lister-hooded rats.

3.6.7.2: Striatum

There was no effect of PCP/housing ($F_{1, 21)}$=0.249, p=0.623) or $GlyT_1$ inhibitor treatment ($F_{(1, 21)}$=0.035, p=0.853) on dopamine overflow within the striatum. There was however a PCP/housing x treatment interaction ($F_{(1, 21)}$=4.327, p=0.05; Figure 3.14; B). Analysis of dopamine metabolites DOPAC and HVA also revealed no significant main effect of PCP/housing or treatment (p>0.05) on pmol/ml concentrations at any time point throughout the study. There was also no PCP/housing x treatment interaction; data not shown. Therefore, it appears unlikely that acute systemic administration of the $GlyT_1$ inhibitor alters basal dopamine in the striatum within 3h in adult Lister-hooded rats.

Table 3.3: Basal Levels (pmol/ml) of dopamine and metabolites

PFC	SAL-GH		PCP-SI	
	VEH	ROx	VEH	ROx
Dopamine	0.75 ± 0.13	2.04 ± 0.26	1.26 ± 0.15	1.48 ± 0.33
DOPAC	186.76 ± 11.80	113.40 ± 8.46	80.47 ± 3.45	116.62 ± 8.25
HVA	169.75 ± 26.25	191.43 ± 33.56	103.26 ± 22.31	97.89 ± 3.18

Striatum	SAL-GH		PCP-SI	
	VEH	ROx	VEH	ROx
Dopamine	0.82 ± 0.34	3.13 ± 0.66	2.02 ± 0.30	1.11 ± 0.17
DOPAC	242.79 ± 9.60	218.40 ± 12.18	231.07 ± 6.88	235.68 ± 10.77
HVA	1002.81 ± 39.75	658.11 ± 48.48	885.70 ± 19.09	656.73 ± 34.86

Basal levels of dopamine and its metabolities in the PFC and striatam (pmol/ml; values as mean ± SEM) in SAL-GH and PCP-SI treated Lister-hooded rats following acute vehicle (VEH; 4ml/kg) or $GlyT_1$ inhibitor RO4993850 (ROx; 3mg/kg). Basal levels calculated from three baseline samples taken in 20min intervals over a 1h time period using HPLC-ECD.

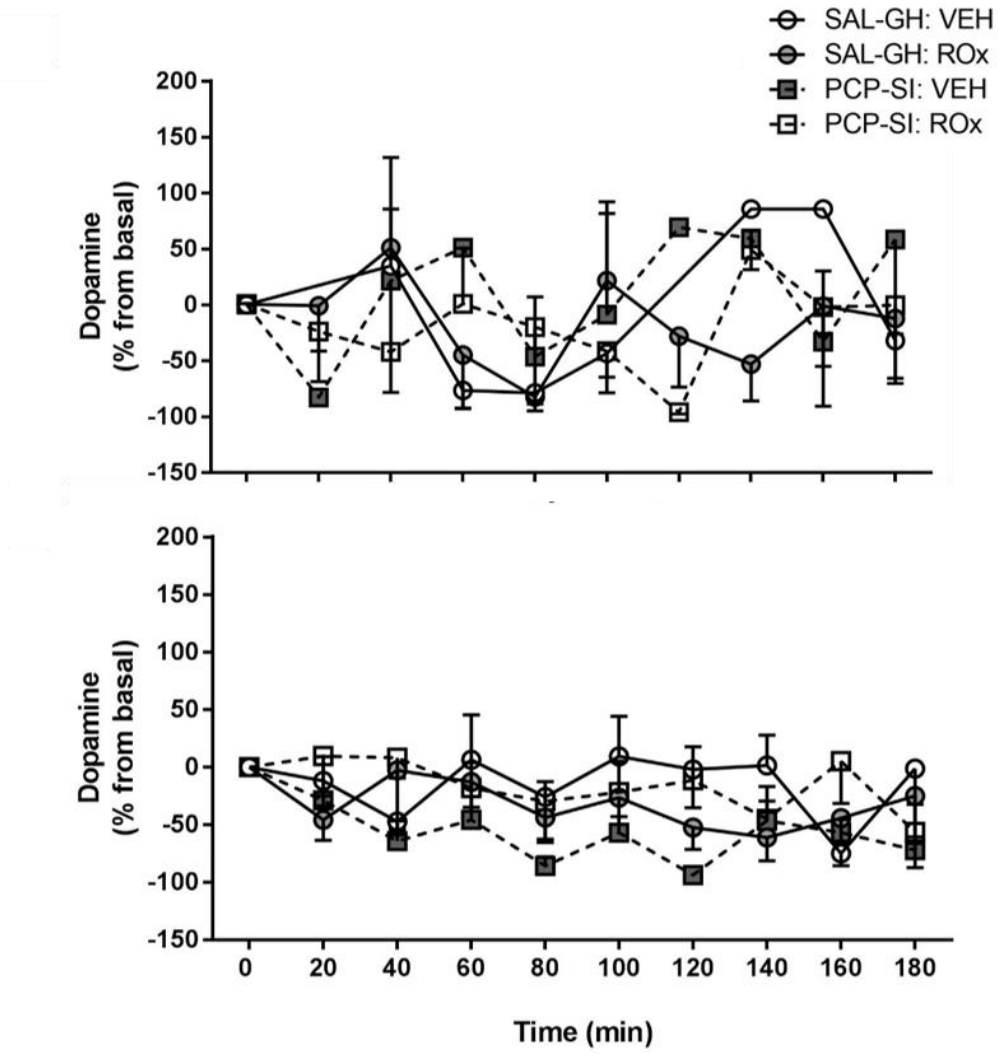


Figure 3.14: Changes to dopamine overflow in the PFC and striatum. Comparative differences (mean ± S.E.M., pmol/ml) on dopamine overflow in the A; PFC and B; striatum following microdialysis in freely-moving adult male Lister-hooded rats administered with either saline (SAL; 0.9% NaCl; 2ml/kg) or phencyclidine (PCP; 10mg/kg) s.c. PND 7, 9 and 11, followed by subsequent isolation (PCP-SI) or group-housing (SAL-GH) PND 23, before acute treatment with vehicle (VEH; 4ml/kg) or GlyT$_1$ inhibitor RO4993850 (ROx; 3mg/kg) i.p. 10 weeks later. Treatment administered following 1h basal collection. A; One-way repeated measures ANOVA of dopamine percentage change from basal revealed no significant differences between treatment groups ($p>0.05$). B; Two-way repeated measures ANOVA of dopamine percentage change from basal revealed no significant effect of PCP/housing, treatment or PCP/housing x treatment interaction ($p>0.05$). PFC; n=2 SAL-GH: VEH, n=5 SAL-GH: ROx; n=4 PCP-SI: VEH; n=5 PCP-SI: ROx. Striatum; n=8 SAL-GH: VEH, n=8 SAL-GH: ROx; n=5 PCP-SI: VEH; n=5 PCP-SI: ROx. NB. Some values missing at each time-point due to dopamine concentrations being below the HPLC detection limit. Where SEM bars are not shown, they lie within the symbol.

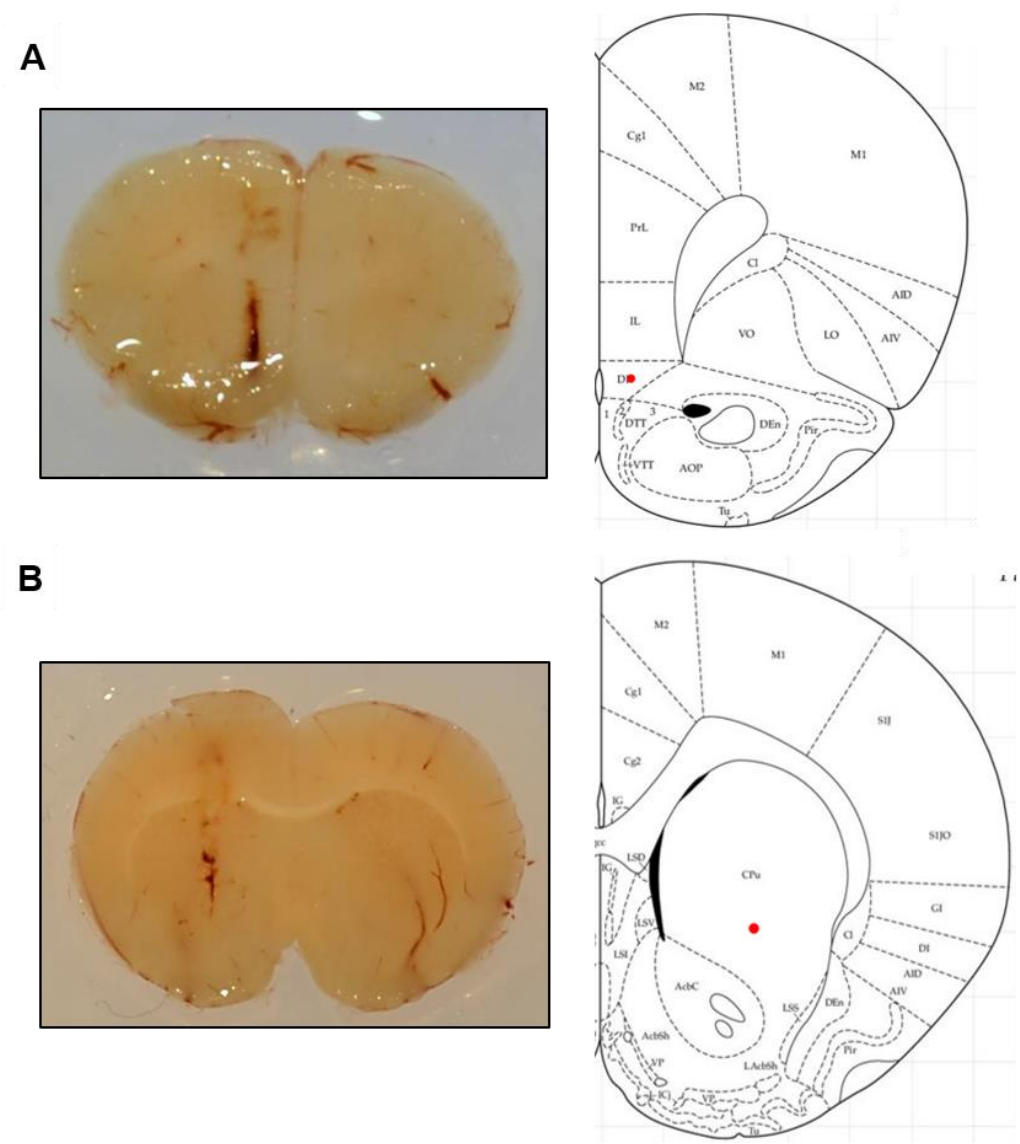


Figure 3.15: Probe placement for stereotaxic surgery for microdialysis. Example pictures of rat brain slices to confirm probe placement in the A; PFC (AP: +3.2, L: -0.7, V: -2.3) and B; Striatum (AP: +0.48, L: +2.5, V: -4.0) from Bregma, according to the rat brain atlas of Paxinos and Watson (1986).

3.7: Discussion

3.7.1: Study 1:

Part 1: Effects of the $GlyT_1$ inhibitor RO4993850 on behaviour

3.7.1.1: Novel-arena induced drug-free LMA

Total ambulatory activity

Neonatal-PCP followed by post-weaning social isolation for five weeks significantly increased LMA in a novel arena compared to saline-treated group-housed controls. This increase in activity (i.e. reduction in the rate of acclimatisation) is consistent with other findings using the "two-hit" neonatal-PCP and isolation model and is also seen with isolation alone (Gaskin et al., 2014, Watson et al., 2016). It is likely that this effect is primarily due to isolation-rearing as extensive evidence has shown this environmental manipulation to contribute to the onset of 'isolation syndrome' and consistently induces locomotor hyperactivity in Lister-hooded and Sprague-Dawley rat strains following 2-8 weeks social isolation from weaning (Bakshi and Geyer, 1999, Silva-Gomez et al., 2003, Hellemans et al., 2004, Fone and Porkess, 2008b, Ashby et al., 2010, Fabricius et al., 2011c, Jones et al., 2011a, Watson et al., 2012a). By contrast, neonatal-PCP fails to produce strong observable effects, or inconsistent effects on LMA. For example, Sprague-Dawley rats repeatedly administered PCP (10mg/kg s.c.) PND 7, 9 and 11 showed no change to baseline activity compared to controls when tested PND 42 (Boctor and Ferguson, 2010), nor did Lister-hooded rats treated with

repeated PCP (20mg/kg s.c.) on the same days post-birth when tested 8 weeks later (Kaalund et al., 2013b). Although repeated neonatal-PCP administration on PND 7, 9 and 11 can consistently cause locomotor sensitisation in rats following an acute challenge with PCP (Anastasio and Johnson, 2008a, Boctor and Ferguson, 2010). Additionally, increased LMA was reported in 4-week old Sprague-Dawley rats treated with PCP (10mg/kg s.c. administered consecutively PND 2-15), suggesting a longer dosing regimen might be necessary for neonatal-PCP alone to induce locomotor hyperactivity in a novel arena as opposed to a higher dose (Zhang et al., 2012). Additionally, administration of MK-801 (0.2mg/kg s.c.) PND 7, 9 and 11 combined with isolation-rearing PND 21, resulted in hyper-locomotion in adult male Sprague-Dawley rats tested PND 77 but not when MK-801 was administered alone (Lim et al., 2012), supporting this theory.

The enhanced LMA in a novel environment is thought to reflect mesolimbic dopaminergic hyperactivity in rodents. For instance, isolation-reared Lister-hooded rats have increased burst firing pattern activity in VTA dopaminergic neurons as well as increased dopamine overflow in response to 0.5mg/kg amphetamine, particularly within the NAc (Jones et al., 1990b, Fone and Porkess, 2008a). In humans, despite previous belief that hyperdopaminergic tone in the NAc/ventral striatum i.e. areas of the mesolimbic pathway were the major components relating to psychosis and therefore positive symptoms (Epstein et al., 1999), recent clinical data implicates a more complex dysfunction of dopaminergic signalling within the fronto-striatal-thalamic

circuit, as extensively reviewed in Dandash et al. (2017). In particular, the associative and sensorimotor striatum are implicated to play a larger role (Howes et al., 2009, Kegeles et al., 2010a, Egerton et al., 2013). As hyperactivity in rodents is believed to be primarily due to increased dopamine release in the NAc, these findings from neuroimaging studies do highlight a common problem with using animal models to decipher the complex circuitry disrupted within schizophrenia. However, as antipsychotics can attenuate locomotor hyperactivity in rodents and attenuate positive symptoms in patients (Fabricius et al., 2011b, Le Pen et al., 2011), the degree of predictive, if not construct, validity in using this behaviour as an assessment of positive symptoms in rodents retains some translational value.

Total rearing activity

Within this study PCP-SI rats also exhibited greater rears than SAL-GH controls. This is consistent with previous findings that isolation-reared Sprague-Dawley rats weaned on PND 21 had increased rears over 1h in a novel-arena compared to socially-reared or environmentally-enriched reared rats tested 8 weeks post-weaning (Varty et al., 2000). Although many studies fail to report effects on rearing activity in rodents, recent findings have speculated the involvement of the GABAergic system, in particular the $GABA_A$ receptor containing the α_1 subunit isoform. For example, it was shown by Alves et al (2005, 2012) that high-rearing adult male Wistar rats had a higher density of $GABA_A$ receptors within the hippocampus compared to low-rearing rats, suggesting a role for GABAergic inhibitory neurotransmission in modulating rearing activity.

Rearing activity is also thought to involve similar brain regions and signalling pathways as those responsible for mediating ambulation, in particular the NAc and VTA. For example, microinjection of NMDA receptor antagonist AP-5 (2-amino-5-phosphonovaleric acid; 0.05-1mg/ml) into the NAc core significantly reduced rears in an open field (Maldonado-Irizarry and Kelley, 1994) as did the administration of glutamate into the same region (Alvarez and Ruarte, 2001). Furthermore, VTA-lesions have been shown to decrease rears in an open field (Stinus et al., 1978) and local VTA infusions with a range of compounds including neurotensin, NMDA and D-Ala2-Met-enkephainamde (DALA) also modulate rearing (Kelley et al., 1980, Cador et al., 1985, 1988, Hillegaart et al., 2000, Ikemoto, 2004), suggesting that glutamatergic neurotransmission may play a role although the precise mechanisms mediating these effects remains unknown. Despite this, it has been suggested that the NAc may be the site of conflict between novelty-exploration and neophobia; a mildly aversive response to a novel environment leading to an inclination to escape (Lever et al., 2006).

3.7.1.2: LMA following acute treatment with $GlyT_1$ inhibitor RO4993850

Total ambulatory and rearing activity

LMA tested on PND 68 following an acute systemic dose of $GlyT_1$ inhibitor RO4993850 showed locomotor hyperactivity in PCP-SI rats to be decreased from the week prior, with no key difference found between all treatment groups. This is somewhat inconsistent to previous results with this compound

(McIntosh, 2014) and so there may be a role of habituation to the arena after the first exposure. Although chamber allocation was pseudo-random with no rat placed into the same chamber twice, the same experimental room was used. Hence the environment was not completely novel on repeated testing and this may account for the lack of difference in ambulatory activity following re-exposure to the arena. For example, some studies have reported that isolation-induced effects on PPI can be lost by repeated testing in Sprague-Dawley and Wistar rat strains (Domeney and Feldon, 1998, Bakshi and Geyer, 1999, Cilia et al., 2001, Krebs-Thomson et al., 2001), so it is possible that repeated testing of ambulatory activity attenuates responsivity. Additionally, Fone and Porkess (2008a) stated that elevated LMA is seen in up to 88% of adult male Lister-hooded cohorts following a 5 week isolation period and the repeated testing of LMA one week later may not be as robust unless another entirely novel arena is also used.

However, the total number of rears accrued over the 1h study maintained an overall effect of PCP/housing between PCP-SI rats and SAL-GH controls. The reason why the effects of PCP/housing would remain consistent on rearing but not overall ambulatory activity remains unclear, although it could be linked to different neurological mechanisms underlying these behaviours. Nevertheless, these findings suggest that neonatal-PCP isolation-rearing from weaning can induce behavioural changes that impact on LMA and induces hyperactivity in a novel environment.

3.7.1.3: Other $GlyT_1$ inhibitors and LMA

The effects of $GlyT_1$ inhibitors on locomotor hyperactivity are also inconsistent in the literature. For example, the $GlyT_1$ inhibitor SSR103800 reduced hyperactivity in juvenile male OFA rats (induced by PCP 4mg/kg i.p.) and in adult male Swiss mice following MK-801 (0.2mg/kg i.p. (Boulay et al., 2008)). Furthermore, the $GlyT_1$ inhibitor SSR504734 attenuated PCP (5mg/kg i.p.) and MK-801-induced (0.2mg/kg i.p.) hyperactivity in male C57BL/6 and Swiss mice respectively (Depoortere et al., 2005, Singer et al., 2009, Boulay et al., 2010). The mechanisms underlying the suppression of LMA is unclear but one possibility is $GlyT_1$ inhibitor-induced potentiation of NMDA receptors located on GABAergic neurons (which are responsible for controlling dopamine release from midbrain dopaminergic neurons projecting to the NAc (Alberati et al., 2012)). In contrast, $GlyT_1$ inhibitor VU0410120 failed to alter novel-arena induced locomotor hyperactivity in Balb/c mice, and TASP0315003 did not change novel-arena induced locomotor hyperactivity in adult male Sprague-Dawley rats (Burket et al., 2015, Chaki et al., 2015), consistent with results observed herein.

3.7.2: Social Interaction

3.7.2.1: Effects of neonatal-PCP isolation-rearing

The social interaction test is often used as a measure of social withdrawal/asociality (typical of negative symptoms) in rodents by observing their interactive behaviour when introduced to a novel conspecific, particularly

focussing on component behaviours including ano-genital sniffing, body sniffing, crawling over-and-under, following, lying side-by-side, pinning and boxing/biting. Typically, all of these (excluding boxing/biting) are classed as pro-social behaviours in adult rats (Palagi et al., 2016) and SAL-GH rats spent significantly more time engaging in these pro-social interactive behaviours than PCP-SI rats, suggesting that social interaction (and therefore arguably social withdrawal) between two conspecifics matched for housing conditions, treatment and weight can be influenced by exposure to early-life adversity.

Although the combination of these developmental manipulations appears to be sufficient to induce significant impairments in social interaction in the adult rat, it is unclear which of the two manipulations is primarily responsible. Isolation-rearing has shown conflicting results; it can increase and decrease the total time spent engaging in social interaction in both Wistar and Sprague-Dawley rat strains (Hol et al., 1999, Ferdman et al., 2007, Han et al., 2011, Moller et al., 2011), but the increase in aggressive behaviour is reproducible in other studies (Wongwitdecha and Marsden, 1996). Furthermore, isolation-rearing has been shown to alter neural activity in the PRL and IL cortical sub-regions of the medial PFC; areas thought to play a role in sociability (Minami et al., 2017).

With regards to neonatal-PCP there is very limited data available to make comparisons, although one study showed neonatal-PCP administered PND 7, 9 and 11 (10mg/kg s.c.) to have no apprarent influence on social interaction in adult female Sprague-Dawley rats (du Bois et al., 2008). PCP has been well

documented to induce deficits in social behaviour, as discussed in Chapter 1, section 1.9.1.2-3, although the long-term impact on behaviour appears to be dependent on rat strain, age, duration of PCP administration and the time of testing following cessation of treatment (Sams-Dodd, 2004, Lee et al., 2005, Egerton et al., 2008, Jenkins et al., 2008, Seillier and Giuffrida, 2009, Seillier et al., 2010, Metaxas et al., 2014). It could be the case therefore, that both manipulations (neonatal-PCP combined with isolation-rearing) work as an additive effect on each other or interact with each other to result in the significant social impairment observed in adult male rats. There is sparse data investigating the effects of combined 'two-hit' models on adult rat social behaviour, although deficits in social interaction have been observed in PCP-SI rats on at least two other occasions in our laboratory (McIntosh, 2014, Watson et al., 2016), suggesting this is a reproducible change in the Lister-hooded rat strain.

3.7.2.2: Effects of treatment with $GlyT_1$ inhibitor RO4993850

This social deficit in PCP-SI rats was ameliorated following chronic treatment with $GlyT_1$ inhibitor RO4993850, which increased the amount of time PCP-SI rats spent in pro-social behaviours. In contrast, SAL-GH rats showed no change in behaviour after RO4993850. Focussing on the individual behaviours scored, SAL-GH rats engaged more in ano-genital and less body sniffing behaviour, the reverse of which occurred in PCP-SI rats. Treatment with the $GlyT_1$ inhibitor RO4993850 did not appear to significantly influence ano-genital sniffing, but it did significantly increase body sniffing in PCP-SI rats.

The ability of the $GlyT_1$ inhibitor to reverse social deficits is consistent with previous studies administering a single acute systemic dose of this compound (McIntosh, 2014) and other published work on the effects of $GlyT_1$ inhibitors on social behaviour in rodents. For example, social interaction deficits induced by sub-chronic PCP (10mg/kg s.c. for 12 days followed by a two-day wash-out period) in adult male ICR mice were reversed following both acute and chronic (8 consecutive days) treatment with $GlyT_1$ inhibitor TASP0315003 (Chaki et al., 2015). Additionally, Balb/c mice administered $GlyT_1$ inhibitor VU0410120 spent more time in a social compartment containing another mouse compared to a non-social compartment (considered a test of sociality and potentially a desire to interact (Burket et al., 2015)). Each of these studies induced deficits in social interaction by chronic PCP administration in adult rats, or repeated neonatal-PCP treatment PND 7, 9 and 11, and so this study is unique in determining the ability of $GlyT_1$ inhibitors to ameliorate social deficits in a 'two-hit' model. Only a few other compounds have been tested in this model for their effects on social behaviour, including clozapine, which failed to improve social deficits (McIntosh, 2014), and cariprazine, which showed minor improvement to social behaviour (attenuating the body sniffing induced in isolation-reared rats (Watson et al., 2016)).

3.7.2.3: Effects on latency to social behaviour

Comparisons of the length of time taken for each rat pair to undergo social interaction revealed that treatment with the $GlyT_1$ inhibitor decreased the total time taken for PCP-SI rats to engage in any of the pro-social behaviours scored

in the trial, especially ano-genital sniffing. Interestingly, treatment with the compound had no obvious effect on SAL-GH rats, suggesting that a deficit in social behaviour must be present for the $GlyT_1$ inhibitor to mediate an effect. It is also important to note however, that the arena used in the social interaction trial was a novel environment for both rats. Therefore the time taken to engage in a specific behaviour may not only reflect patterns of activity, but potentially motivation to interact as there was competition between exploring the novel environment and the novel conspecific. It could also be possible that one rat from each pair was more willing to engage in pro-social behaviour than the other as interaction between both rats are co-dependent, although the nature of the experiment makes this difficult to determine. The total distance moved during the social interaction test is also considered to be an indicator of LMA, and whilst PCP-SI rats generally had slightly higher activity than SAL-GH controls, it did not reach significance.

3.7.3: USVs during social interaction

3.7.3.1: Effects of neonatal-PCP isolation-rearing

The number of USVs emitted by each rat pair were recorded during the social interaction trial as markers of communication and categorised into pro-social (50 kHz) and aversive (22 kHz) type calls. Overall, very few 22 kHz calls were emitted and these were predominantly recorded from PCP-SI vehicle-treated rats during periods of aggressive (boxing/biting) behaviour. Categorisation of the 50 kHz calls into flat, step and trill type calls based on their pattern analysis

revealed that PCP-SI rats emitted the highest numbers of all three USV subtypes, especially flat and trill type calls, compared to SAL-GH rats. A deeper analysis of differences in call parameters between treatment groups also revealed that the USVs emitted by PCP-SI rats were longer in duration and had a greater change in bandwidth compared to those recorded from SAL-GH pairs for all three call subtypes.

Currently very few studies have determined the effects of neonatal-PCP combined with isolation-rearing on USV emissions however, the increase in USVs could be primarily due to isolation-rearing. For example, adult male Sprague-Dawley rats isolated for 3 weeks emitted more calls, often longer in duration, when two novel conspecifics were placed into a novel arena (Hamed et al., 2009), consistent with results obtained herein. This alteration in interactive behaviour could be due to the lack of normal 'social skills' experienced between cage-mates during development, resulting in increased vocal communication instead of physical interaction following introduction to a novel conspecific. In contrast however, male Wistar rats isolated for 6 months elicited fewer 50 kHz calls in response to sexually receptive and non-receptive females (Inagaki et al., 2013), suggesting a differential vocal response following exposure to a female as opposed to male conspecific which is also impaired in isolated rats. In addition, isolation-rearing in the juvenile, but not post-adolescent period has been shown to reduce approach behaviour and LMA in male Wistar rats isolated PND 21 for 4 weeks in a 50 kHz radial arm maze play-back paradigm (Seffer et al., 2015), further highlighting how long-term isolation

could impair social function and communication in adult rats, potentially as a compensatory mechanism from lack of physical contact.

The effects of NMDA antagonists on USV emissions have been less well studied although MK-801 microinjections into the intrapreoptic-anterior hypothalamus were found to significantly decrease the number of USVs in adult rats (Brudzynski and Pniak, 2002), as did acute challenge with PCP (2mg/kg) in rats pre-treated with sub-chronic PCP (2mg/kg daily, 7 days (Swalve et al., 2016)). The significance of these changes in USV emissions and their acoustic parameters is largely unknown. The three main prevailing theories regarding the cause of USVs is as an affective expression (such as positive or negative emotional states), as a social signal and/or a physiological mechanism associated with movement (Portfors, 2007, Burgdorf et al., 2008, Wohr et al., 2008, Burke et al., 2017). For example, separated cage-mate rats show an increase in USVs, particularly flat-type calls, supporting the notion that USVs can be emitted to establish social contact (Wohr et al., 2008) and are not solely linked to experiencing positive affective states such as reward (Brenes and Schwarting, 2015, Avvisati et al., 2016).

Research over the past decade has aimed to increase our understanding behind different call subtypes i.e. flat and frequency-modulated calls. USVs can be characterised into 14 different categories based on their pattern analysis (Wright et al., 2010). This was done for the current study although several call subtypes rarely occurred so categorisation into flat, step and trill was deemed more informative. The few studies that have focussed on USVs during social

interaction examined calls emitted immediately before social contact, during social contact and just after to assess the USVs emitted during interaction itself. Interestingly, trill type calls were the most predominant (77%) in adult male Long-Evans rats, and USVs were emitted more frequently immediately prior to nape contact (Himmler et al., 2014). The emission of trill type calls herein therefore could be linked to pro-social interaction. However, in the study by Himmler et al. (2014), details of USV emissions outside of social contact are not included and so it is possible that trill type calls were also emitted outside of physical interaction. Although not practical to do within this study, one way to fully understand how social behaviour and USVs are interlinked would be to associate each USV emission with rat behaviour i.e. sniffing, following or exploratory/rearing behaviour to the surrounding environment.

Another factor to consider is the effects of coordinated calling between each rat pair, which may be necessary for successful physical contact to occur and thus facilitate social interaction. In primates, social play is more likely and lasts longer when two partners match each other's facial signals prior to contact (Davila Ross et al., 2008, Mancini et al., 2013). If PCP-SI rats have deficits in coordinated calling, it might contribute to increased USVs and reduced social interaction. However, this hypothesis remains untested until it can be determined which rat was responsible for each type of call emitted during the trial.

3.7.3.2: Effects of treatment with RO4993850

Chronic treatment with RO4993850 did not alter pro-social 50 kHz calls or their characteristics in SAL-GH rats, and the reduction in flat and trill call emissions in PCP-SI rats did not reach significance. Additionally, RO4993850 reduced the extent of bandwidth change for all call subtypes, as well as the duration of flat type calls in PCP-SI rats. Therefore the $GlyT_1$ inhibitor may reverse the preference of PCP-SI rats to engage in vocal rather than physical contact when introduced to a novel conspecific, resulting in fewer USV emissions but an increase in total interaction. Alternatively, RO4993850 might have restored effective coordinated calling which contributed to the increase in social interaction observed herein. The number of aversive 22 kHz USVs associated with boxing/biting behaviour was also reduced in PCP-SI rats since they expressed less aggressive behaviour. This is consistent with other studies where significant increases in 22 kHz emissions are observed following exposure to an aversive environment or stimulus, reviewed in (Wohr and Schwarting, 2013).

The ability of $GlyT_1$ inhibitors to alter USV emissions could involve the activation of inhibitory glycine GlyA receptors. For example, RO4993850 (unpublished in-house data at F. Hoffmann-La Roche), like $GlyT_1$ inhibitors SSR504734 (30mg/kg p.o.) and ALX5407 (1mg/kg p.o.) decreased maternal separation anxiety-induced USV emissions in PND 10 Sprague-Dawley rat pups, an effect which was ameliorated in the presence of GlyA receptor antagonist strychnine (Komatsu et al., 2015). The localisation of GlyA receptors to NMDA receptors,

combined with previous evidence indicating the functional role of the NR2B NMDA receptor subunit in mediating responses to play-induced hedonic 50 kHz USVs (Burgdorf et al., 2011), suggests the importance of targeting these receptors to restore communication-deficits in adult rats. This is further supported by evidence demonstrating peripheral administration of GLYX-13; a partial agonist for the NMDA receptor glycine site, to significantly increase the rate of hedonic (defined as step and trill) 50 kHz calls in adult male low-USV emitting Long-Evans rats, an effect which was attenuated by the NMDA receptor NR2B subunit antagonist ifenprodil (Burgdorf et al., 2011, Moskal et al., 2011).

Although the NMDA receptor is postulated to play a functional role in USV emissions, direct comparisons between these studies cannot be made without further work. The effects of maternal separation on rat pups and the placement of a single rat placed in an open field cannot be directly correlated with effects on a social interaction paradigm between two adult rats. Additionally, rat strain differences have been shown to influence USV emissions. For example, Wistar rats have been found to vocalise significantly more than Long-Evans rats when singly-tested in a home cage or novel arena (Wohr et al., 2008). As there is a lack of published work utilising Lister-hooded rats, there could be an underlying rat strain factor within this study and chronic treatment with the $GlyT_1$ inhibitor RO4993850 may result in more significant changes to USV emissions if tested in Long-Evans rats specifically bred for low vocalisation.

Part 2: Effects of the $GlyT_1$ inhibitor RO4993850 on cortical connectivity

3.7.4: C-fos immunoreactivity

Identification of neuronal activation (using c-fos immunoreactivity) showed minimal changes induced by PCP/housing, with small subtle effects observed in the rostral ventral OFC, dorsal striatum and NAc shell. The $GlyT_1$ inhibitor significantly decreased neuronal activation in the caudal PRL and IL cortices of both PCP-SI and SAL-GH rats, but only decreased neuronal activation in the rostral dorsolateral OFC, BMA and BLA brain regions in SAL-GH rats.

There was therefore some overlap in the sub-regions targeted by RO4993850, namely in the PRL and IL. Although c-fos activation in the home cage (chapter 2) indicated an increase in neuronal activation in these areas, there was a decrease following social interaction with a novel conspecific. Additionally, results from Chapter 2 indicated no concomitant c-fos immunoreactivity in the NAc, striatal or amygdala regions although a subtle potential effect was observed following social interaction in the BMA and BLA. This contrariety is not wholly surprising however as direct comparisons between the two studies cannot be made due to key differences. Although RO4993850 was administered equal time prior to transcardial tissue perfusion, the animals had vastly different housing conditions (drug-naïve vs neonatal-PCP isolation-reared rats), and c-fos production herein was influenced not only by the $GlyT_1$ inhibitor but also from placement into a novel arena with a novel conspecific,

as opposed to returning to the homecage. Therefore the differences in c-fos production within prefrontal areas observed herein and in Chapter 2 are most likely due to combined effects of the behaviour test and drug treatment, both of which can alter c-fos immunoreactivity alone. It is possible to compare c-fos neuronal activation with the results in Chapter 2 if additional groups of animals are used which receive chronic RO4993850 and no social interaction i.e. return to the homecage. This would determine whether $GlyT_1$ inhibitor administration shows a similar pattern of c-fos expression in PCP-SI rats.

Comparisons of changes to c-fos expression post-social interaction are varied. For example, adult male Sprague-Dawley rats isolated from weaning (PND 20) for four weeks showed reduced c-fos expression in the medial PFC when placed in an open field, whilst adult male Wistar rats treated with sub-chronic PCP (5mg/kg i.p. bi-daily for 7 days) showed no difference to c-fos expression compared to control vehicle-treated rats (Levine et al., 2007, Matricon et al., 2016). However, other studies using sub-chronic PCP treated rats have shown decreased c-fos in the IL, OFC, dorsomedial striatum and central amygdala and increased IL and OFC c-fos expression and decreased expression in the central amygdala of vehicle-treated rats (Matricon et al., 2016). C-fos immunoreactivity has also been shown to be significantly increased in the cingulate cortex, BLA and NAc shell and core sub-regions of both group-housed and isolation-reared Sprague-Dawley rats following a 15min social encounter with a novel conspecific, but not when placed alone in a novel arena (Ahern et al., 2016). Although another study using the same rat strain and isolation-

rearing protocol found increased c-fos immunoreactivity in the medial PFC (particularly the PRL and IL) in group-housed, but not isolated rats (Wall et al., 2012).

The effects of neonatal-PCP on c-fos expression have been less well studied, although chronic administration (PND 1-14) also revealed reduced c-fos mRNA expression in the NAc shell and VTA in adult rats following systemic methamphetamine administration PND 42 (Semba et al., 2001), indicating how both isolation-rearing and neonatal-PCP administration can result in long-lasting functional changes in the adult rat.

3.7.4.1: Brain region activation following social interaction

Although neuronal activation following social interaction does not appear to be specific to certain brain regions within this study, the OFC, IL and posterior central amygdala are implicated to play a role during this behavioural test. This is not surprising as these regions are considered key structures for motivation and decision-making (Bechara et al., 2000, Quirk and Beer, 2006, Balleine et al., 2007, Balleine et al., 2011, Schoenbaum et al., 2011). The ability of RO4993850 to reduce neuronal activation in the PRL and IL is expected as c-fos immunoreactivity is altered in these brain regions following social interaction. For example, adult male Sprague-Dawley rats isolated for 3 weeks showed increased c-fos expression in the PRL, IL and anterior cingulate cortex following social interaction with an aggressive rat (Goodell et al., 2017), although c-fos expression was decreased in these same regions following social interaction

with a normal rat (Ahern et al., 2016). Therefore, the reduced c-fos immunoreactivity in these brain regions following $GlyT_1$ inhibitor administration may be linked to the compound's ability to influence social behaviour, particularly within PCP-SI rats.

3.7.5: Parvalbumin immunoreactivity

Following social interaction, one rat from each pair was also used to determine parvalbumin immunoreactivity in the hippocampus and PFC. Neonatal-PCP isolation-rearing significantly reduced the number of parvalbumin expressing neurons selectively in the CA1, CA2/3 and DG regions of the hippocampus, but not in rostral PFC sub-regions. However, the $GlyT_1$ inhibitor did not influence parvalbumin expression in either brain region.

The 'two-hit' neurodevelopmental rat model used herein resulted in similar changes to parvalbumin expression as other rodent models using either isolation-rearing or neonatal-PCP although there are variations in the extent of parvalbumin reduction. For example, isolation-rearing of female Sprague-Dawley rats for 11 weeks decreased parvalbumin expression in the DG and CA2/3 hippocampal sub-regions (Harte et al., 2007b), while in male Sprague-Dawley rats isolated for 9 months, parvalbumin expression was reduced in the CA1 and CA2/3 but unaltered in the DG, PRL or OFC (Powell et al., 2015). By contrast, adult male Lister-hooded rats isolated for 8 weeks showed no change to parvalbumin immunoreactivity in either the medial PFC or hippocampus (Kaalund et al., 2013b).

Results from neonatal-PCP studies are also inconsistent; adult male Sprague-Dawley rats given PCP (10mg/kg PND 7) had reduced CA1 and ACC parvalbumin expression (Wang et al., 2008), whilst Lister-hooded rats given PCP (20mg/kg) PND 7, 9 and 11 had reduced parvalbumin expression in the medial PFC but not the CA or DG (Kaalund et al., 2013b). In other two-hit models, adult male Lister-hooded rats treated with MK-801 (1mg/kg, PND 7 and isolated for 8 weeks) showed significantly reduced IL cortical parvalbumin expression but no change in the hippocampus compared to controls (Gilabert-Juan et al., 2013). Therefore the extent of parvalbumin alteration in adult rats appears to be dependent on rat strain, gender, and most likely, the length of isolation and duration of PCP administration.

As parvalbumin expression has been shown to be decreased in hippocampal neurons in schizophrenia patients (Zhang and Reynolds, 2002, Konradi et al., 2011), the reduced expression observed within PCP-SI rats increases the translational validity of the 'two-hit' model as a model for schizophrenia. The reduction in parvalbumin expression is believed to be associated with impaired GABAergic neurotransmission, leading to reduced inhibitory control of excitatory pyramidal neurons. The implications for altered GABAergic neurotransmission is developed from observations that Glutamate Decarboxylase 1 (GAD_{67}); codes for an enzyme responsible for glutamate to GABA conversion, and GAT-1 mRNA levels are substantially reduced in post-mortem hippocampal and prefrontal tissue from schizophrenia patients (Hashimoto et al., 2003, Lewis et al., 2005), an effect which has since been

replicated in several rodent models for schizophrenia (Kinney et al., 2006, Wang et al., 2008, McKibben et al., 2010, Gilabert-Juan et al., 2012, Curley et al., 2013). Decreased parvalbumin mRNA also strongly correlates to the decreased density of GAD_{67} mRNA positive neurons in schizophrenia patients (Guidotti et al., 2000, Hashimoto et al., 2003, Benes et al., 2007). Although it was not possible within this study to observe immunohistochemical changes to GAD_{67} and its co-localisation on parvalbumin-positive interneurons (due to a lack of visible dual-stain on our tissue with currently available antibodies), microarray studies in the same 'two-hit' model (neonatal-PCP combined with isolation-rearing) have shown reduced expression of several hippocampal genes associated with GABAergic neurotransmission including SLC6A1; encodes GAT-1, and PVALB; encodes parvalbumin (Gaskin et al., 2016).

Studies on post-mortem tissue make it difficult to determine whether such cellular changes are a consequence of disease progression or a contributory factor. However the ability of environmental or drug-manipulations to induce these changes in animal models, particularly those with a developmental element, suggests they contribute to disease pathophysiology.

3.7.5.1: The potential influence of oxidative stress on parvalbumin-immunoreactivity

One of the ways in which parvalbumin immunoreactivity is thought to be altered in schizophrenia and in animal models for schizophrenia is through mitochondrial dysfunction and oxidative stress. The resultant defects in

GABAergic signalling may therefore lead to altered glutamatergic signalling, manifesting in strong behaviour deficits in adulthood (Kann et al., 2014).

Mitochondria are essential for a number of vital processes and in particular, energy production. The by-products from these bio-energetic reactions include the generation of reactive oxygen (ROS) or nitrogen (RNS) species such as superoxide and hydroxyl radicals, collectively known as oxidative stress. These toxic by-products are converted into less reactive molecules including hydrogen peroxide and water by a range of enzymatic and non-enzymatic anti-oxidants such as glutathione (GSH) and superoxide dismutase (SOD), classified as REDOX reactions. However, a loss of homeostatic balance either by an overproduction of ROS and/or a deficiency in anti-oxidant defence mechanisms can result in cell damage, particularly to lipids, proteins and DNA (Valko et al., 2007). This cellular damage is thought to contribute to the pathological changes observed in a number of psychiatric diseases, including schizophrenia, potentially at two key stages. Firstly, genetic or environmental risk factors such as hypoxia or infection could induce oxidative stress during foetal development and secondly, during brain maturation in adolescence e.g. synaptic pruning (Koga et al., 2016).

Clinical research has shown a number of endogenous anti-oxidants to be altered in schizophrenia patients compared to controls. For example, MRI studies have shown a 52% reduction in GSH levels in the medial PFC of drug-free schizophrenia patients (Do et al., 2000). Additionally, positive correlations have been found between GSH levels and executive function (Martinez-

Cengotitabengoa et al., 2012), extensively reviewed in (Smaga et al., 2015, Koga et al., 2016). One leading theory is that the altered parvalbumin expression observed within schizophrenia patients is due to REDOX dysregulation as these immature neurons require high energy levels during development and potentially have less robust anti-oxidant defence mechanisms than mature interneurons, making them more susceptible targets.

Therefore the altered parvalbumin expression observed within PCP-SI rats in the 'two-hit' model could be due to both neonatal-PCP and isolation-rearing inducing REDOX dysregulation. This is supported by studies determining changes to oxidative stress biomarkers in developmental animal models. For example, repeated neonatal-PCP (10mg/kg s.c.) PND 2, 6, 9 and 12 was shown to decrease GSH, SOD and lipid peroxidase levels in the frontal cortex and hippocampus of adult Wistar rats (Radonjic et al., 2010). Additionally, levels of PGC-1α (Peroxisome Proliferator-activated receptor gamma 1-alpha); a transcriptional co-activator that regulates genes involved in energy metabolism and one of the main regulators of mitochondrial biogenesis, were reduced in a NMDA receptor hypofunction mouse model of schizophrenia (Jiang et al., 2013b), reviewed in (Jiang et al., 2013a).

Study 2:

3.7.6: Effects of $GlyT_1$ inhibitor RO3994850 on monoamines

There were considerable difficulties in obtaining and analysing the microdialysis samples used within the study due to levels being close to the detection limit, and as such, sample sizes in each treatment group are quite small. Overall, SAL-GH and PCP-SI rats had similar basal PFC and striatal dopamine levels and acute systemic administration of the $GlyT_1$ inhibitor RO4993850 did not influence dopamine overflow in either brain region over the 3h sampling period in either PCP-SI or SAL-GH rats.

No previous studies have examined the impact of $GlyT_1$ inhibitors on increased dopamine overflow in the adult medial PFC. However, several antipsychotics (such as clozapine and olanzapine) increase dopamine in this brain region following acute systemic administration in adult male Sprague-Dawley rats (Tanda et al., 2015). By comparison, $GlyT_1$ inhibitors may act to increase dopaminergic neurotransmission, but through indirect mechanisms to compensate for hypo-functional glutamate neurotransmission (Lewis and Lieberman, 2000a, Abi-Dargham and Moore, 2003).

As there may be increased changes to dopamine overflow during particular behaviours, as opposed to overnight acclimatisation in an arena, it may be more informative to monitor changes to dopamine levels in the PFC and striatum following a specific activity such as social interaction. Although to do this the paradigm may need to be altered, such as using a nose-poke to indicate

the desire to interact (Gururajan et al., 2012), as access of the novel conspecific to the microdialysis probes and subsequent tubing may confound the study outcome. Nonetheless, the ability of the $GlyT_1$ inhibitor to attenuate social deficits in neonatal-PCP isolation-reared rats suggests the value of monitoring changes to dopamine overflow during social interaction to better understand how the compound mediates its effects. It is also worth noting that many studies comparing dopamine overflow in the NAc and medial PFC use Sprague-Dawley rats and aCSF containing 1.2-2.2mM $CaCl_2$; substantially higher than what was used within this study. As the presence of $CaCl_2$ can increase the extent of dopamine overflow a higher concentration may have increased basal dopamine levels, resulting in fewer samples being below the detection limit and allowing better observation of subtle changes to dopamine overflow in these regions which may have otherwise been missed.

3.8: Future Work

One limitation of this study is that in the clinical setting $GlyT_1$ inhibitors are likely to be used as an adjunctive therapy to antipsychotics, and so determining how co-administration of these compounds alters behaviour in the adult rat would be of interest. Additionally, one major concern is that the diagnosis and treatment for schizophrenia occurs too late in disease progression to significantly improve negative symptoms which often go unnoticed in high-risk groups until positive symptoms become more apparent. It may be useful therefore to determine $GlyT_1$ inhibitor-induced changes in juvenile rats, which

would also help to further validate the model as a way to predict the therapeutic potential of administering antipsychotics in high-risk individuals for developing schizophrenia.

It would also be of interest to explore how deficits to parvalbumin-positive interneurons occur. As discussed earlier, recent clinical research has implicated a potential role for mitochondrial dysfunction within schizophrenia (Clay et al., 2011, Hardingham and Do, 2016) which can result in oxidative stress. Analysis of how ROS and anti-oxidant defence pathways are altered within the 'two-hit' model would increase its face and construct validity. It would also allow further study as to the mechanisms which contribute to this homeostatic imbalance and how they can contribute to disease symptomatology. For example, it has been postulated that antipsychotics such as haloperidol and clozapine are able to alter and restore the imbalance to ROS production and anti-oxidant defence mechanisms in some animal models, as reviewed in (Smaga et al., 2015).

3.9: Summary

Therefore, to summarise, neonatal-PCP combined with isolation-rearing from weaning induced behavioural changes in adult Lister-hooded rats, particularly locomotor hyperactivity and social deficits including reduced social interaction and increased USV communication. The 'two-hit' model also selectively decreased parvalbumin expression in the hippocampus. Treatment with the $GlyT_1$ inhibitor RO4993850 showed no effect on locomotor hyperactivity, parvalbumin expression in either the PFC or hippocampus nor dopamine

overflow in the PFC or striatum following an acute systemic dose. However, chronic treatment improved social deficits in the social interaction test and altered both USV emissions and call characteristics.

Based on the findings of this study, the 'two-hit' model has some potential to identify novel therapeutics that have efficacy for the negative symptoms of schizophrenia. The strong behavioural differences in PCP-SI rats in terms of pro-social interaction and USV emissions also suggests this model could be used for other disorders manifesting social deficits, such as ASD although further work is yet required to fully validate this. The neuropeptide oxytocin is one such compound shown to influence social behaviours in both animals and humans, making it a suitable candidate for use within the two-hit model used herein. Therefore the next chapter will focus on the role of oxytocin in modulating social behaviours.

Chapter 4: Effects of oxytocin on social behaviours in drug-naïve rats

4.1: Introduction

4.1.1: Effects of oxytocin on social behaviour

Oxytocin is a nine amino acid neuropeptide hormone with a short half-life (10-12min) and synthesised in the PVN and SON of the hypothalamus (Neumann et al., 1993). Since discovering its ability to induce or facilitate uterine contractions in childbirth (Styles and Sullivan, 1962), further research has implicated a role in brain development and social behaviours including relationship formation and social interaction as discussed later in chapter 4 section 4.1.4.1. For example, 0.4µg oxytocin i.c.v. induced maternal grooming behaviour (pup-licking, nest-building and pup-retrieval) in female Sprague-Dawley rats (Pedersen and Prange, 1979), whilst administration of oxytocin antagonist d(CH2)5-8-ornithine-vasotocin i.c.v. delayed the response to pup-grooming in new Wistar rat mothers (van Leengoed et al., 1987). Furthermore, dams demonstrating higher levels of pup-grooming had increased oxytocin immunoreactivity in the medial pre-optic nucleus and parvocellular region of the PVN, as well as increased oxytocin-VTA projections compared to rat mothers with lower pup-grooming activity (Shahrokh et al., 2010).

The hypothesis is that oxytocin not only promotes social attachments, but also plays a role in maintaining and developing trust. For example, female Prairie voles (often used to study the formation of social relationships due to their

tendency to form monogamous relationships and social attachments, considered to have translational relevance to humans (Fisher et al., 2005)), were shown to exhibit partner preference behaviour, choosing to spend more time with a familiar than novel male following oxytocin (0.5ng/h i.c.v.). This behaviour was attenuated by administration of oxytocin receptor antagonist [1-D(CH2)5,-Tyr(ME)2,Thr4,Tyr-NH2(9)]ornithine vasotocin (Insel and Hulihan, 1995).

Oxytocin can also influence social behaviours in humans. For example, healthy male volunteers treated with 24IU oxytocin showed an increased tendency to cooperate as a group to receive financial gain, and in moral conflict situations displayed a decreased willingness to sacrifice targets within the group compared to outside members (De Dreu et al., 2010, De Dreu et al., 2011). In addition, 24-40IU oxytocin increased generosity, trusting behaviour and 'promoted optimism' in healthy human volunteers (Kosfeld et al., 2005, Zak et al., 2007). Although the exact mechanisms underlying trust, generosity, and other complex social behaviours are not entirely known, there is thought to be a link between these social traits and an individual's emotive state. However, such results are not always reproducible. For example, 32IU oxytocin was sufficient to promote trusting behaviour in an envelope task paradigm, but this effect failed to be replicated by the same group (Lane et al., 2015). Therefore it is possible that the effects of oxytocin may be both subtle and variable in healthy individuals and context related. Additionally, intranasal oxytocin may

not be the most effective and reliable route for facilitating oxytocin transport across the BBB, as discussed in Leng and Ludwig (2016).

4.1.2: Oxytocin: route of administration

Concern over poor oxytocin permeability across the BBB following systemic administration has been addressed by using intranasal administration in the clinic. By contrast, both intranasal and intracerebral (i.c.v.) oxytocin administration routes are used in animal studies, reviewed in (Feifel et al., 2016). There remains considerable debate however as to how effectively intranasal oxytocin penetrates the brain (Leng and Ludwig, 2016) and whether central and peripherally administered oxytocin can elicit similar responses in behaviour. For example, fMRI BOLD studies in rats have suggested that systemic oxytocin does not activate oxytocin receptor rich areas similar to that of i.c.v. administration (Ferris et al., 2015) and so it may modify neuronal activity through peripheral input. However, oxytocin levels were increased in microdialysates from the amygdala and hippocampus within 30-60min of subcutaneous injection in rats (Neumann et al., 2013).

A handful of studies have begun to explore the effects of oxytocin in plasma and aCSF following both peripheral and intranasal routes. For example, subcutaneous and intravenous administration of 5.0µg oxytocin into adult male Wistar rats elevated CSF levels after 10min, and up to 1h before these returned to baseline (Mens et al., 1983). Meanwhile, intranasal oxytocin in adult Wistar male rats (20µl/rat) and C57BL/6 mice (12µl/mouse) elevated

plasma and aCSF oxytocin levels in the dorsal hippocampus and amygdala, reaching peak concentrations 30-60min later (Neumann et al., 2013). The peripheral administration of oxytocin in mice at the same dose (i.p.) was shown to increase peak levels of oxytocin in both regions 30min after treatment indicating that although plasma levels of oxytocin increase within ~10min post-injection, it takes longer for peripherally administered oxytocin to cross the BBB (Neumann et al., 2013, Kent et al., 2016). The normal physiological concentration of circulating oxytocin in humans (and the rat) is ~1-10pg/ml, as cited by Leng and Ludwig (2016), and the considerably higher concentrations found in both plasma and aCSF following peripheral or intranasal administration suggests that at least some of oxytocin penetrates the BBB in these studies.

4.1.3: Oxytocin: mechanism of action

Oxytocin, following its production in the SON and PVN of the hypothalamus, is released via exocytosis in response to a wide range of physiological stimuli. Oxytocin binds primarily to its specific oxytocin receptor; a G-protein coupled receptor (GPCR) but has additional affinity for the arginine vasopressin (AVP) V_{1A} and V_{1B} receptors located centrally and peripherally, as well as renal V_2 receptors (Borthwick, 2010). Oxytocin receptors are coupled to G_q, G_i and G_o G-proteins, as are central V_{1A} receptors (Busnelli et al., 2012). Stimulation of either the oxytocin or V_{1A} receptor can therefore induce numerous cell signalling cascades to mediate subsequent central effects (Viero et al., 2010).

Overall, it is generally believed that the affinity and efficacy of agonists and antagonists for the oxytocin and vasopressin receptors (in particular the V_{1A} receptor) vary on the subtypes present in different species (Chini et al., 2008).

Oxytocin and its primary receptor are both relatively well conserved across species although some oxytocin peptide variations have been reported in New World monkeys (Wallis, 2012, Vargas-Pinilla et al., 2015). Ligand binding studies in the rat have highlighted the presence of oxytocin and V_{1A} receptors to have a complementary expression. In particular, strong oxytocin receptor expression is observed in the lateral central amygdala and NAc shell whilst V_{1A} receptors have been observed more strongly in the medial part of the central amygdala and NAc core (Veinante and Freund-Mercier, 1997), which may underlie the role of these neuropeptides in modulating the fear response, as discussed later in section 4.5.2.

It is generally accepted that oxytocin/AVP signalling contributes to the regulation of social-decision-making including aggressive, sexual, parental and salient behaviours in mammals, as reviewed in (Grinevich et al., 2016). As shown in Figure 4.1, rodents have high levels of oxytocin expression within the olfactory system which is unsurprising considering the use of olfactory inputs in this species in promoting social behaviours. A similar pattern is seen within primates, suggesting that oxytocin receptors act at the most relevant sensory modalities (e.g. visual attention) to regulate social approach and behaviour (Freeman et al., 2014, Grinevich et al., 2016) via different signalling pathways. For example, fMRI data has illustrated the parallels between the processing of

negative emotional stimuli and dorsolateral PFC activity (Herwig et al., 2007). Additionally, the intricate links between the HPA axis, oxytocin and 'emotional processing' is of key interest (reviewed in (Neumann, 2002, Phelps and LeDoux, 2005, Laviolette, 2007), particularly as the BLA is able to induce transient increases in dopamine in both the NAc and PFC in specific behavioural states (Rosenfeld et al., 2011).

The central and peripheral localisation of oxytocin receptors however, means the effects of oxytocin are not restricted to the CNS. In particular oxytocin can induce confounding cardiovascular effects including hypothermia and bradycardia in rats. Despite this, very few studies have examined effects of exogenous oxytocin on these parameters when observing behavioural responses. For example, high doses of oxytocin (2mg/kg i.p.) can cause sedation in Sprague-Dawley rats (Uvnas-Moberg et al., 1994). It has been proposed that the concomitant cardiovascular effects of oxytocin may be mediated through V_{1A} receptor activation in the rat. For example, both core body temperature and heart rate were reduced in adult Wistar rats following a high dose of oxytocin (1mg/kg, but not 0.01 or 0.1mg/kg i.p.). However, these effects were only ameliorated following co-administration with non-peptide V_{1A} receptor antagonist SR49059 and not by the oxytocin receptor antagonist C25 (Hicks et al., 2014). Oxytocin displays high affinity for both receptor types and its effects at the V_{1A} receptor may become apparent at higher doses, making dose selection in behavioural studies extremely important.

Despite this, it is not yet certain whether the effects on social behaviours are influenced more by vasopressin or oxytocin receptor activation. For example, both oxytocin (0.1-1mg/kg i.p.) and vasopressin (0.001-0.1mg/kg i.p.) decreased general investigation (body sniffing) and ano-genital sniffing between two novel conspecifics at the highest doses given in adult male Long Evans rats. Conversely in this study, lying side-by-side behaviour was increased following administration of 0.5mg/kg oxytocin and 0.005-0.01mg/kg AVP (Ramos et al., 2013). As body temperature was not recorded, it is possible that a resultant cardiovascular/hypothermic response contributed to the observed behavioural responses at the doses used. However, as both neuropeptides elicited changes to social behaviour within social interaction, it suggests that activation of oxytocin and/or AVP receptors can influence rodent behaviour. There may also be a crossover in downstream signalling effects (Ramos et al., 2013).

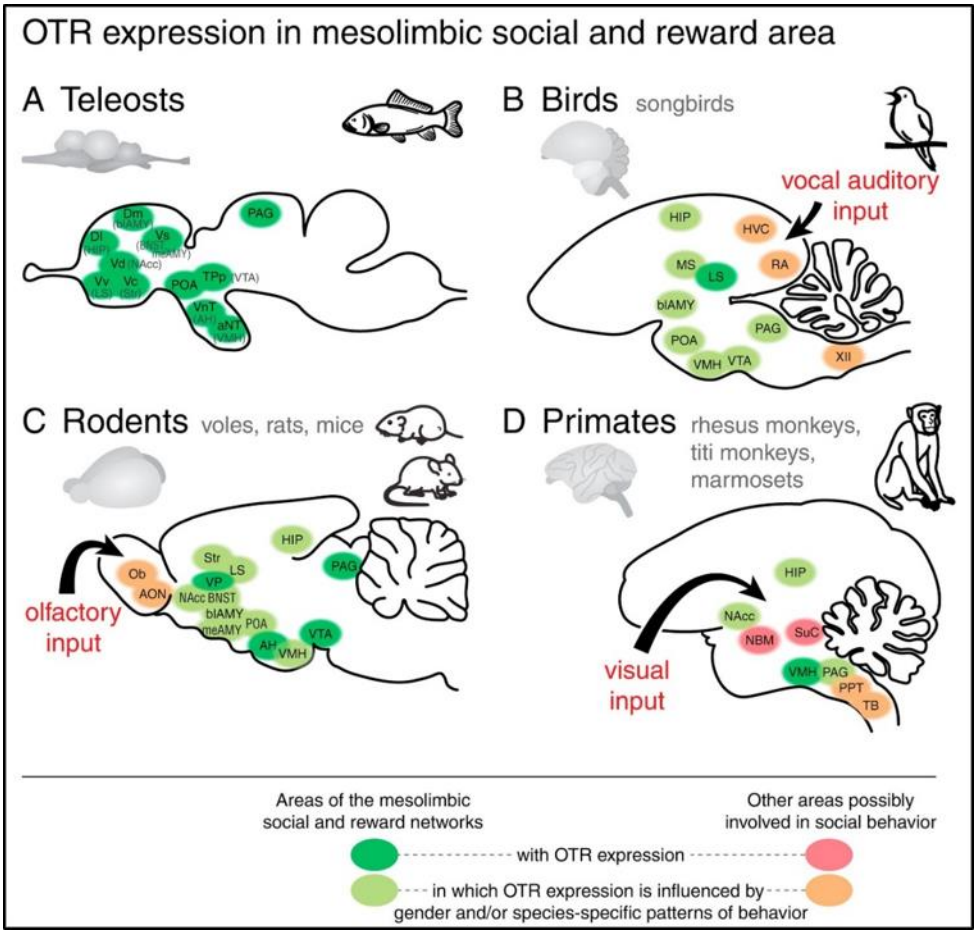


Figure 4.1: Oxytocin receptor expression in the social decision-making network across four vertebrate classes. Figure taken from (Grinevich et al., 2016), summarises oxytocin receptor expression for teleosts, songbirds, rodents and primates. Authors defined areas of the mesolimbic social decision making network to include: nucleus accumbens (NAcc); ventral pallidum (VP); lateral septum (LS); preoptic area (POA); ventromedial nucleus of hypothalamus (VMH); basolateral amygdala (blAMY); medial amygdala, (meAMY); bed nucleus of the stria terminalis (BNST); anterior hypothalamus (AH); hippocampus (HIP); ventral tegmental area (VTA); periaqueductal gray (PAG); and striatum (Str). Teleost areas of the network (A) include: anterior nucleus tuberal (aNT), lateral pallial (dorsal) part of the telencephalon (Dl); medial pallial (dorsal) part of the telencephalon (Dm); periventricular nucleus of the posterior tuberculum (TPp); central part of subpallial (ventral) telencephalon (Vc); dorsal nucleus of subpallial (ventral) telencephalon (Vd); ventral tuberal nucleus (VnT); supracommissural nucleus of subpallial (ventral) telencephalon (Vs); ventral part of subpallial (ventral) telencephalon (Vv). Other areas depicted to be involved in social behavior include: medial septum (MS); high vocal center (HVC); robust nucleus of the arcopallium (RA); and motor nucleus of the XII cranial nerve (XII) in panel (B); olfactory bulb (Ob) and anterior olfactory nucleus (AON) in panel (C); nucleus basalis of Meynert (NBM); superior colliculus (SuC); pedunculopontine tegmental nucleus (PPT); and trapezoid body (TB) in panel (D).

4.1.4: Therapeutic potential of oxytocin in neuropsychiatric disorders

The observed effects of oxytocin have highlighted its potential to restore social and communicative deficits in schizophrenia, as discussed in Chapter 1 section 1.8.5.1, but also other neuropsychiatric illnesses such as anxiety and mood disorders, or neurodevelopmental disorders including ASD. For example, in William's syndrome, where abnormal social behaviour is potentially due to the deletion of ~26-28 genes on chromosome 7q11.23, patients often display hyper-sociability such as over-trust towards strangers and decreased reactivity to negative facial expressions (Plesa Skwerer et al., 2009, Martens et al., 2012). Interestingly, William's syndrome patients are reported to have altered oxytocin and vasopressin signalling, including three times more oxytocin circulating in the blood (Dai et al., 2012) and significant overexpression of the oxytocin receptor gene *OXTR* (Haas and Smith, 2015).

4.1.4.1: Preclinical studies

The effects of acute and chronic oxytocin in various species including rats, mice, voles and rhesus monkeys (reviewed in (Feifel et al., 2016) on behavioural tasks associated with social and cognitive function such as the forced-swim test and social interaction supports the viability of oxytocin as a potential therapeutic agent. Chronic oxytocin (0.4µl/vole s.c. for 14 days) treatment of female Prairie voles significantly attenuated isolation-induced immobility in the forced swim test (Grippo et al., 2009). In addition, adult Groningen male rats administered

oxytocin (0.5µl/h for 7 days) show decreased offensive behaviours (lateral threat and upright posture) and increased social explorative behaviours (general investigation, ano-genital sniffing, crawling and social grooming) in a resident-intruder paradigm, even up to one week after cessation of treatment. The opposite was observed following administration of oxytocin receptor antagonist 'desGly-NH2(9),d(CH2)5[Tyr(Me)2,Thr4]OVT' (Calcagnoli et al., 2014).

Oxytocin also influences rodent behaviour in social interaction paradigms although results in these studies are dependent on strain, species, gender and the route of drug administration. For example, drug-naïve group-housed Wistar rats given oxytocin during adolescence (1mg/kg i.p. daily for 10 days PND 33-42) showed increased proximity to a novel conspecific and an increased number of social contacts (Bowen et al., 2011). Conversely, another study found 0.15IU intranasal oxytocin to facilitate social behaviours following an acute dose, but not following chronic treatment for 7, 15 or 21 days in wildtype C57BL/6J adult mice during male-female social interaction (Huang et al., 2014).

A few select studies have also explored the effects of oxytocin in models for schizophrenia. Adult male Sprague-Dawley rats administered PCP (3mg/kg i.p. for 14 days) displayed reduced social interaction, spending considerably less time in contact behaviours compared to saline controls, and had reduced oxytocin mRNA expression in the PVN. However a bilateral infusion of oxytocin at a high dose of 1000ng (but not 10 or 100ng) into the central nucleus of the amygdala increased the total time spent engaging in proximal close-contact

behaviours (Lee et al., 2005). In another study by the same research group, discrete injection of oxytocin into the amygdala PND 56 reversed social interaction deficits in prenatally-stressed (GD 14-22) adult male Sprague-Dawley rats (Lee et al., 2007). Effects have also been seen in mice lacking the oxytocin gene (*Oxt* -/-) where infusion of oxytocin (1ng/4µl CSF) into the medial amygdala ameliorated social recognition deficits, whilst administration of the oxytocin antagonist ornithine vasotocin (0.01ng/4µl CSF) into the same brain region reinstated these deficits (Ferguson et al., 2001).

4.1.4.2: Clinical studies

Based on preclinical work, clinical trials have evaluated the therapeutic efficacy of oxytocin to restore social deficits in psychiatric diseases including ASD, depression, obsessive-compulsive disorder, post-traumatic stress disorder and schizophrenia, as extensively reviewed in (Bakermans-Kranenburg and van, 2013, Rich and Caldwell, 2015, Shilling and Feifel, 2016). Despite the implicated relationship between oxytocinergic signalling and social behaviour in schizophrenia however, clinical studies have shown some inconsistencies, particularly for negative and cognitive symptoms. For example, plasma and CSF oxytocin levels have been negatively correlated with the severity of negative symptoms presented in patients (Sasayama et al., 2012, Modabbernia et al., 2013, Jobst et al., 2014) but Glovinsky et al. (1994) found no difference in CSF oxytocin levels between schizophrenia patients and healthy individuals. Additionally, increased plasma oxytocin levels have been associated with enhanced social cognition, including more pro-social behaviour, and appear to

be related to the menstrual cycle in females (Rubin et al., 2010, Pedersen et al., 2011, Walss-Bass et al., 2013). Meanwhile elevated plasma levels of oxytocin have been associated with increased positive symptom severity (Walss-Bass et al., 2013, Rubin et al., 2014). However in many of these studies, the schizophrenia patients tested were receiving antipsychotic medication which may have influenced the observed CSF/plasma levels of oxytocin. Oxytocin may also have differential effects in sub-group populations of schizophrenia such as individuals presenting with prominent negative symptoms or first-episode psychosis patients. Thus the exact role of oxytocin in schizophrenia aetiology and symptom severity remains unclear although this is likely due to several factors as the pharmacodynamics of intranasal oxytocin administration are not fully understood, and the modulation of social interaction and social behaviour in humans is highly context dependent (Bartz et al., 2011).

A number of studies have adjunctively administered exogenous oxytocin with antipsychotics to yield mixed results. Some studies reported improvements to negative (and sometimes positive) symptoms, whilst other studies reported no change to symptom severity, as extensively summarised in (Bakermans-Kranenburg and van, 2013, Shilling and Feifel, 2016, Bradley and Woolley, 2017). However, it is often difficult to directly compare such trials due to small sample sizes, different dosing/treatment schedules, different behavioural tasks/context, the effects of antipsychotics on dopaminergic signalling, general inter-individual variation e.g. oxytocin receptor variants, antipsychotic medication and its effects on the dopaminergic system, as well as the symptom

rating scale used within the trial (Bradley and Woolley, 2017). For example, intranasal oxytocin (40-80IU) has been shown to significantly decrease positive and negative symptoms scores on the PANSS and SANS subscales following 2-8 weeks treatment (Feifel et al., 2010, Modabbernia et al., 2013, Feifel et al., 2016) but not using the BPRS subscale (Lee et al., 2013). In fact, the PANSS and SANS subscales, although the most frequently used, are limited in their ability to encompass all negative symptoms and newer second-generation scales such as the Brief Negative Symptom Scale (BNSS) and the Clinical Assessment Interview for Negative Symptoms (CAINS) recently developed are not yet commonly used (Marder and Kirkpatrick, 2014, Garcia-Portilla et al., 2015). Therefore, oxytocin may enhance social behaviour and improve social interaction in schizophrenia patients but the optimal therapeutic dose, relationship between peripheral (plasma) and central oxytocin levels, and effectiveness of intranasal or peripheral administration on behaviour are yet to be addressed.

4.2: Aims

Based on oxytocin's potential as a therapeutic to modulate social behaviour, it would be interesting to determine its ability to restore deficits in neurodevelopmental models such as the 'two-hit' model used in chapter 3 (neonatal-PCP combined with isolation-rearing). However, few if any studies have reported the effect of oxytocin in the Lister-hooded rat strain. Therefore this study initially examined the effect of systemic oxytocin on LMA and social behaviour, as well as any confounding thermoregulatory effects. The overall aims of this study were to determine a dose of oxytocin following peripheral administration which was able to influence social behaviours, but not induce hypothermia or sedative effects in drug-naïve Lister-hooded rats.

The study was divided into two parts. Experiment 1 was a dose-response experiment monitoring the effects of oxytocin (0.03-0.3mg/kg s.c.) on body temperature and LMA. Experiment 2 involved acute administration of one selective dose of oxytocin (determined from experiment 1) to explore its effect on PCP-induced hyperactivity, social interaction and USV emissions. Additionally, microdialysis was used to measure dopamine overflow in the PFC and NAc following acute oxytocin to determine the potential role of the neuropeptide as a key player in the dopaminergic system.

4.3: Methods

4.3.1: Animals

Drug-naïve adult male Lister-hooded rats (Experiment 1: n=12; Experiment 2: n=32; Charles River UK), weighing 150-200g on arrival, were housed in groups of four in individually ventilated cages until surgery where they were then housed in individual metal-grid cages for the duration of the experiment. The holding room was maintained at a controlled temperature (21 ± 2^{o}C) and humidity (45 ± 15%), with a 12h light/dark cycle (lights on 06:00-18:00h). All experiments were performed in the light phase, with food (Beekay standard diet) and water available *ad libitum*. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986 and the ARRIVE guidelines (Kilkenny et al., 2010), with approval of AWERB.

4.3.2: Experiment Design

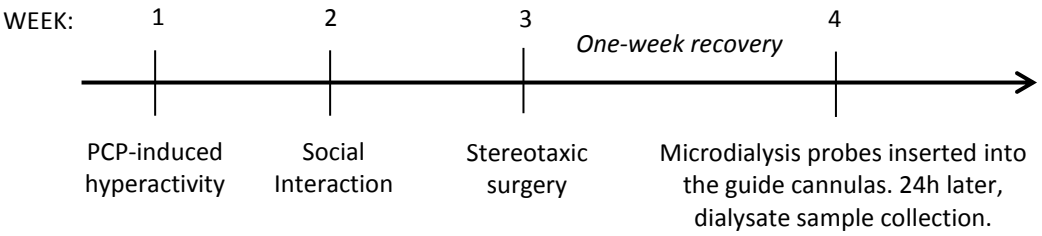
<u>Experiment 1: Oxytocin dose-response on LMA and body temperature.</u>

Each rat received s.c. oxytocin (0.03 or 0.3mg/kg) or saline (1ml/kg) in a random cross-over design such that every rat received all treatments across a three-week test period (n=12 injections at 7d intervals). A high-dose of oxytocin induced hypothermia in rats despite showing no effect on overall activity. As a result, a lower intermediate dose of 0.1mg/kg oxytocin was the highest dose used in all subsequent experiments.

To confirm that a dose of 0.1mg/kg did not induce hypothermia or hyper/hypo-activity in the Lister-hooded rat strain, the experiment was repeated by BBSRC DTP postgraduate student Adele Edwards and Professor Kevin Fone. Twelve rats received four treatments (oxytocin; 0.03, 0.1 and 0.3mg/kg and vehicle) in a random cross-over design across a four week period.

Experiment 2: Oxytocin, behaviour and oxytocin-dopamine interactions.

This study was split into two parts. In the first half, 32 experimentally naïve rats were treated with oxytocin to determine effects on body temperature, PCP-induced hyperactivity (4 treatment groups; n=8 per treatment group; PCP + 0.03mg/kg oxytocin, PCP + 0.1mg/kg, PCP + vehicle, vehicle + vehicle), as well as social interaction and USV emissions (2 treatment groups; n=16 rats per treatment group; n=8 trials per treatment group; oxytocin 0.1mg/kg or vehicle). In the second half of the experiment, one rat from each pair was used for stereotaxic surgery (7d after social interaction) and microdialysis probe implantation (7d post-surgery) to determine effects of oxytocin on dopamine overflow in the PFC and NAc.



4.3.3: Drugs

For all studies, oxytocin (0.03-0.3mg/kg; H2510-0025; Bachem) was dissolved in 0.154M saline vehicle and stored on ice until s.c. injection at 1ml/kg. PCP (5.6mg/kg; Sigma Aldrich UK) was dissolved in 0.154M saline vehicle and administered i.p. at 2ml/kg. Saline vehicle (0.9% w/v NaCl) was administered either s.c. or i.p. at 1 or 2ml/kg. Treatment groups were kept blind until data analysis was complete.

4.3.4: Measuring body temperature

Temperature microchips (Bio-Therma iDENTICHIP; AnimalCare Ltd) were implanted s.c. 48h before behavioural testing using sterile injectors. Body temperature was recorded at baseline and 15min intervals post-injection, both during LMA and microdialysis trials, using a digital chip reader with minimal disturbance to the animal. For social interaction, temperature was recorded at baseline (30min prior) and immediately before and after the trial. Body temperature data were analysed using two-way repeated measures ANOVA, with treatment and antagonist or PCP as between group factors and time as repeated measure, followed by multiple comparison post hoc tests where appropriate. Data are presented as mean ± SEM and $p<0.05$ considered significant.

4.3.5: Locomotor activity

LMA was assessed as described previously in chapter 3, section 3.3.1. LMA was recorded for 2h, after which animals were returned to their home cage. In experiment 1, for the dose response study, rats were habituated to the arena for 30min before injection with either oxytocin or vehicle. Activity was then recorded for a further 90min. In experiment 2, rats were again habituated to the arena for 30min before injection with oxytocin or vehicle, and received a second injection of PCP or vehicle 25min later. Activity and rearing were calculated as mean ± SEM for every consecutive 5min epoch during the experiment time course and analysed using one-way or two-way repeated measures ANOVA, followed with Sidak post-hoc analysis where appropriate. Statistical significance was set to $p<0.05$.

4.3.6: Social interaction

Social interaction was assessed as described previously in chapter 3, section 3.3.3. Twenty-four hours prior to the social interaction test, rats were isolated into individual cages to increase motivation to interact during the test. Rats from different litters (specifically requested from CRUK) were matched for weight (<30g to prevent dominance) and the treatment received during LMA one-week prior. Oxytocin 0.1mg/kg or saline vehicle 1ml/kg was administered 45min prior to the test. After the trial, rats were removed from the arena and returned to their home cage. Half the rats were then kept for stereotaxic surgery and microdialysis. Total time spent interacting was analysed by

Student's unpaired *t*-test and time spent interacting in individual behaviours was analysed via two-way ANOVA, with treatment and behavioural component as between group factors. Differences in specific behaviours was determined by appropriate post-hoc analysis.

4.3.7: USVs

USVs were recorded during social interaction and categorised by pattern analysis into flat, step and trill type calls as described previously in chapter 3, section 3.3.4., and as shown in Figure 3.8. The total number of calls for each category, as well as call parameters (changes in duration and call bandwidth) were $\log_{10}$ transformed prior to two-way ANOVA analysis for each subtype.

4.3.8: Stereotaxic surgery

Stereotaxic surgery was performed as described previously in chapter 3, section 3.5.1. CMA 12 guide cannulas (Linton Instrumentation, Diss, UK) were implanted relative to Bregma into the PFC (AP: +3.2, L: -0.7, V: -2.3) and NAc core (AP: +1.7, L: +1.6, V: -3.8); as outlined in the Paxinos and Watson rat brain atlas (1986). The guide cannulas, two copper-wire tethers and four skull-embedded stainless steel screws were cemented in place with dental acrylic.

4.3.9: Microdialysis probe implantation

Microdialysis probe implantation was the same as described in chapter 3, section 3.5.2 however the aCSF was altered to contain 125mM NaCl, 1.25mM

KCl, 0.5mM $MgCl_2$, 13.5mM $NaHCO_3$, 0.2mM NaH_2PO_4, 0.90mM Na_2HPO_4, 0.30mM Na_2SO_4 and 1.2mM $CaCl_2$.

4.3.10: Collection of dialysate samples

The collection of dialysate samples into vials, brain dissection and confirmation of probe placement were the same as described in chapter 3, section 3.5.2-3. Three baseline samples were collected at 20min intervals before injection with either oxytocin 0.1mg/kg s.c. or saline vehicle 1mg/ml. Samples continued to be collected in 20min intervals for a further 2h. The PFC samples collected from one rat were excluded from the study due to incorrect probe placement. PFC samples were also unable to be collected in two rats; one from each treatment group, although all samples were able to be collected in the NAc.

4.3.11: Analysis of monoamines

Microdialysis samples were analysed in the same way as described in chapter 3, section 3.5.4. Both dopamine and 5-HT were able to be measured along with their major metabolites. Due to low detection limits for dopamine, samples collected from the NAc region of one rat were unable to be included in the overall analysis; n=6/7 per treatment group in the PFC, and n=7/8 per treatment group in the NAc. Microdialysis data, presented as mean ± SEM, were analysed using two-way repeated measures ANOVA, with $p<0.05$ considered significant.

4.4: Results

Experiment 1: Oxytocin dose-response on LMA and body temperature

4.4.1: Effect of oxytocin on LMA

Over the 2h time course, rats became habituated to the arena and subsequently decreased their activity ($F_{(23,1012)}$=86.20, p<0.0001). Treatment with acute oxytocin (0.03, 0.1 or 0.3mg/kg) however, revealed no significant effect ($F_{(3, 44)}$=0.6133, p=0.6100; two-way repeated measures ANOVA). The overall ambulatory activity count over 90min (post-injection with oxytocin) also showed no significant main effect of treatment on LMA ($F_{(3, 44)}$=1.293, p=0.2888; one-way repeated measures ANOVA; Figure 4.2; A).

4.4.2: Effect of oxytocin on body temperature

Over the 2h time course, rat core body temperatures naturally decreased ($F_{(7, 308)}$=31.82, p<0.0001), but treatment with the highest dose of oxytocin (0.3mg/kg) significantly reduced body temperature compared to the lower oxytocin doses (0.03 and 0.1mg/kg) and saline control ($F_{(3, 44)}$=23.73, p<0.0001) groups. There was also a treatment x time interaction ($F_{(21, 308)}$=14.29, p<0.0001; two-way repeated measures ANOVA). Further analysis revealed the change in core body temperature to reach significance (p<0.05, 0.01 or 0.001) at the 15-90min post-injection time points; Tukey post-hoc (Figure 4.2; B).

Based on the findings from this study, 0.1mg/kg oxytocin was the highest dose used in the subsequent experiments (experiment 2) as this dose produced no observable hypothermia or LMA.

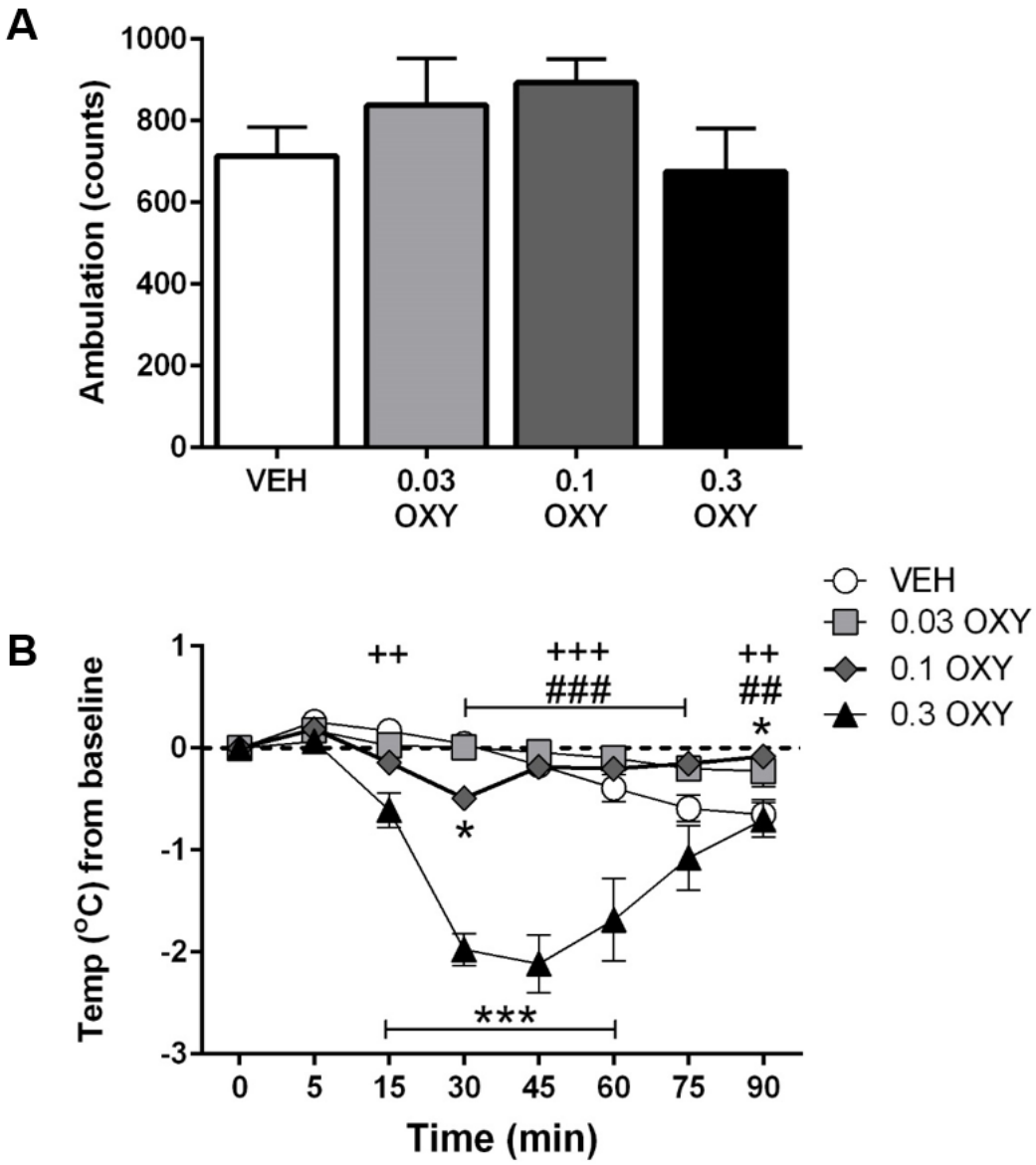


Figure 4.2: Effects of oxytocin on locomotor activity and core body temperature. Comparative effect of oxytocin (OXY, 0.03-0.3mg/kg; s.c.) and vehicle (VEH, 0.154M saline 1ml/kg) on the A; total locomotor activity (LMA, cumulative ambulatory counts every 5min, mean ± S.E.M., n=12) and B; change in body temperature (from basal recorded 5min prior to injection, mean ± S.E.M., °C) in adult Lister hooded rats placed in a computerised activity box. A; One-way ANOVA of total LMA revealed no significant main effect of treatment ($F_{(3,44)}$=1.293, p=0.2888). B; Two-way repeated measures ANOVA of the change in body temperature revealed both a significant effect of time ($F_{(7,308)}$=31.82, p<0.0001) and treatment ($F_{(3,44)}$=23.73, p<0.0001) together with a treatment x time interaction ($F_{(21,308)}$=14.29, p<0.0001). *p<0.05, ***p<0.001 VEH vs. 0.3 or 0.1mg/kg OXY; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 0.3mg/kg OXY vs. 0.1mg/kg OXY; $^{++}$p<0.01 0.3mg/kg OXY vs. 0.03mg/kg OXY; Tukey post-hoc.

Experiment 2: Oxytocin, behaviour and oxytocin-dopamine interactions

4.4.3: Effect of oxytocin on PCP-induced hyperactivity and temperature

Two-way repeated measures ANOVA of ambulatory activity over the 90min time course following injection with oxytocin or vehicle (Injection 1) revealed a significant main effect of time ($F_{(17, 476)}$=19.86, p<0.0001), treatment ($F_{(3, 28)}$=25.88, p<0.0001) and a treatment x time interaction ($F_{(51, 476)}$=3.428, p<0.0001; Figure 4.3; A). The overall ambulatory count of LMA following the second injection with PCP or saline (Injection 2) also revealed a significant main effect of treatment ($F_{(3, 28)}$=24.34, p<0.0001; one-way ANOVA). Further post-hoc analysis showed only the highest dose of oxytocin to significantly attenuate PCP-induced hyperactivity compared to vehicle-PCP treated rats (p<0.01; Tukey post-hoc; Figure 4.3; B).

Temperature measurements recorded during the 2h time-course revealed that rats had a general reduction in their overall core body temperature ($F_{(7, 189)}$=20.17, p<0.0001; two-way repeated measures ANOVA). However, there was no significant effect of oxytocin treatment at either dose; ($F_{(3, 27)}$=1.325, p=0.2868). There was also no treatment x time interaction ($F_{(21, 189)}$=0.6389, p=0.8860; Figure 4.3; C).

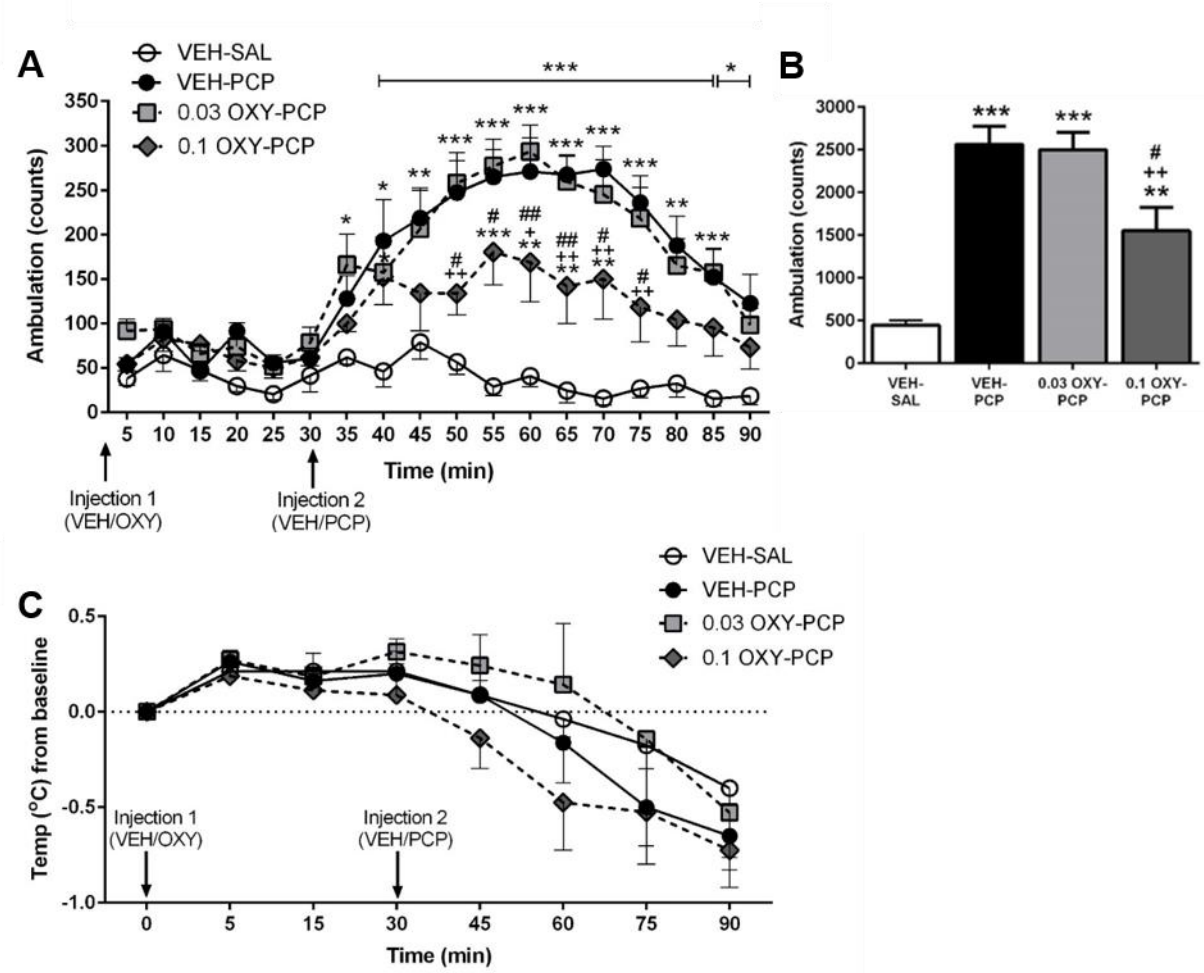


Figure 4.3: Effects of oxytocin on PCP-induced locomotor activity and core body temperature. Comparative effect of oxytocin (OXY, 0.03-0.1mg/kg; s.c) and vehicle (VEH, 0.154M saline 1ml/kg) on (mean + S.E.M.) the A; time course of phencyclidine- (PCP, 5.6mg/kg; i.p.) induced locomotor activity (LMA; cumulative counts/5min epoch, n=12), B; total ambulatory counts (following injection 2 with PCP/VEH), and C**;** change in body temperature (from basal, 5min prior to injection, ^{o}C) in adult Lister hooded rats placed in an activity box. A; Two-way repeated measures ANOVA of the LMA time course on all values recorded following injection with OXY/VEH over 90min revealed a significant main effect of time ($F_{(17, 476)}$=19.86, p<0.0001), treatment ($F_{(3, 28)}$=25.88, p<0.0001) and a treatment x time interaction ($F_{(51, 476)}$=3.428, p<0.0001). B; One-way ANOVA of total LMA following the second injection (PCP) revealed a significant main effect of treatment ($F_{(3, 28)}$=24.34, p<0.0001). C; Two-way repeated measures ANOVA showed a significant main effect of time ($F_{(7, 189)}$=20.17, p<0.0001) on core body temperature, but there was no significant effect of oxytocin at either dose ($F_{(3, 27)}$=1.325, p=0.2868) nor any treatment x time interaction ($F_{(21, 189)}$=0.6389, p=0.8860). *p<0.05, **p<0.01, ***p<0.001 compared to VEH-SAL; $^{+}$p<0.05, $^{++}$p<0.01 compared to VEH-PCP; $^{\#}$p<0.05, $^{\#\#}$p<0.01 compared to 0.03mg/kg OXY-PCP; Tukey post-hoc) VEH=saline vehicle, OXY=oxytocin; n=8 per group as indicated VEH-VEH; VEH-PCP; OXY 0.03mg/kg-PCP; OXY 0.1mg/kg-PCP.

4.4.4: Effect of oxytocin on social interaction in drug-naïve rats

Socially interactive behaviours were scored manually post-trial including ano-genital sniffing, body sniffing, crawling over-and-under, following, pinning, lying side-by-side, and boxing/biting. Comparison of vehicle and oxytocin-treated rats revealed a significant main effect of both treatment ($F_{(1, 98)}$=7.001, p=0.0095) and behavioural component scored ($F_{(6, 98)}$=123, p<0.0001). There was also a treatment x behaviour interaction ($F_{(6, 98)}$=3.241, p=0.006; two-way ANOVA). Further post-hoc analysis revealed that oxytocin-treated rats engaged in significantly more body sniffing (p=0.0160) and ano-genital sniffing (p=0.0008; Sidak post-hoc) compared to saline controls (Figure 4.4; A).

Treatment with oxytocin also significantly increased the total time spent in social interaction (all individual behaviours collated; (Mean=71.6 ± 4.3)), compared to those treated with saline vehicle (Mean=56.9 ± 4.1; $t_{(14)}$=2.446, p=0.0282; Student's unpaired *t*-test (Figure 4.4; B)).

Comparison of temperature changes from baseline, before and after social interaction revealed that core body temperature increased during the trial ($F_{(2, 60)}$=23.3, p<0.0001). However, treatment with oxytocin reduced the extent of body temperature increase ($F_{(1, 30)}$=5.011, p=0.0328). There was also a treatment x time interaction ($F_{(2, 60)}$=2.41, p=0.0985; two-way repeated measures ANOVA). Comparison of the temperature change from baseline between vehicle and oxytocin-treated rats was close to post-hoc significance before the social interaction trial (p=0.0954) and reached significance post-trial (p=0.0416; Sidak post-hoc (Figure 4.4; C).

Comparison of distance travelled during the social interaction also revealed no significant effect of oxytocin treatment (Mean=6806 ± 368.9) on general ambulatory activity during the social interaction trial compared to saline controls (Mean=6812 ± 451.5; $t_{(14)}$=0.009455, p=0.9926; Student's unpaired *t*-test; data not shown).

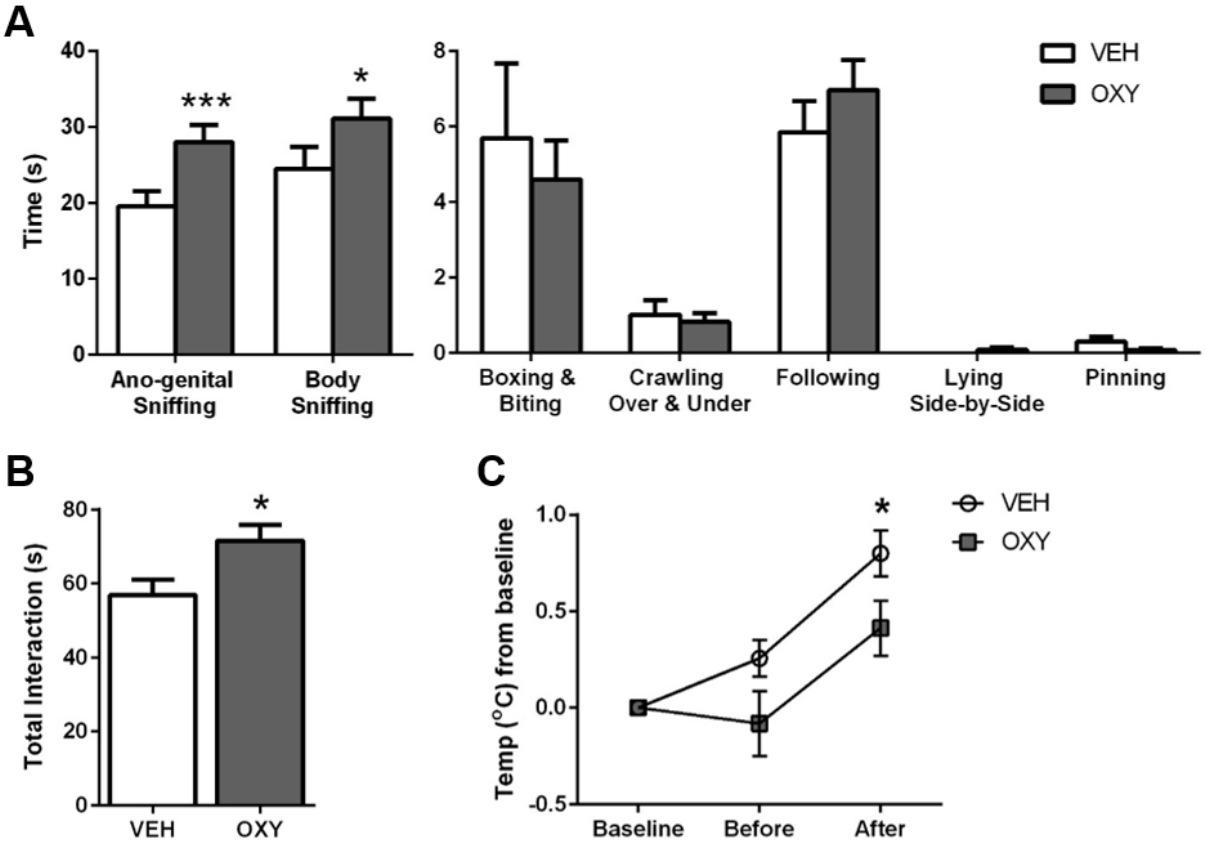


Figure 4.4: Effects of oxytocin on social interaction. Effect of oxytocin (OXY, 0.1mg/kg; s.c.) and vehicle (VEH, 0.154M saline 1ml/kg) on social interaction between two male Lister hooded rats from different litters paired by similar weight and the same previous drug treatment recorded over 10min. In order, the figures show A; individual behaviours (s), B; total time in social interaction (s) and C; body temperature change from baseline (immediately prior to injection), before (45min post-injection) and after the interaction trial (55min post-injection as indicated; ^{o}C). All data is presented as mean ± S.E.M. A; Two-way ANOVA of time spent in individual social behaviours showed a significant main effect of treatment ($F_{(1, 98)}$=7.001, p=0.0095), and behavioural component ($F_{(6, 98)}$=123, p<0.0001) together with a treatment x behaviour interaction ($F_{(6, 98)}$=3.241, p=0.006). B; *p=0.05; Student's unpaired *t*-test. C; Two-way repeated measures ANOVA of body temperature revealed a significant effect of time ($F_{(2, 60)}$=23.3, p<0.0001) treatment ($F_{(1, 30)}$=5.011, p=0.0328) and a significant treatment x time interaction ($F_{(2, 60)}$=2.41, p=0.0985). *p<0.05 ***p<0.001 VEH vs. OXY; Sidak post-hoc; VEH=saline vehicle, OXY=0.1mg/kg oxytocin; n=16 per group. NB. A different time scale is used to show ano-genital and body sniffing from other, less frequent, behaviours.In graph C, the error bars at baseline are shorter than the size of the symbol.

4.4.5: Effect of oxytocin on USVs

The number of pro-social 50 kHz USVs emitted from each rat pair during social interaction were defined into three subtypes; flat, step and trill, following Fast Fourier transformation and pattern analysis. Differences in call parameters were determined following a $\log_{10}$ transformation of the data.

Two-way ANOVA of total USVs revealed a significant main effect on the type of call ($F_{(2, 42)}$=18.75, p<0.0001) emitted, with more flat type calls emitted than any other call subtype. The number of USVs emitted were however unaltered by treatment ($F_{(1, 42)}$=0.8109, p=0.3730) for any of the call subtypes (Figure 4.5; A).

Analysis of USV duration between treatment groups and bandwidth showed a similar pattern. Two-way ANOVA of USV duration revealed a significant main effect on the type of call ($F_{(2, 28)}$=38.36, p<0.0001) emitted, with trill type calls being the longest in duration. There was however no significant effect of oxytocin treatment on the duration of any call subtype ($F_{(1, 14)}$=0.059, p=0.8202; Figure 4.5; B).

Two-way ANOVA of USV bandwidth (the change between minimum and maximum peak frequencies) once again revealed a significant main effect on the type of call ($F_{(2, 28)}$=53.72, p<0.0001) emitted, with step and trill type calls showing a greater change in call bandwidth than flat type calls, which was not influenced by oxytocin treatment ($F_{(1, 14)}$=0.1498, p=0.7045; Figure 4.5; C).

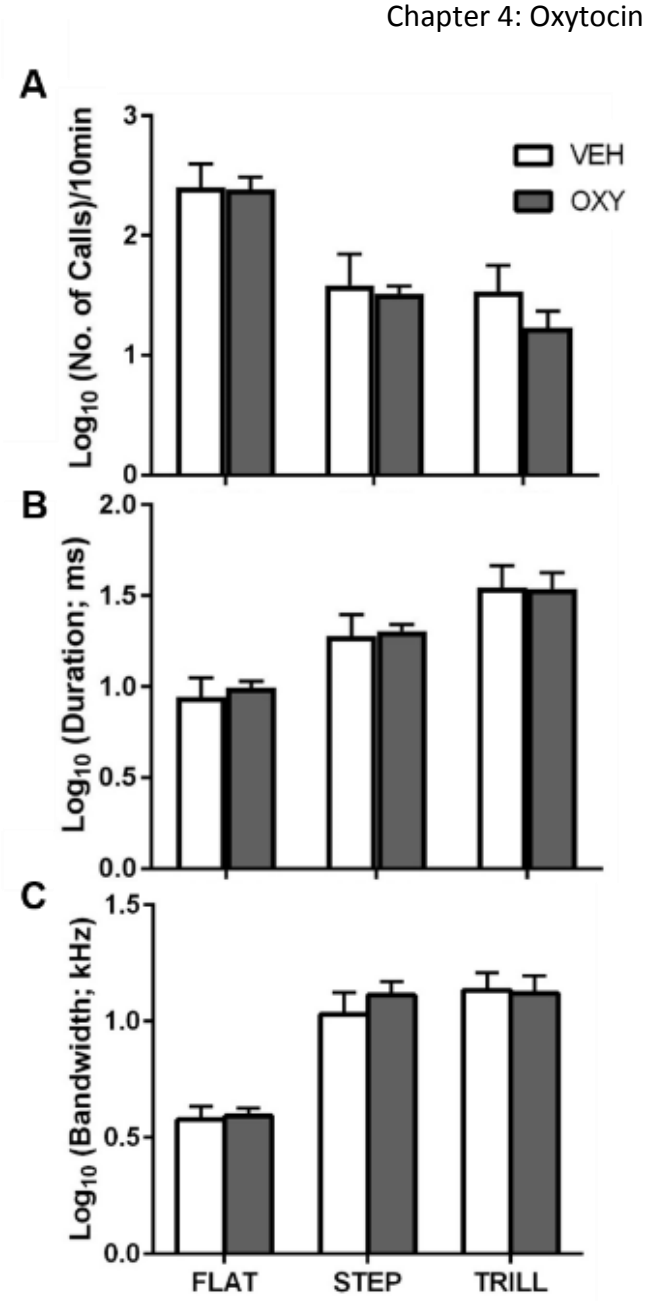


Figure 4.5: Effects of oxytocin on pro-social 50 kHz ultrasonic vocalisations. Effects of oxytocin (OXY, 0.1mg/kg; s.c.) and vehicle (VEH, 0.154M saline 1ml/kg) on pro-social 50 kHz ultrasonic vocalisations (USVs) emitted from rat pairs during social interaction between two male Lister hooded rats from different litters paired by similar weight and the same previous drug treatment recorded over 10min. USVs categorised into three subtypes; flat, step and trill, following Fast Fourier transformation and pattern analysis In order, the figures show A; the number of pro-social 50 kHz USVs emitted, B; the total duration (ms) and C; the change in call bandwidth (difference between minimum and maximum peak frequencies, kHz). All data is presented as mean ± S.E.M. A-C; Following log_{10} transformation, two-way ANOVA of total USV emissions, USV duration and USV change in bandwidth revealed no significant main effect of oxytocin treatment on any of the call subtypes ($p<0.05$). VEH=saline vehicle, OXY=0.1mg/kg oxytocin; n=16 per group.

4.4.6: Effect of oxytocin on monoamines

Monoamines, including dopamine and 5-HT, were determined in the PFC and NAc using HPLC-ECD. Baseline values were averaged and the percentage change in concentration from baseline were calculated for each compound of interest. Two-way repeated measures ANOVA of changes to dopamine from baseline in the PFC (0.0728 pmol/ml) and NAc (0.176 pmol/ml) over the 2h period revealed a significant main effect of treatment ($F_{(1, 12)}$=7.983, p=0.0153) within the NAc, and a trend towards significance in the PFC ($F_{(1, 11)}$=4.254, p=0.0634; Figure 4.6; A), indicating increased dopamine overflow following oxytocin administration.

In contrast, 5-HT levels were unaltered from baseline in the PFC (2.212 pmol/ml) and NAc (3.677 pmol/ml), revealing no significant main effect of oxytocin treatment in either the PFC ($F_{(1, 11)}$=0.06091, p=0.8096) or NAc ($F_{(1, 14)}$=0.08213, p=0.7786; Figure 4.6; B).

Core body temperatures decreased over the 2hr time course ($F_{(8, 104)}$=11.05, p<0.0001) but was unaltered by oxytocin treatment ($F_{(1, 13)}$=3.129, p=0.1003). There was however, a significant time x treatment interaction ($F_{(8, 104)}$=2.482, p=0.0167; two-way repeated measures ANOVA). Further post-hoc analysis also revealed a significant difference in temperatures between vehicle and oxytocin-treated rats at a single time-point (45min; p=0.0143; Sidak post-hoc; Figure 4.6; C).

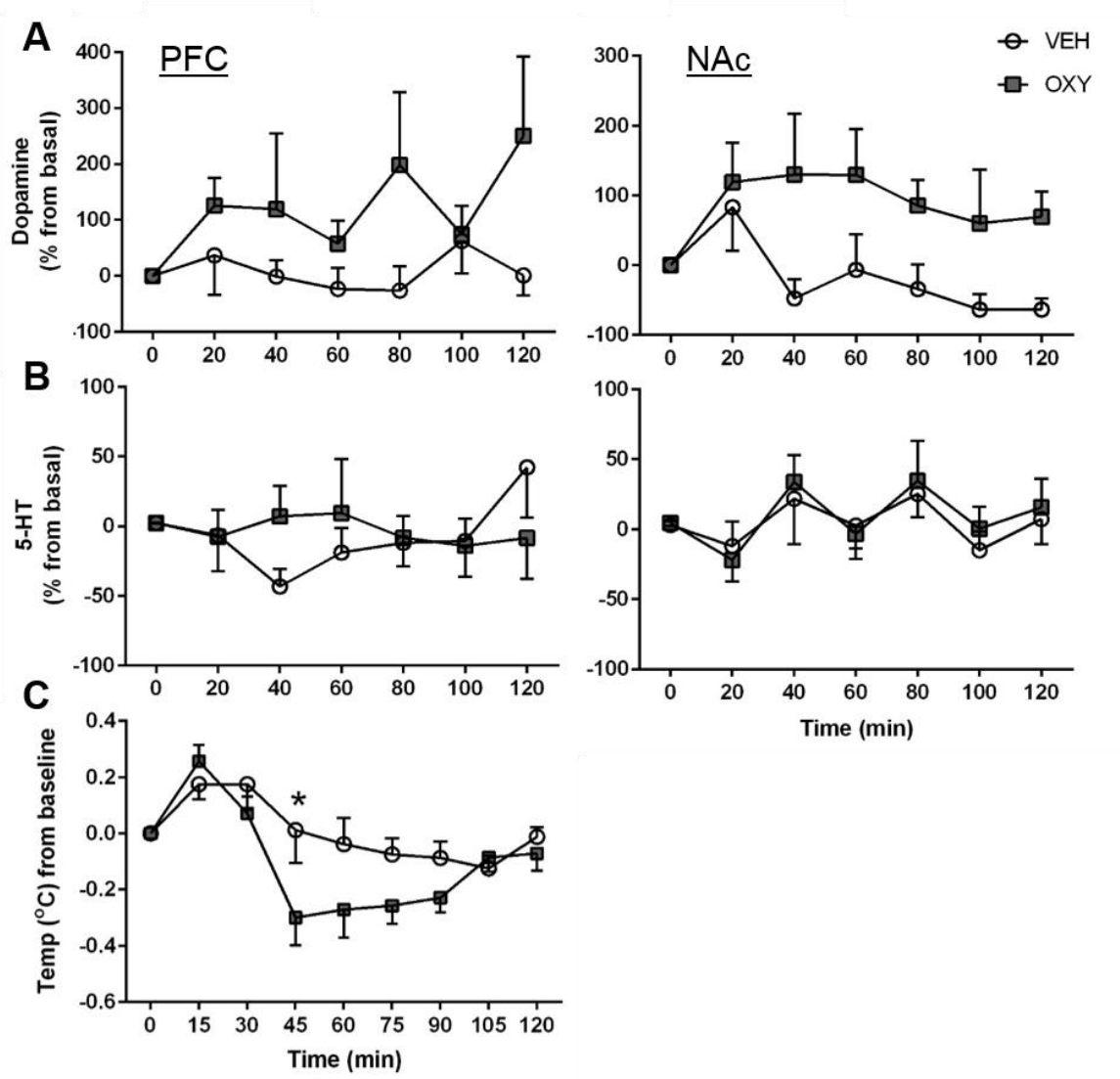


Figure 4.6: Effects of oxytocin on monoamines. Comparison of the effects of oxytocin (OXY, 0.1mg/kg; s.c.) or vehicle (VEH, 0.154M saline 1ml/kg) on A; dopamine and B; serotonin (5-HT) overflow (mean ± S.E.M., pmol/ml) in the prefrontal cortex (PFC) and nucleus accumbens (NAc) measured by microdialysis, and concomitant C; core body temperature (mean ± S.E.M.; °C) in freely-moving rats. A; Two-way repeated measures ANOVA of changes to dopamine from baseline in the PFC (0.0728 pmol/ml) and NAc (0.176 pmol/ml) over the 2h period revealed a significant main effect of treatment ($F_{(1, 12)}$=7.983, p=0.0153) within the NAc, and a trend towards significance in the PFC ($F_{(1, 11)}$=4.254, p=0.0634). B; In contrast 5-HT levels were unaltered from baseline in the PFC (2.212 pmol/ml) and NAc (3.677 pmol/ml) such that the ANOVA found no main effect of treatment in either the PFC ($F_{(1, 11)}$=0.06091, p=0.8096) or NAc ($F_{(1, 14)}$=0.08213, p=0.7786). PFC: Dopamine: n=7 VEH, n=6 OXY, 5-HT: n=7 VEH, n=6 OXY; NAc: Dopamine: n=7 VEH, n=7 OXY, 5-HT: n=8 VEH, n=8 OXY; VEH=saline vehicle, OXY=0.1mg/kg oxytocin; PFC; Prefrontal Cortex, NAc; Nucleus Accumbens. C; Core body temperature decreased with time ($F_{(8, 104)}$=11.05, p<0.0001; two-way repeated measures ANOVA), but was unaltered by treatment ($F_{(1, 13)}$=3.129, p=0.1003). There was however, a significant time x treatment interaction ($F_{(8, 104)}$=2.482, p=0.0167). *p<0.05 VEH vs OXY; Sidak post hoc. VEH=saline vehicle, OXY=0.1mg/kg oxytocin; n=8 per treatment group; 2 treatment groups.

4.5: Discussion

Experiment 1: Oxytocin dose-response on LMA and body temperature

In an open field, the highest dose (0.3mg/kg) of oxytocin significantly decreased core body temperature in Lister-hooded rats, despite having no significant effect on overall activity. By comparison, a lower dose of 0.03mg/kg or 0.1mg/kg showed little change to core body temperature or LMA.

Based on ligand binding studies *in vitro,* oxytocin exhibits greater selectivity for its own receptor, binding to the AVP receptor with approximately 100-fold less affinity (Mouillac et al., 1995, Manning et al., 2012, Stoop, 2012). Therefore the observed hypothermic response following high dose (0.3mg/kg) administration suggests the dose is high enough to start activating V_{1A} receptors, resulting in a subsequent decrease in core body temperature. This proposal is supported in a subsequent antagonist study completed in our laboratory by BBSRC DTP student Stuart Williams and Professor Kevin Fone, where administration of V_{1A} receptor antagonist SR49059; (2S)-1-[[(2R,3S)-5-Chloro-3-(2-chlorophenyl)-1-[(3,4-dimethoxyphenyl)sulfonyl]-2,3-dihydro-3-hydroxy-1H-indol-2-yl]carbon-yl]-2-pyrrolidinecarboxamide (1mg/kg i.p.), but not oxytocin receptor antagonist (L-368,899, 2mg/kg i.p.), attenuated the hypothermic response following oxytocin (0.3mg/kg s.c.) administration, similar to findings by (Hicks et al., 2014). However, neither antagonist reversed the modest decrease in ambulatory activity observed in the oxytocin-treated rats (data not shown),

suggesting the hypoactivity to occur via a different pathway. For example, it could be mediated by oxytocin acting at distinct receptors, such as the V_{1B} for which it has modest affinity, or allosteric modulation of dopamine binding at oxytocin-D_2 dopamine receptor heterodimers (Romero-Fernandez et al., 2013).

Some studies have reported low doses of oxytocin to increase locomotion. For example, oxytocin (0.05mg/kg i.p.) increased LMA in adult male Wistar rats in an open field (Klenerova et al., 2009). However, only higher doses of oxytocin (0.25-1mg/kg but not 0.06mg/kg s.c.) reduced LMA in adult male Sprague-Dawley rats (Uvnas-Moberg et al., 1994), consistent with results presented herein. A potential discrepancy between these studies may be due to lack of habituation to the arena before injection, as rats within our study and (Uvnas-Moberg et al., 1994) received prior habituation before recording the locomotor effects of oxytocin, and so the arena was no longer completely novel by the time of injection. Despite this, the antagonist study supports findings that higher doses of oxytocin have 'sedative-like' effects in rats. For example, significant decreases in activity were seen in Sprague-Dawley rats at a high dose of 2mg/kg i.p. (Angioni et al., 2016).

Experiment 2: Oxytocin, behaviour and oxytocin-dopamine interactions

4.5.1: Effect of oxytocin on PCP-induced hyperactivity and temperature

Similar to findings in the dose response study (experiment 1) oxytocin (0.1mg/kg s.c.) did not alter core body temperature. In contrast, this dose significantly attenuated PCP-induced hyperactivity, although a lower dose of 0.03mg/kg had no effect. No previous study has examined the effect of oxytocin on PCP-induced activity. However, oxytocin (2mg/kg i.p. or 5µg s.c.) has been shown to decrease methamphetamine-induced hyperactivity in both rats and mice (Qi et al., 2008, Carson et al., 2010) and cocaine-induced hyperactivity in mice (Kovacs et al., 1990). These two stimulants are known to enhance dopamine overflow, particularly within the striatum and NAc (Rothman et al., 1989, Mansour et al., 1992, Mele et al., 1997, Whitton, 1997, Fisher et al., 1998, Kalinichev et al., 2008) which contributes to the hyperactivity seen.

As studies have shown the ability of oxytocin infusion in the VTA, ventral subiculum of the hippocampus and amygdala to elicit dopamine release (Shahrokh et al., 2010, Love, 2014), it is perhaps contradictory that oxytocin can attenuate PCP-induced hyperactivity given that both PCP and oxytocin enhance dopamine release, particularly within the NAc. On the other hand, acute PCP-induced hyper-locomotion is mainly due to glutamate rather than

dopamine release in NAc (Takahata and Moghaddam, 2003), suggesting a potential impact of oxytocin on glutamate release. Interestingly in transgenic OTR-Cre mice, approximately half of the oxytocin receptor expressing cells in the VTA were shown to be glutamatergic neurons while less than 10% were tyrosine hydroxylase dopaminergic neurons (Peris et al., 2017), thereby suggesting a potential role for glutamatergic signalling. Thus it may be that dopamine release to the NAc is due to glutamate release, which in turn activates dopamine neurons in the VTA (see additional discussion in section 4.5.2)**.** Furthermore, in the substantia nigra (SN), oxytocin receptors were suggested to be mainly expressed on glutamatergic terminals which arise from the subthalamic nucleus (Angioni et al., 2016). Stimulation of the oxytocin receptors would result in increased glutamate release to GABA neurons in the SN pars reticula and internal Global Pallidum (GPi), and ultimately lead to increased GABA release to the thalamus/cortex, decreasing locomotion and thus reversing the effects of PCP-induced hyperactivity, as hypothesised in Figure 4.7 (Angioni et al., 2016).

By contrast, the serotonergic system is unlikely to contribute the attenuation of PCP-induced hyperactivity. PCP can markedly elevate 5-HT levels within the NAc, and antagonism of the 5-HT_{2A} receptor has been shown to attenuate PCP-induced hyperactivity (Millan et al., 1999). However, as oxytocin did not alter 5-HT release within the NAc or PFC in the current microdialysis study (as discussed later), it is unlikely that direct modulation of serotonergic function contributes to the attenuation of PCP-hyperactivity observed herein.

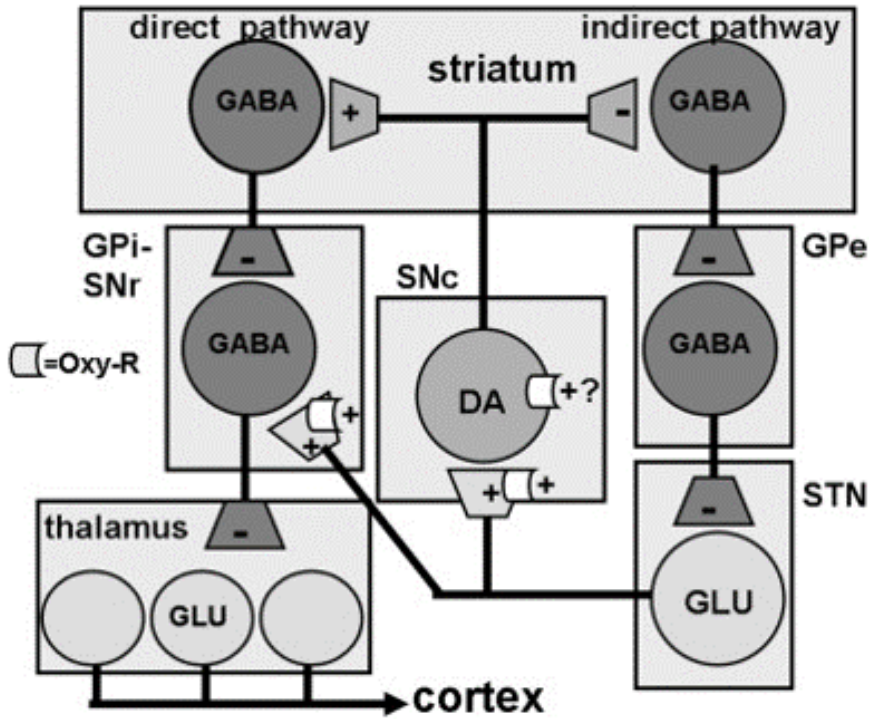


Figure 4.7: Mechanism by which oxytocin can inhibit locomotor activity. Figure, taken from (Angioni et al., 2016), summarises a potential way in which oxytocin inhibits locomotor activity. Oxytocin acting on glutamatergic neurons originating from the STN could lead to increased glutamate release to GABA neurons in the SN pars reticula and internal Global Pallidum (GPi). stimulation of OXY receptors in GLU nerve endings causes an increase in nigral GLU tone, which leads to an increase in the tone of nigral/pallidal (SNr/GPi) GABAergic efferents to the thalamus (and other nuclei of the basal ganglia). This would hypothetically increase GABA release to the thalamus/cortex, decreasing locomotion. GLU; Glutamatergic, STN; Subthalamic Nucleus, SN; Substantia Nigra; SNc; SN compacta, SNr; SN reticulate, OXY-R; Oxytocin receptors, DA; dopamine.

4.5.2: Effect of oxytocin on social interaction

Oxytocin at a dose of 0.1mg/kg was also shown to increase social interaction between two novel conspecifics, specifically increasing time spent in body and ano-genital sniffing behaviours, which may provide further insight as to how rats engage within social environments (Wesson, 2013). This is consistent to effects on social behaviours observed in both mice and rats (Lee et al., 2005, Bowen et al., 2011, Ramos et al., 2013, Calcagnoli et al., 2014, Huang et al., 2014). Additionally, engaging in social interaction increased core body temperature between the two conspecifics. Although the extent of core body temperature increase was reduced in oxytocin-treated rats, it does not appear to confound behaviour as oxytocin treated rats engaged in more social behaviour compared to saline controls.

Although not possible to determine whether the effect of oxytocin on social behaviours in rats was due to a predominantly vasopressin- or oxytocin-receptor mediated mechanism within this study, it is likely that both play a role via a cross-over in downstream signalling (Ramos et al., 2013). In support of this theory, the only reported opposing effects of oxytocin or vasopressin with regards to social behaviours appears in modulation of the fear response. For example, oxytocin infusions (100ng) into the amygdala of adult male Sprague-Dawley rats has shown anxiolytic properties in response to mild stress in the elevated plus-maze (Windle et al., 1997), whilst administration of AVP (100µM infusions) decreased open-arm preference, an effect which was not attenuated following co-administration with a oxytocin receptor antagonist (L-371,257,

100 µM; (Cragg et al., 2016)). These opposing effects are likely due to both neuropeptides differentially activating the central amygdala and inhibiting corticosterone release in response to stress (Roozendaal et al., 1993, Windle et al., 1997), as shown in electrophysiology studies (Huber et al., 2005).

Experiments showing that both peripheral i.p. and central administration of oxytocin receptor antagonist L-368, 899 (5mg/kg) into the NAc can reduce social preference in adult male C57BL/6 mice in a social reward paradigm (Dolen et al., 2013), combined with the lack of change to aggressive-type behaviour within this study, suggests the oxytocin receptor to be the primary mediator influencing this behaviour response. One way to test this would be to co-administer oxytocin in the presence of an oxytocin or V_{1A} receptor antagonist and determine subsequent effects on social interaction between two novel conspecifics. It may also be useful to determine the ability of oxytocin to attenuate the fear response in a contextual fear or PPI paradigm in the presence of antagonists for both receptor subtypes, which may provide further insight into how differential receptor activation can influence behavioural outcomes in rats with sensorimotor-gating deficits.

The mechanism by which acute oxytocin facilitates social interaction between two novel conspecifics remains unclear, but the increased release of dopamine in the NAc and potentially enhanced dopaminergic D_1 receptor activation as a consequence, indicates a potential mechanism as activation of this receptor subtype appears necessary to facilitate specific social behaviours. For example, administration of D_1 receptor antagonist SCH23390 disrupted pup-retrieval in

nulliparous female rats (Numan et al., 2005) and oxytocin has been shown to facilitate this behaviour (Marlin et al., 2015). Furthermore, rat mothers displaying high maternal behaviour (such as pup-grooming and licking) have the greatest increase in dopamine overflow in the NAc and increased oxytocin-VTA projections. Maternal behaviours are attenuated following 6-hydroxydopmaine-induced VTA-lesions and following VTA-infusion of an oxytocin receptor antagonist (β-mercapto-β,β cyclopentamethylenepropionyl; (Shahrokh et al., 2010)). Additionally, electrophysiology studies in female mice using optogenetic and applied fibre photometry techniques have shown increased VTA neuronal activity during social interaction with a novel conspecific, with VTA cell bodies reaching peak fluorescence during approach and active investigation (Gunaydin et al., 2014). The same research group also showed that NAc-infusion of a D_1 (SCH23390), but not D_2 (raclopride) dopamine receptor antagonist attenuated the pro-social effect elicited by VTA-dopamine neuron stimulation (Gunaydin et al., 2014), further illustrating the potential impact of oxytocin-dopamine interactions in modulating social responses.

4.5.3: Effect of oxytocin on USVs

In contrast to effects on social behaviour, oxytocin had no influence on the number of USV emissions, duration or bandwidth for any of the call categories examined herein, suggesting oxytocin to have little influence on USV production during social interaction at the doses used. There is little evidence comparing the effects of oxytocin administration on USV production in adult

rats, but studies in infant pups have suggested that oxytocin receptor activation does not influence USV emissions. For example, a high dose of oxytocin (1000 but not 100, 500 ng/pup i.c.v.) decreased USV emissions in Sprague-Dawley rat pups but there was no change to USV emissions following administration of oxytocin receptor antagonist (d(CH,),[Tyr(Me)*,Thr4, Tyr-NH;IOVT; 500ng/pup (Insel and Winslow, 1991)). Additionally, stimulation of the vasopressin system is unlikely to play a role as juvenile rats treated with AVP produced a similar number of 50 kHz calls compared to vehicle-treated rats during social play (Lukas and Wohr, 2015). Interestingly however, the same study showed that blockade of the AVP system following administration of V_{1A} receptor antagonist ($d(CH_2)_5[Tyr(Me)^2]AVP$; 0.75 µg/2.5 µl; i.c.v.) into the lateral ventricles reduced social play behaviour and USV emissions (Lukas and Wohr, 2015), suggesting a potential role in facilitating social behaviours in rats. However, these responses may also partly be due to confounding cardiovascular effects which were not examined. Despite this, the lack of change to USV frequency or pattern suggests that the change in social interaction is due to activation of mesocorticolimbic circuits and not the consequence of altered communication or coordinated calling between rat pairs (Wohr and Schwarting, 2013).

4.5.4: Effect of oxytocin on monoamines

One of the more interesting findings of this study was the supporting evidence for oxytocin acting as a substrate within the central dopaminergic system. At 0.1mg/kg s.c. oxytocin significantly increased dopamine overflow in the NAc

with a more subtle effect in the PFC over a 2h time course, without any concomitant change in serotonin levels in either the NAc or PFC. Therefore, acute systemic oxytocin is able to selectively increase dopamine release in drug-naïve Lister-hooded rats and does not appear to specifically target the serotonergic pathway.

Although dopamine appears essential for synergistic oxytocin-mediated responses, the specific mechanisms by which they interact remain unclear. For example, the NAc and striatum are abundant with both dopamine and oxytocin receptors but it is not known whether the receptors are co-expressed or co-regulated within these regions (Baskerville and Douglas, 2010). Despite this, the hypothesised localisation of oxytocin receptors on glutamatergic terminals (Angioni et al., 2016) could lead to further glutamate release following receptor activation. This in combination with dopamine release acting at dopamine D_1 receptors could enhance GABA medium spiny neuron activation within the direct pathway of movement (striatal input leads to reduced GPi firing which increases activity in the thalamus and cortex; (Schroll and Hamker, 2013, Calabresi et al., 2014)), and thus promote social approach behaviour. It has also been suggested that the ability of both oxytocin and AVP to activate brain areas rich in dopamine receptors could contribute to an enhanced hedonic value of specific behaviours such as social interaction and sexual activity (Meisel et al., 1993, Pfaus et al., 1995, Skuse and Gallagher, 2009). This is based on localisation studies showing the areas of greatest oxytocin receptor density to lie in brain regions associated with reward, including the VTA, NAc and PRL

(Insel, 1997, Shahrokh et al., 2010, Love, 2014). Despite this, it is unknown whether oxytocin plays a specific role in rewarding behaviour, can influence motivation to participate in rewarding behaviour, or whether its release is a consequence of mesolimbic reward pathway activation (Liberzon et al., 1997, Kent et al., 2013, Love, 2014). Nonetheless, dopamine turnover in the NAc is thought to be pivotal for the attribution of salience to reward, to shape social motivation, and to express appropriate affiliative behavioural responses, consistent with findings herein.

4.5.5: Future Studies

Based on the ability of oxytocin to attenuate PCP-induced hyperactivity and increase social interaction between two novel conspecifics, it would be of interest to determine its ability to restore social deficits within the 'two-hit' neonatal-PCP isolation-rearing model' used within chapter 3. It would also be of interest to examine the effects of oxytocin on other behaviours that are impaired in PCP-SI Lister-hooded rats including PPI, CER and specific learning and memory tasks such as the Morris Water Maze, allowing further corroboration between other animal models and clinical data.

To further elucidate the mechanisms by which oxytocin is able to mediate its effects, it may also be of interest to determine how neonatal-PCP isolation-rearing alters oxytocin receptor binding/density in key brain areas and whether treatment with oxytocin alone or adjunctively with antipsychotics can alter these expression profiles. For example, clozapine was not shown to improve

social deficits in the 'two-hit' model (McIntosh, 2014) and so co-administration of oxytocin may increase social interaction in neonatal-PCP isolation-reared rats. Oxytocin receptor profiling in future studies may also help to untangle the complex signalling pathways which underlie social behaviours and potentially provide evidence for how compounds such as clozapine can improve negative symptoms in subsets of schizophrenia patients (Shu et al., 2014).

4.5.6: Summary

The experiments conducted within this study established a dose of oxytocin which when administered peripherally, can attenuate PCP-induced hyperactivity, increase pro-social behaviour and selectively enhance dopamine release in the NAc without decreasing core body temperature in Lister-hooded rats. This study is one of very few to correlate effects on social behaviour with changes to core body temperature and activity, and provides further evidence in support for regionally-specific oxytocin-dopaminergic interactions within the mesocorticolimbic circuits responsible for regulating associative and rewarding behaviour.

Nevertheless, the relationship between oxytocin and dopamine and the precise neuronal circuitry involved is yet to be determined, potentially by the use of fMRI or c-fos immunohistochemistry following social behaviour tests to identify the brain regions involved. Increasing our understanding of the complex underlying interactions following oxytocin could prove useful in identifying why oxytocin often yields controversial and unreproducible results in clinical trials,

and provide insight into the exact dosing and administration routes most effective for use in treating psychiatric disorders alongside current medications.

Chapter 5: General Discussion

Schizophrenia is a complex and debilitating neurodevelopmental disorder with compelling evidence for both genetic and environmental influences on disease progression. These influences can lead to altered neuronal connectivity throughout the crucial development stages, from the neonate through adolescence. Although the positive symptoms of schizophrenia are relatively well treated by currently available antipsychotics, there is an unmet clinical need for better treatments that target the negative and cognitive symptom domains. The likelihood of advances in this area would be improved if there was a better understanding of the underlying neurobiology. This thesis has aimed to establish specific brain loci and signalling pathways that may be involved in the mechanism of action of a $GlyT_1$ inhibitor RO4993850 and oxytocin, to better understand how they may mediate effects on social behaviours in the adult rat, with the potential to translate this to effects on human behaviours.

5.1: Summary of findings

The studies reported herein have investigated the effects of a $GlyT_1$ inhibitor and oxytocin on social interaction in Lister-hooded rats, to evaluate their potential to treat the negative symptoms of schizophrenia and to further investigate the neurobiological mechanisms by which they can influence social behaviour in the rat. Using c-fos immunoreactivity as a marker for neuronal

activation, the $GlyT_1$ inhibitor RO4993850 (1 and 3mg/kg i.p.) was shown to specifically activate key brain regions including the OFC and cingulate cortices (Chapter 2). This data suggests that acute administration of the compound targets areas within the PFC and may subsequently influence glutamatergic signalling due to the presence of $GlyT_1$ within glutamatergic synapses (Gibert-Rahola and Villena-Rodriguez, 2014).

The 'dual-hit' neonatal-PCP combined with post-weaning social isolation model was then used to assess the ability of this brain penetrant $GlyT_1$ inhibitor, RO4993850, to attenuate LMA and social interaction following chronic administration (3mg/kg i.p. 14d), based on previous findings that this model produces more robust behavioural and neurochemical changes in adult rats than isolation rearing alone (Gaskin et al., 2014, McIntosh, 2014, Watson et al., 2016). As before, neonatal-PCP isolation-reared rats showed hyperactivity to a novel arena and had a deficit in social interaction. A particularly novel discovery was that PCP-SI rats also had altered USV characteristics, emitting a greater number of 50 kHz pro-social calls that were longer in duration and had a greater change in call bandwidth compared to those of SAL-GH littermates in all three USV subtypes examined (Chapter 3). This may be a very useful index of communication deficits, of relevance to features seen in schizophrenia. Treatment with the $GlyT_1$ inhibitor failed to influence LMA however it did improve social interaction behaviours that were reduced in PCP-SI rats. Specifically it increased total pro-social interaction, increased the time spent engaging in sniffing behaviour, and altered both USV emissions and call

characteristics, although the latter failed to reach significance. Furthermore, the compound had little effect in control SAL-GH rats, suggesting that a developmental behavioural deficit needed to be present before a reversal could be observed.

The neonatal-PCP isolation-rearing model was also further validated by demonstration of a decrease in the number of parvalbumin positive neurons in the hippocampal (CA1, CA2/3 and DG) sub-regions, but not in the rostral PFC (of the regions examined), comparable to changes seen in several other pre-clinical models used for schizophrenia (Harte et al., 2007b, Powell et al., 2015) and as outlined in Table 1.1. By contrast, basal dopamine levels in the PFC and striatum (measured by microdialysis) were similar in SAL-GH and PCP-SI rats, and acute systemic administration of the $GlyT_1$ inhibitor RO4993850 did not influence dopamine overflow in either brain region over the 3h sampling period in either group.

Finally, experiments herein examined the effects of oxytocin on social behaviour in drug-naïve group-housed Lister-hooded rats (Chapter 4). Specifically, a subcutaneous dose of 0.3mg/kg (but not 0.03 or 0.1mg/kg) oxytocin significantly decreased core body temperature despite having no effect on overall exploratory LMA. However, 0.1mg/kg s.c. oxytocin did significantly attenuate PCP-induced hyperactivity and increased social interaction between two novel conspecifics, specifically increasing time spent in sniffing (body and ano-genital) behaviours, but had no concomitant effect on USV emissions or call characteristics. The act of engaging in social

interaction was accompanied by an increased core body temperature between the two conspecifics but the extent of increase was reduced in oxytocin-treated rats. Nonetheless the dose administered did not appear to confound behaviour as rats treated with oxytocin engaged in more social behaviour compared to saline controls. By using microdialysis to simultaneously measure neurotransmitter overflow in two brain regions, oxytocin at the same dose, (0.1mg/kg s.c.) specifically increased dopamine overflow in the NAc, producing a more subtle effect in the PFC which did not reach significance over a 2h time course, without any concomitant effect on serotonin overflow in either region.

5.2: Altered signalling within schizophrenia

As discussed in previous chapters herein, goal-directed behaviour is thought to play a large role in mediating social responses through the convergence of dopamine, glutamate and GABA signalling. Disruption to the neural networks that regulate motivation therefore, could contribute to negative symptoms in schizophrenia patients (Gold et al., 2012, Brown et al., 2014). The main regions implicated include the amygdala (involved in emotional behaviour/processing), PFC (modulates behavioural flexibility), ventral hippocampus (provides contextual information) and the NAc where overlapping projections from these regions converge (Grace et al., 2007), summarised in Figure 5.1.

There are therefore, a potential number of pathways which could mediate the effects of $GlyT_1$ inhibitors like RO4993850 on social responses. For example, inhibition of $GlyT_1$ increases NMDA receptor activity, which can facilitate

approach behaviour by acting in synergy with dopamine D_1 receptors (direct pathway), or via indirect pathway (expresses dopamine D_2 receptors) activation. As the NAc inhibits the ventral pallidum, the subsequent release of glutamate from the BLA and ventral subiculum of the hippocampus to the NAc would further facilitate NMDA receptor activation of the direct pathway; dopaminergic neurons would be released from inhibition leading to subsequent output to limbic and motor regions. The resultant increase in dopaminergic phasic firing could also contribute to hippocampal association with reward-related cues and impact upon reward-directed behaviour (Lisman and Grace, 2005), as input from the ventral subiculum of the hippocampus is modulated by dopamine D_1 receptors.

In addition, the OFC can also contribute to mediating the computational value of stimuli (Padoa-Schioppa and Cai, 2011, Rudebeck and Murray, 2011) and integrating their value into goal-directed and possibly reward-seeking behaviour, thought to involve the dorsolateral PFC (Levy and Dubois, 2006, Millan et al., 2014). Therefore the specific activation/implication of the OFC determined herein by the $GlyT_1$ inhibitor is also of interest, as this region is closely linked to the NAc, amygdala and hippocampus (Levy and Dubois, 2006, Kring and Barch, 2014, Strauss et al., 2014).

In a similar fashion to $GlyT_1$ inhibitors, oxytocin may interact within the same signalling pathways, as their assumed co-localisation on glutamatergic terminals would also facilitate further glutamate release and act on dopamine D_1 to enhance GABA medium spiny neuron activation via the direct pathway

(Schroll and Hamker, 2013, Calabresi et al., 2014, Angioni et al., 2016). Furthermore, aberrant oxytocinergic signalling within the amygdala is thought to underpin asociality (Gradin et al., 2011, Kring and Barch, 2014, Millan et al., 2014).

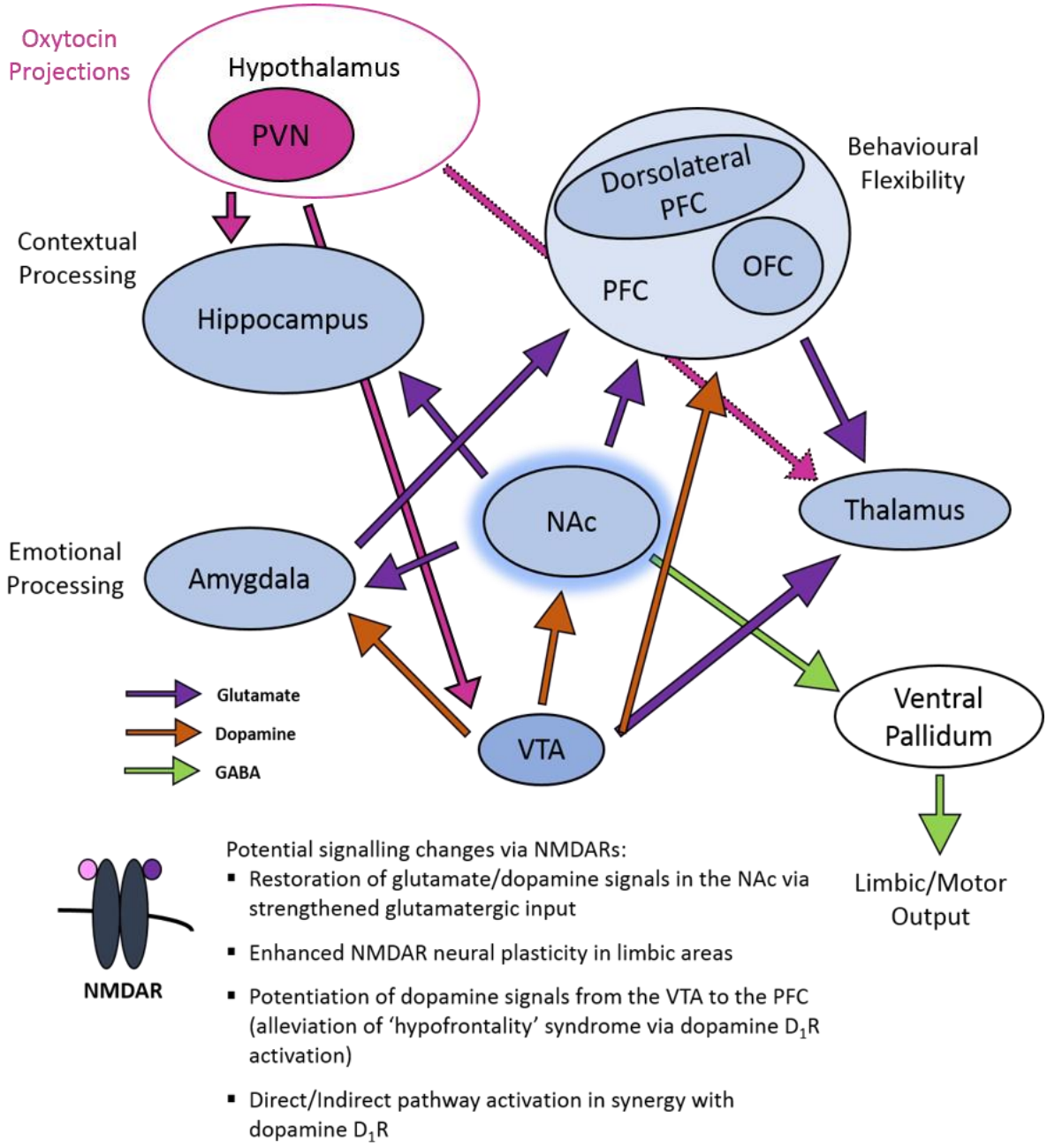


Figure 5.1: Altered signalling within schizophrenia. Potential signalling pathways involved in modulating motivation and social behaviour. PFC; Prefrontal Cortex, OFC; Orbital frontal cortex, NAc; Nucleus Accumbens, VTA; Ventral Tegmental Area, PVN; Paraventricular Nucleus, NMDAR, N-Methyl-D-Aspartate Receptor; D_1R; Dopamine D1 Receptor. Image adapted from (Grace et al., 2007, Millan et al., 2014).

5.3: Translational relevance of the 'dual-hit' neonatal-PCP isolation-rearing model

Improved pre-clinical models that better mimic schizophrenia symptomatology have better potential to aid research into disease aetiology and lead to improved therapy. One approach aims to produce and validate dual-hit models, such as that used within this thesis (neonatal-PCP and isolation-rearing) to utilise the strengths of each individual manipulation alone, and combine their effects to induce long-term changes in the adult rat that have translational value to core aspects of schizophrenia. In particular PCP-SI rats have demonstrated greater deficits in social interaction, akin to the negative symptoms they are considered to be an index for.

The experiments conducted within this thesis have aimed to gain some insight as to the underlying mechanisms by which the $GlyT_1$ inhibitor RO4993850 and oxytocin mediate their effects *in vivo*, and how this correlates to their effects on social behaviours. Although the social interaction paradigm is a commonly used, non-invasive and straightforward method for measuring sociability in rodents, social withdrawal is only one aspect of this symptom domain in humans. Negative symptoms also include poverty of speech (alogia) and avolition (Foussias and Remington, 2010). Although typically harder to assess and quantify the former in rodents, there have been new paradigms created to measure goal-directed behaviours (Markou et al., 2013, Salamone et al., 2016b). Future studies could therefore investigate the effects of neonatal-PCP isolation-rearing on paradigms thought to assess motivation, such as

progressive ratio learning tests. This would help to determine whether components of the motivation/reward system are also affected in the 'dual-hit' model. For example, Rygula et al. (2015) used a progressive-ratio schedule where motivation to receive reward (0.1 ml of 20% sucrose solution) and motivation to avoid punishment (an electric shock; 0.5 mA, 10s) were both assessed in Sprague-Dawley rats.

Although the $GlyT_1$ inhibitor used herein increased social behaviour between two novel conspecifics in the 'dual-hit' model, it is worth noting that a close analogue, Bitopertin, failed to significantly improve negative symptoms in recent phase III clinical trials compared to placebo (Bugarski-Kirola et al., 2017), questioning the predictive validity of such rodent two-hit models. Post-hoc results from these trials have suggested that failure could possibly be due a high placebo response rate, a common problem in such trials, but also due to a weaker response to Bitopertin than previously achieved (Hofmann et al., 2011, Umbricht et al., 2011). However further increasing our knowledge of the alterations to neuropathology and physiological mechanisms e.g. REDOX dysregulation within the 'dual-hit' model, could provide insight into additional mechanisms/pathways that may be activated by this class of compound, and provide an additional measure of how the animal model used herein differs from other models of schizophrenia (Radonjic et al., 2010, Jiang et al., 2013a, Jiang et al., 2013b).

Another avenue worthy of exploration is determining how USVs are altered in isolated rats as there is little data available to compare the results found herein.

Although changes to USV emissions are more commonly used as a communication index in preclinical models of ASD, it could provide further insight into the effects lack of social play (isolation-rearing) and neonatal-PCP have on other aspects of social behaviour and whether it can provide another marker by which to improve the model's validity.

5.4: Future studies

As highlighted within Chapter 4, oxytocin retains potential as a therapeutic for schizophrenia based on its role in deficits associated with the disease. However inconsistent findings in clinical trials, as well as the difficulty posed in comparing results between studies which use different measures and treatment populations to assess oxytocin's efficacy on negative symptoms, limits the conclusions which can be drawn regarding its use. As already discussed, intranasal oxytocin administration, although commonly used in both pre-clinical and clinical trials, would be difficult to perform in neonatal-PCP isolated rats, and it would be difficult to determine the exact dose required to reach the CNS. Therefore, peripheral administration of the compound in the 'dual-hit' model combined with an *ex vivo* assessment of oxytocin receptor binding/expression studies may shed light into the pharmacodynamics of the neuropeptide within the rat, which could then be correlated to changes in social behaviour. Oxytocin receptor profiling in future studies may also help to untangle the complex signalling pathways that underlie social behaviours.

Activation of specific pathways as well as changes in signalling, particularly between glutamate, oxytocin and dopamine also need to be determined to establish the complex circuitry underlying oxytocin's action. Potentially through the use of fMRI or c-fos immunohistochemistry following social behaviour tests, activity of the specific brain regions involved could be evaluated. Increasing our understanding of the complex underlying interactions following oxytocin could potentially prove useful in identifying why oxytocin often yields controversial and unreproducible results in clinical trials, and provide insight into the exact dosing and administration routes most effective for use in treating psychiatric disorders alongside current medications.

5.5: Conclusions

Based on the findings of this study, the 'two-hit' model has some potential to identify novel therapeutics that have efficacy for the negative symptoms of schizophrenia. The strong behavioural differences in PCP-SI rats in terms of pro-social interaction and USV emissions also suggests this model could be used for other disorders manifesting social deficits such as ASD, although further work is yet required to fully validate this.

It is also essential to untangle the complex neurobiology underlying how compounds such as oxytocin work in the brain to help establish how dysfunctional signalling may contribute to the onset of schizophrenia

throughout development, and secondly, help determine its potential as an adjunctive therapy to patients with predominant negative symptoms.

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